Regulation and function of p38 protein kinase in isolated canine gastric parietal cells

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Pausawasdi, N., S. Ramamoorthy, V. Stepan, J. Del Valle, and A. Todisco. Regulation and function of p38 protein kinase in isolated canine gastric parietal cells. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G24–G31, 2000.—We examined the regulation and functional role of p38 kinase in gastric acid secretion. p38 kinase was immunoprecipitated from cell lysates of highly purified gastric parietal cells in primary culture, and its activity was quantitated by in vitro kinase assay. Carbachol effects were dose- and time-dependent, with a maximal 10-fold stimulatory effect detected after 30 min of incubation. SB-203580, a highly selective inhibitor of p38 kinase, blocked carbachol induction of p38 kinase activity, with maximal inhibition at 10 µM. Stimulation by carbachol was unaffected by preincubation of parietal cells with the intracellular Ca²⁺ chelator BAPTA-AM, but incubation of cells in Ca²⁺-free medium led to a 50% inhibition of carbachol induction of p38 kinase activity. Because some of the effects of carbachol are mediated by the small GTP-binding protein Rho, we examined the role of Rho in carbachol induction of p38 kinase. We tested the effect of exoenzyme C3 from Clostridium botulinum (C3), a toxin known to ADP-ribosylate and specifically inactivate Rho. C3 led to complete ADP-ribosylation of Rho, and it inhibited carbachol induction of p38 kinase by 50%. We then tested the effect of SB-203580 and C3 on carbachol-stimulated uptake of [14C]aminopyrine (AP). Inhibition of p38 kinase by SB-203580 led to a dose-dependent increase in AP uptake induced by carbachol, with maximal (threefold) effect at 10 µM SB-203580. Similarly, preincubation of parietal cells with C3 led to a twofold increase in AP uptake induced by carbachol. Thus carbachol induces a cascade of events in parietal cells that results in activation of p38 kinase through signaling pathways that are at least in part dependent on Rho activation and on the presence of extracellular Ca²⁺. p38 kinase appears to inhibit gastric acid secretion.

gastric acid secretion; protein kinases; mitogen-activated protein kinase/extracellular signal-regulated protein kinase; GTP-binding proteins; Rho proteins; cellular cytoskeleton.
gastrointestinal hormone CCK (25, 34, 35). Activation of p38 kinase has been linked to regulation of programmed cell death, skeletal muscle differentiation, organization of the cellular cytoskeleton, and phosphorylation of transcription factors (25, 34, 36).

Because the function of p38 kinase in the stomach is currently unknown, we took advantage of SB-203580, a specific p38 kinase inhibitor (18, 25), to investigate the regulation and the functional relevance of p38 kinase in carbachol-stimulated gastric acid secretion. In addition, because members of the Rho family of GTPases are known to activate p38 kinase (34), we examined whether, in the parietal cells, carbachol targets p38 kinase through Rho-dependent mechanisms and whether Rho exerts regulatory actions on gastric acid secretion. For these experiments, we used exoenzyme C3 from Clostridium botulinum (C3), a toxin known to ADP-ribosylate and specifically inactivate Rho (26, 28).

In this report, we demonstrate that carbachol induces a cascade of events in the gastric parietal cells that results in the activation of p38 kinase through signaling pathways that are at least in part dependent on the small GTP-binding protein Rho and on the presence of extracellular Ca2+. Furthermore, the functional effect of both Rho and p38 kinase appears to be inhibition of gastric acid secretion.

MATERIALS AND METHODS

Plasmids. Bacteria transformed with the expression plasmid for transcription factor GST-ATF2 (1–109) were a gift from Dr. J. Han (Scripps Research Institute, La Jolla, CA). Plasmid pET3a/C3 was a gift from S. Narumiya (Kyoto, J apan).

Primary parietal cell preparation and culture. For preparation of primary parietal cells, we utilized a modification of the method of Soli et al. (5, 21, 27, 30). 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% 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 J E-6B elutriation rotor. 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 p = 1.05 consisted of virtually all parietal cells, as determined by staining with a specific mouse monoclonal antibody to gastrin (Sigma, St. Louis, MO) and with 12- and 25-ml cell culture dishes (Corning, Cambridge, MA) coated with 150 ml of H2O-diluted 10% BSA. The parietal cells were counted in a Coulter Counter (Coulter Electronics, Hialeah, FL) and analyzed for morphology by light microscopy.

Immunoprecipitations and p38 kinase assay. Immunoprecipitations and p38 kinase assays were performed according to previously described techniques (22, 25) with minor modifications. The parietal cells were lysed in 500 µl of lysis buffer [50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1.5 mM MgCl2, 1 mM Na2VO4, 10 mM NaF, 10 mM Na2PO4·2H2O, 1 mM 4-(2-aminomethyl)benzenesulfonic acid (AEBSF, ICN-Biomedicals, Aurora, OH), 1 µg/ml leupeptin, and 1 µg/ml aprotinin]. The lysates were transferred into Microfuge tubes and spun at 16,000 g for 20 min at 4°C. Equal amounts of protein from each treatment group (300 µg) were incubated with an anti-p38 kinase antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Protein concentrations were measured by the Bradford method (2). Aliquots of protein-A-Sepharose (50 µl) (Pharmacia Biotech), were then added and the solutions mixed for an additional 1 h. After centrifugation, the pellets were washed once with lysis buffer and twice with kinase buffer (18 mM HEPES, pH 7.4, 10 mM magnesium acetate, 50 µM ATP, and 2.5 µCi/sample [32P]ATP). Immunoprecipitated p38 kinase was used to phosphorylate 2 µg of GST-ATF2 (1–109). Kinase reactions were carried out in 20 µl kinase buffer at 30°C for 30 min. Reactions were terminated by addition of 20 µl 5x electrophoresis buffer (for 5 ml: 2.5 ml glycerol, 1.25 ml 2-mercaptoethanol, 0.5 g SDS, 1.043 ml of 1.5 M Tris, pH 6.8, and 1.25 mg bromophenol blue). The samples were then boiled for 5 min and applied to a 10% SDS-polyacrylamide gel, followed by staining with Coomassie blue and destaining (22) to ensure that identical amounts of proteins were loaded on the gel. Labeled phosphorylated proteins were visualized by autoradiography and quantitated by scanning densitometry. GST-ATF2 (1–109) was expressed and purified from Escherichia coli as previously described (22).

Western blots. Parietal cell lysates (80 µg) were loaded on 10% SDS-polyacrylamide minigels and run at 20 A for 1 h. The gels were transferred on an Immobilon-P transfer membrane (Millipore, Bedford, MA) in 25 mM Tris, 150 mM glycine, and 20% methanol. After transfer, the membranes were blocked in 10 ml TBST (20 mM Tris, 0.15 M NaCl, 0.3% Tween) for 1 h and then incubated for 16–18 h at 4°C in 10 ml TBST and 5% dry milk, containing either a specific anti-phospho-p38 antibody that recognizes phosphorylated tyrosine 182 of p38 kinase (1:1,000) or an antibody recognizing p38 kinase independent of its phosphorylation state (1:1,000) (New England Biolabs, Beverly, MA). At the end of the incubation period, the membranes were washed in TBST for 30 min at room temperature and then incubated for 1 h in TBST and 5% dry milk, containing protein A directly conjugated to horseradish peroxidase (Amersham Life Science, Arlington Heights, IL) (1:2,500). The membranes were washed in TBST for 30 min at room temperature and then exposed to the Amersham enhanced chemiluminescence detection system according to the manufacturer’s instructions.

ADP-ribosylation of Rho in gastric parietal cells. Recombinant C3 was expressed in E. coli using a plasmid encoding C3 (pET3a/C3) and purified as previously described (28). To verify the effectiveness of ADP-ribosylation of Rho by C3, the parietal cells were either left untreated or incubated with increasing concentrations of the toxin (10–100 µg/ml) for,
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24 h. The cells were then extensively washed with PBS and lysed by three cycles of freezing and thawing in 100 µl of hypotonic lysis buffer (20 mM Tris, pH 8.0, 3 mM MgCl₂, 0.4 mM AEBSF, 5 µg/ml aprotinin, 2 µg/ml trypsin inhibitor, and 20 µM leupeptin). Cell debris was separated by centrifugation at 14,000 rpm at 4°C for 20 min, and the supernatant was subjected to ADP-ribosylation assay. Cell lysates containing equal amounts of protein were incubated with 4 µg/ml recombinant C3 and 1 µCi [³²P]nicotinamide adenine dinucleotide (NAD) (Amersham Life Science) in 20 µl of buffer, containing 10 µM [³²P]NAD, 50 mM triethanolamine hydrochloride, pH 7.5, 2 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM AEBSF, 10 mM thymidine, and 100 µM GTP at 37°C for 60 min. Protein concentrations were measured by the Bradford method (2). Samples were resolved by SDS-PAGE on 15% gels, and the ADP-ribosylated Rho was visualized by autoradiography.

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. After a 20-min incubation at 37°C, the cells were centrifuged and the radioactivity of the pellet was quantified in a liquid scintillation counter as previously reported (31). In some experiments, the parietal cells were cultured in Ham’s F-12-DMEM (1:1) (6, 18, 25) with or without C3 (40 µg/ml) for 24 h before the addition of carbachol. At the end of the incubation period, the cells were washed once with EBSS, preincubated with 0.1 µCi [¹⁴C]aminopyrine for 30 min, and then stimulated with carbachol (100 µM) for 30 min. Parietal cells were lysed with 500 µl of 1% Triton X-100, and the radioactivity of lysate was quantified in a liquid scintillation counter.

Data analysis. Data are expressed as means ± SE. Statistical analysis was performed using Student’s t-test. P values <0.05 were considered significant.

RESULTS

We examined the effect of carbachol on p38 kinase activity. As depicted in Fig. 1, carbachol induced p38 kinase activity in a dose-dependent fashion with a maximal 10-fold stimulatory effect detected at a dose of 100 µM. In addition, carbachol induction of p38 kinase activity was detectable after 5 min of incubation, it reached a maximum effect between 30 and 60 min, and it decreased by 120 min (Fig. 2). Because activation of p38 kinase requires phosphorylation of the kinase on tyrosyl residues (34), we examined the effect of carbachol on p38 kinase phosphorylation using Western blots with a specific anti-phospho-p38 kinase antibody. As shown in Fig. 3, carbachol induced p38 kinase phosphorylation after 30 min of incubation.

Ca²⁺ is an important mediator of the action of carbachol in the gastric parietal cells (11, 12). Thus we undertook studies to examine the role of both intracellular and extracellular Ca²⁺ on carbachol induction of p38 kinase activity. Chelation of intracellular Ca²⁺ by BAPTA-AM (100 µM) failed to affect the stimulatory action of carbachol. In contrast, deprivation of extracellular Ca²⁺ by incubation of the cells in Ca²⁺-free medium containing 1 mM EGTA inhibited carbachol induction of p38 activity by 50%, suggesting that carbachol induces p38 activation through signaling pathways that are at least in part dependent on the presence of extracellular Ca²⁺ (Fig. 4).

We established the dose of the highly specific p38 kinase inhibitor SB-203580 that would effectively inhibit carbachol induction of p38 kinase activity in the gastric parietal cells. SB-203580 dose-dependently inhibited carbachol-stimulated p38 kinase activity with a maximal 60% inhibitory effect detected at the dose of 10 µM (Fig. 5).

Because some of the effects of carbachol are mediated by the small GTP-binding protein Rho (17, 29), we examined whether carbachol induction of p38 kinase would require Rho activation. For these experiments, we tested the effect of recombinant C3, a toxin known to ADP-ribosylate and specifically inactivate Rho (26, 28). We first performed ADP-ribosylation assays to monitor the dose of toxin required to ADP-ribosylate Rho in vivo. As shown in Fig. 6, the parietal cells were either left untreated (lanes 1 and 6) or incubated with increasing concentrations of C3 (lanes 2–5). After 24 h, the cells were lysed and equal amounts of protein were subjected to an in vitro ADP-ribosylation assay using GST-ATF2 as substrate. A: representative assays obtained with a single parietal cell preparation. B: linear transformation of densitometric analysis of autoradiograms. Data are multiples of induction over control (means ± SE). Numbers in parentheses indicate number of separate dog preparations from which parietal cells were obtained. O.D., optical density.
data indicate that 40 µg/ml C3 can completely ADP-ribosylate Rho in vivo.

We then examined whether Rho is involved in carbachol induction of p38 kinase. C3 (40 µg/ml), inhibited carbachol-stimulated p38 kinase activity by 40%, indicating that, in the gastric parietal cells, carbachol induces p38 kinase through signaling pathways that are at least in part dependent on activation of Rho (Fig. 7).

To investigate the functional significance of p38 kinase in the acid secretory function of parietal cells, we tested the effects of SB-203580 on carbachol-stimulated uptake of [14C]aminopyrine. Inhibition of p38 kinase by SB-203580 led to a dose-dependent increase in aminopyrine uptake induced by carbachol, with a maximal threefold stimulatory effect observed in the presence of 10 µM SB-203580. Thus p38 kinase activation is not important for stimulation of gastric acid secretion by carbachol, but, on the contrary, it appears to exert a significant inhibitory effect (Fig. 8).

Because Rho appears to be involved in carbachol signaling to p38 kinase, we examined the effect of C3 on carbachol-stimulated uptake of [14C]aminopyrine. As depicted in Fig. 9, inhibition of Rho activation by 40 µg/ml C3 led to a twofold increase in aminopyrine uptake induced by carbachol, suggesting that Rho has a negative modulatory effect on gastric acid secretion.
DISCUSSION

The gastric parietal cell secretes gastric acid in response to a broad range of physiological stimuli. Numerous studies have previously reported that carbachol is the most potent inducer of gastric acid secretion in canine parietal cells (11). In our study, we have examined the regulation of p38 kinase in response to carbachol and the physiological role of this kinase in the process of gastric acid secretion. Carbachol induces p38 kinase at doses and with kinetics that are similar to those observed in the case of the ERKs and the JNKs. However, the intracellular signal transduction pathways involved in the activation of these kinases appear to exhibit considerable mechanistic differences. Mobilization of intracellular and extracellular Ca\(^{2+}\) is an important event in carbachol signaling in the gastric parietal cells, in which it plays a pivotal role in the process of gastric acid secretion (11). In our study, we noted that carbachol targets p38 kinase through signaling pathways that do not require mobilization of intracellular Ca\(^{2+}\) but that are affected by changes in extracellular Ca\(^{2+}\) levels. In fact, carbachol induction of p38 kinase was not inhibited by the intracellular Ca\(^{2+}\)
chelator BAPTA-AM, whereas it was significantly diminished in the presence of Ca\(^{2+}\)-free medium. Interestingly, we observed that carbachol targets JNK1 through signaling pathways that require mobilization of intracellular Ca\(^{2+}\) (22), whereas it stimulates ERK2 through Ca\(^{2+}\)-independent mechanisms (31). The specificity of these observations was further confirmed by experiments in which we measured the activity of both JNK1 and p38 kinase in the same parietal cell lysates. In these studies, we noted that, although JNK1 was significantly inhibited by the addition of BAPTA-AM, the activity of p38 kinase was unaffected by this compound (data not shown). Accordingly, in the gastric parietal cells, carbachol induces multiple protein kinases through signaling pathways that exhibit different and specific requirements for Ca\(^{2+}\) mobilization.

To analyze the functional relevance of p38 kinase induction in the parietal cells, we took advantage of the recent discovery of SB-203580, a highly selective p38 kinase inhibitor (18). As in other cellular systems, SB-203580 did not have any effect on both JNK1 and ERK2 activity (data not shown), confirming the notion that, in the parietal cells, this agent specifically inhibits p38 kinase and not other members of the superfamily of MAPKs. By the use of this compound, we were able to examine the functional importance of p38 kinase activation in the process of gastric acid secretion. In this study, we noted that SB-203580 dose-dependently potentiated carbachol induction of aminopyrine uptake, suggesting that p38 kinase has a negative regulatory effect on gastric acid secretion. Interestingly, inhibition of ERK2 by the specific MAPK/ERK kinase inhibitor PD-98059 led to a similar, although weaker, stimulatory effect on carbachol induction of aminopyrine uptake (31), indicating that both the ERKs and p38 kinase have acute inhibitory actions on gastric acid production.

The mechanisms responsible for these observations are currently unknown. Because p38 kinase is known to play an important role in the organization of the actin cytoskeleton (25, 34), another mechanism responsible for p38 kinase-induced inhibition of gastric acid secretion might involve changes in the actin cytoskeleton of the gastric parietal cells. The cytoskeleton is an important element in secretagogue-stimulated gastric acid secretion because, during this process, the parietal cell undergoes dramatic morphological modifications that lead to the translocation of the H\(^{+}\)-K\(^{+}\)-ATPase from cytoplasmic tubulovesicular structures to the apical plasma membrane (3, 11, 13). Accordingly, induction of p38 kinase might result in phosphorylation of cytoskeletal proteins and significant changes in the organization of the actin cytoskeleton. These events could be responsible for alterations in the process of H\(^{+}\)-K\(^{+}\)-ATPase insertion into the parietal cell apical membrane and for inhibition of gastric acid production. In addition, because cessation of gastric acid secretion requires the internalization of the H\(^{+}\)-K\(^{+}\)-ATPase into an intracellular storage compartment (11), it is possible that p38 kinase-induced changes in the cellular cytoskeleton might also be involved in the attenuation system involved in the recycling of the proton pump in the gastric parietal cells.

The Rho GTPases are important molecular switches in the cellular activation process (14, 17, 19, 24, 26, 29). Rho proteins include Rac, Cdc 42, and RhoA, B, and C (19). These small GTP-binding proteins have been implicated in the regulation of numerous cellular functions, such as activation of JNK and p38 kinase, organization of the cellular cytoskeleton, and induction of gene transcription (14, 19, 34). In this study, we examined the physiological role of Rho in the gastric parietal cells. In particular, we investigated the function of this small GTP-binding protein in carbachol signaling to p38 kinase and in the regulation of gastric
acid secretion. In these experiments, we employed a well-known and specific inhibitor of Rho function, C3, a toxin known to ADP-ribosylate and to specifically inactivate Rho (26, 28). Because C3 was added to intact parietal cells, we confirmed by ADP-ribosylation assays that the dose of C3 used in the study was able to induce complete ADP-ribosylation of Rho. Furthermore, we reported that Rho is a crucial element in carbachol signaling to p38 kinase because C3 significantly blocked the stimulatory effect of carbachol on p38 kinase activity. Interestingly, this inhibition was not complete, thus suggesting the possible involvement of Rho-independent pathways. Additional studies will be necessary to define whether, in addition to Rho, Cdc42, Rac1, or Ras might be also involved in carbachol signaling to p38 kinase. Recent reports have demonstrated that M₃ muscarinic receptors induce Rho activation via both tyrosine kinase- and protein kinase C-dependent pathways and that this process plays an important role in the organization of cytoplasmic myosin and in the regulation of cell morphology, adhesion, and proliferation (17, 24, 29). Although in this study we did not examine the role of tyrosine kinase and protein kinase C in carbachol induction of p38 kinase, it is conceivable that these signaling pathways, which mediate some of the physiological actions of carbachol in the stomach, could be involved in a signaling cascade linking M₃ muscarinic receptors to Rho and p38 kinase in the gastric parietal cells.

The physiological relevance of these observations is underscored by the finding that inhibition of Rho by C3 leads to potentiation of carbachol-stimulated aminopyrine uptake, indicating that Rho activation leads to inhibition of gastric acid secretion. Because Rho is an important regulator of cytoskeletal function (14, 19, 26), we hypothesize that this effect of Rho could be mediated by changes in the parietal cell cytoskeleton. A recent report has indicated that inhibition of Rho in the G cells of the canine stomach leads to potentiation of both basal and bombesin-stimulated gastrin release (26). In that study, the authors postulated that C3 could be responsible for increased gastrin release through disruption of the G cell actin cytoskeleton. Thus similar Rho-dependent mechanisms could affect the secretory function of the G cells and the parietal cells regulating the release of both gastrointestinal hormones and gastric acid. Because Rho appears to be upstream of p38 kinase and because inhibition of p38 kinase leads to stimulation of gastric acid secretion, we postulate that, in the gastric parietal cells, carbachol might activate a signaling cascade that targets Rho and p38 kinase, leading to changes in the actin cytoskeleton that result in inhibition of gastric acid secretion.

To document more precisely the occurrence of modifications in the actin filaments of the parietal cells in response to C3, SB-203580, or carbachol in D, a known and potent inhibitor of cytoskeletal function, we performed histochemical studies using phallolidin staining and confocal fluorescence microscopy. In these experiments, we did not detect significant and reproducible changes in the organization of the actin cytoskeleton in response to either C3 or SB-203580, whereas we observed perinuclear actin clumps in parietal cells treated with carbachol in D (data not shown). These findings are in agreement with observations generated in the G cells in which C3 failed to induce any detectable changes in the G cell cytoskeleton (26). Thus we hypothesize that carbachol and bombesin might induce subtle changes in the organization of the actin cytoskeleton of both canine G cells and parietal cells that cannot be detected by conventional imaging techniques. The complexity of these findings is underscored by the observation that treatment of the G cells with carbachol in D leads to increased gastrin release (26), whereas addition of this compound to the parietal cells causes inhibition of gastric acid secretion (Ref. 13 and data not shown). These apparent discrepancies might reflect differences in the organization and in the requirement of the cytoskeleton for the execution of secretory functions between G cells and parietal cells. Furthermore, it is possible that in the parietal cells Rho and p38 kinase might have fine regulatory effects on the organization of the actin cytoskeleton, leading to decreased insertion of the proton pump on the apical membrane. In contrast, carbachol in D, which induces profound and visually detectable alterations in the organization of the actin filaments, causes inhibition of gastric acid secretion.

In conclusion, carbachol induces a cascade of events in the gastric parietal cells that results in the activation of p38 kinase through signaling pathways that are at least in part dependent on the small GTP-binding protein Rho and on the presence of extracellular Ca²⁺. Furthermore, the acute functional effect of both Rho and p38 kinase appears to be inhibition of gastric acid secretion. These actions might be mediated by changes in the organization of the actin cytoskeleton, and they could involve the phosphorylation of yet-unidentified cytoskeletal proteins.

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