JNK mitogen-activated protein kinase limits calcium-dependent chloride secretion across colonic epithelial cells

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Donnellan F, Keating N, Geoghegan P, Murray FE, Harvey BJ, Keely SJ. JNK mitogen-activated protein kinase limits calcium-dependent chloride secretion across colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol 298: G37–G44, 2010. First published October 29, 2009; doi:10.1152/ajpgi.00202.2009.—Neuroimmune agonists induce epithelial Cl− secretion through elevations in intracellular Ca2+ or cAMP. Previously, we demonstrated that epidermal growth factor receptor (EGFR) transactivation and subsequent ERK MAPK activation limits secretory responses to Ca2+-dependent, but not cAMP-dependent, agonists. Although JNK MAPKs are also expressed in epithelial cells, their role in regulating transport function is unknown. Here, we investigated the potential role for JNK in regulating Cl− secretion in T84 colonic epithelial cells. Western blot analysis revealed that a prototypical Ca2+-dependent secretagogue, carbachol (CCh; 100 μM), induced phosphorylation of both the 46-kDa and 54-kDa isoforms of JNK. This effect was mimicked by thapsigargin (TG), which specifically elevates intracellular Ca2+, but not by forskolin (FSK; 10 μM), which elevates cAMP. CCh-induced JNK phosphorylation was attenuated by the EGFR inhibitor, tyrphosatin-AG1478 (1 μM). Pretreatment of voltage-clamped T84 cells with SP600125 (2 μM), a specific JNK inhibitor, potentiated secretory responses to both CCH and TG but not to FSK. The effects of SP600125 on CCh-induced secretion were not additive with those of the ERK inhibitor, PD98059. Finally, in apically permeabilized T84 cell monolayers, SP600125 potentiated CCh-induced K+ conductances but not Na+/K+ ATPase activity. These data demonstrate a novel role for JNK MAPK in regulating Ca2+- but not cAMP-dependent epithelial Cl− secretion. JNK activation is mediated by EGFR transactivation and exerts its antisecretory effects through inhibition of basolateral K+ channels. These data further our understanding of mechanisms regulating epithelial secretion and underscore the potential for exploitation of MAPK-dependent signaling in treatment of intestinal transport disorders.

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JNK MAPK REGULATES INTESTINAL EPITHELIAL SECRETION

mones (6, 10), cytokines (1, 20), and inflammatory mediators (23). In addition, JNK can be activated by exogenous factors, including viruses (19) and pathogenic bacteria, and their toxins (18, 34, 36, 38). Intestinal epithelial JNK activation is also induced by pharmaceuticals, such as nonsteroidal anti-inflammatory drugs (14), and various dietary components with therapeutic activity (9, 28). Although the roles for JNK in regulating epithelial cell function have not been so well defined as they have for other MAPKs, it has been clearly demonstrated to be important in regulating cell cycle progression and apoptosis (6, 16, 30, 36, 41, 42) and is therefore an important regulator of epithelial cell growth. JNK can also regulate epithelial cytokine production and cell migration, thereby contributing to inflammatory responses in the intestine (18, 37).

Indeed, JNK activation has been reported to be elevated in patients suffering from IBD (32–35). Since JNK activation has been shown to be important in regulating cell cycle progression and apoptosis (6, 16, 30, 36, 41, 42) and is therefore an important regulator of epithelial cell function (36), this study set out to investigate the role of JNK in regulating intestinal epithelial Cl− secretion.

MATERIALS AND METHODS

Materials. T84 cells were a kind gift from Dr. Kim Barrett (University of California, San Diego, CA). Carbachol (CCh), thapsigargin (TG), and forskolin (FSK) were obtained from Sigma Chemical (Poole, UK). The JNK inhibitor, SP600125, the JNK inhibitor II positive control, tyrphostin AG-1478, and PD 98059 were obtained from Calbiochem (San Diego, CA). Phospho-SAPK/JNK (Thr183/ Tyr185), phospho-c-Jun (Ser73), phospho-ERK and p38 antibodies, and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA).

Cell culture. T84 cells were grown in Dulbecco’s modified Eagle’s/F-12 media (Sigma) supplemented with 5% fetal calf serum (HyClone, Logan, UT), 1% penicillin/streptomycin, and 1% glutamine, with medium changes every 3 days. Cells were routinely passaged when ~90% confluent by trypsinization. Approximately 106 cells were seeded onto 30-mm Millicell Transwell inserts (Millipore, Bedford, MA) for Western blotting experiments and 5 × 105 cells onto 12-mm Millicell-HA Transwells for Ussing chamber experiments. Cells were cultured on inserts for ~10–15 days before use, and experiments were performed when transepithelial resistance reached 1,000–2,000 Ω·cm2. Under these conditions, T84 cells develop the polarized phenotype of native epithelial cells and are widely considered to be among the best models for reductionist studies of epithelial secretion presently available.

Western blotting. T84 cells were grown on permselective filter supports as described above. Monolayers were washed three times in Ringer solution (comprised of (in mM): 140 Na+, 5.2 K+, 1.2 Ca2+, 0.8 Mg2+, 120 Cl−, 25 HCO3−, 2.4 H2PO4−, 0.4 HPO42−, and 10 glucose) and allowed to equilibrate for 30 min at 37°C. Cells were then treated with agonists (with or without inhibitors) as described in figure legends, and reactions were terminated by washing in ice-cold Ringer solution. The cells were lysed in ice-cold lysis buffer containing 1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain, 100 μg/ml PMSF, 1 mM Na+, vadate, 1 mM NaF, and 1 mM EDTA) for 35 min at 4°C. Cells were scraped into
Eppendorf tubes and centrifuged for 10 min at 15,300 g, and the pellet was discarded. Samples were assayed for protein content using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) and adjusted so they contained equal amounts of protein. Gel loading buffer (2×) containing 50 mM Tris·HCl, 100 mM DTT, 40% glycerol, and 4% SDS was added to each sample, and, after being heated at 95°C for 5 min, samples were separated by SDS-PAGE. Separated proteins were then transferred to PVDF membrane, after which membranes were preblocked using 5% dried skimmed milk (Marvel) for 1 h. Membranes were then incubated with the appropriate dilution of primary antibody in 5% bovine serum albumin overnight at 4°C. This was followed by washing (×5) in Tris-buffered saline with 1% Tween (TBST) followed by incubation with HRP-conjugated secondary antibody for 1 h at room temperature. After further washing (×5) in TBST, immunoreactive proteins were detected using an enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK) and were quantified by densitometry. Equal loading of protein was routinely confirmed by stripping and reprobing blots for β-actin.

Electrophysiological studies. T84 cells were grown on Millicell-HA supports as described above. Cell monolayers were mounted in Ussing chambers (aperture = 0.6 cm²) and bathed in oxygenated Ringer solution at 37°C. The cells were voltage clamped to zero potential difference and monitored for changes in short-circuit current (Î�sc) using EVC 4000 Precision V/I Clamps (Physiological Instruments, San Diego, CA). Under these conditions, changes in Ï�sc are wholly reflective of changes in basolateral K⁺ conductance (Iₖ). The activity of the Na+/K⁺ ATPase was measured on the basis of changes in Ï�sc stimulated with CCh alone (z) and CCh stimulated with CCh and agonist, as a prototypical GqPCR agonist. Cells grown on permeable supports were stimulated basolaterally with CCh (100 μM) for periods ranging from 1–30 min. Cell lysates were then analyzed by Western blotting with an antibody specific for the phosphorylated form of JNK. These experiments revealed that CCh induced phosphorylation of both 46-kDa and 54-kDa isoforms of JNK (Fig. 1, A and B). Phosphorylation of p46 JNK was maximal within ~5 min, whereas p54 JNK phosphorylation was slower in onset with maximal responses occurring after 30-min stimulation. In further experiments, we analyzed the effects of TG, an agent that elevates intracellular Ca²⁺ by inhibiting the endoplasmic reticulum Ca²⁺-ATPase. We found that treatment of T84 cell monolayers with TG (2 μM) mimicked the effects of CCh on JNK phosphorylation, suggesting that elevations in intracellular Ca²⁺ alone are sufficient to induce activation of JNK in colonic epithelial cells.

JNK MAPK negatively regulates Ca²⁺-dependent secretory responses in colonic epithelial cells. We next determined whether stimulation of JNK activity by Ca²⁺-dependent secretagogues has implications for the secretory responses evoked by 10.220.33.1 on June 25, 2017 http://ajpgi.physiology.org/ Downloaded from

RESULTS

GqPCR agonists stimulate JNK activation in colonic epithelial cells. We first set out to determine whether agonists that act at GqPCRs stimulate JNK activation in colonic epithelial cells. For these experiments we used CCh, a M₃ muscarinic receptor agonist, as a prototypical GqPCR agonist. Cells grown on permeable supports were stimulated basolaterally with CCh (100 μM) for periods ranging from 1–30 min. Cell lysates were analyzed by Western blotting with antibodies against phospho c-Jun, JNK, ERK, and p38 MAPK. A: representative blot showing that SP600125 inhibits CCh-stimulated c-Jun phosphorylation in T84 cells. T84 cells were treated bilaterally with SP600125 (20 nM-20 μM) for 30 min before basolateral stimulation with CCh (100 μM; 10 min). Lysates were analyzed by Western blotting with antibodies against phospho c-Jun, JNK, ERK, and p38 MAPK. A: representative blot showing that SP600125 inhibits CCh-induced c-Jun phosphorylation in a concentration-dependent manner. B: densitometric analysis of 4 similar experiments. Asterisks denote significant differences from control cells (**p < 0.01); #significant differences from cells stimulated with CCh alone (#p < 0.05). C: representative blots demonstrating that SP600125 does not inhibit CCh-induced JNK, ERK, or p38 phosphorylation (blots are representative 5 separate experiments).
by these agonists. SP600125 was used as a selective inhibitor of JNK, and we analyzed e-Jun phosphorylation as a measure of JNK activity. Bilateral pretreatment of T84 cell monolayers with varying concentrations of SP600125 (20 nM-20 μM) inhibited CCh-stimulated e-Jun phosphorylation with maximal inhibition occurring at 2 μM (Fig. 2, A and B). However, SP600125 did not inhibit CCh-induced JNK phosphorylation, nor did it inhibit CCh-stimulated ERK or p38 MAPK phosphorylation (Fig. 2C).

To determine whether JNK plays a role in regulation of Ca2+-dependent Cl− secretion, the effect of SP600125 on Isc responses to CCh and TG across voltage-clamped monolayers of T84 cells mounted in Ussing chambers was examined. Pretreatment of T84 cells with SP600125 (2 μM) significantly potentiated Isc responses to both CCh and TG. The maximal response to CCh in SP600125 pretreated cells was 54.2 ± 7.2 μA/cm² compared with 28.9 ± 3.5 μA/cm² in control cells (n = 14, P < 0.01) (Fig. 3A). The maximal response to TG in SP600125-pretreated cells was 44.0 ± 5.5 μA/cm² compared with 28.5 ± 2.4 μA/cm² in paired controls (n = 6; P < 0.05) (Fig. 3B). The effects of SP600125 (200 nM-20 μM) on Isc responses to CCh occurred with a similar concentration dependence to its effects on JNK phosphorylation (Fig. 3C). An inactive analog of SP600125, the negative control JNK II inhibitor (2 μM), did not alter Isc responses to CCh (Fig. 3D). Responses to CCh in the presence of the negative control JNK II inhibitor were 34.6 ± 7.8 μA/cm² compared with 33.3 ± 8.2 μA/cm² in control cells (n = 5).

JNK MAPK does not regulate cAMP-dependent chloride secretion in colonic epithelial cells. The possible role of JNK in regulation of cAMP-dependent secretory responses was examined in cells treated with the adenylate cyclase activator, FSK, to specifically elevate intracellular cAMP. First, we determined the effects of FSK on JNK phosphorylation. Cells were stimulated bilaterally with FSK (10 μM) for periods ranging from 1 to 30 min, and cell lysates were analyzed for JNK phosphorylation by Western blotting. In contrast to Ca2+-dependent agonists, FSK did not stimulate phosphorylation of JNK (Fig. 4, A and B) although it did effectively stimulate ERK MAPK phosphorylation in the same samples (data not shown). Furthermore, pretreatment of voltage-clamped T84 cells with SP600125 (2 μM) did not alter subsequent Isc responses to FSK (Fig. 4C). Maximal responses to FSK in SP600125-pretreated cells were 55.0 ± 4.7 μA/cm² compared with 67.1 ± 4.6 μA/cm² in control cells (n = 8). These results indicate that JNK is not involved as a signaling intermediate in regulation of cAMP-dependent Cl− secretion in colonic epithelial cells.

CCh-induced JNK phosphorylation is mediated by EGFR transactivation. Previous work from this group has demonstrated that Ca2+-dependent Cl− secretion is limited by a signaling pathway mediated by transactivation of the EGFR (22). Because our present data indicate that JNK also exerts a negative influence on Ca2+-dependent secretion, we investigated whether JNK activation is related to the EGFR-mediated signaling pathway previously described. T84 cells were pretreated bilaterally for 10 min with the EGFR inhibitor, tyrphostin AG1478 (1 μM), before basolateral stimulation with CCh (100 μM). Western blotting showed that pretreatment with the EGFR inhibitor significantly attenuated CCh-induced JNK phosphorylation (Fig. 5, A and B). Furthermore, pretreatment of voltage-clamped T84 cells with a combination of both PD 98059 and SP 600125, to simultaneously inhibit ERK and JNK MAPK, did not have an additive effect in potentiating Isc responses to CCh. Responses to CCh in SP600125- and PD98059-pretreated cells were increased by 70.8 ± 14.5% and 121 ± 15.7%, respectively, relative to controls (Fig. 5C). Responses to CCh in presence of both inhibitors increased by 130.4 ± 23.4% compared with controls (n = 5). This was not significantly greater than the Isc responses elicited in the cells pretreated with PD98059 alone. These data suggest that JNK and ERK MAPK share a common signaling pathway to down-regulate Ca2+-dependent Cl− secretion.

Fig. 3. SP600125 potentiates calcium-dependent chloride secretion in T84 cells. Bilateral pretreatment of T84 cells for 10 min with SP600125 (2 μM, ○) significantly potentiated subsequent short-circuit current (Isc) responses to stimulation with CCh (100 μM) (n = 6) (A) and TG (2 μM) (n = 6) (B) compared with controls (●). T84 cells were pretreated with varying concentrations of SP600125 (200 nM-20 μM) for 30 min before addition of CCh (n = 6 for each concentration tested). D: T84 cells were pretreated bilaterally with either SP600125 (2 μM) or its inactive analog, the JNK inhibitor II negative control (2 μM) before stimulation with CCh (100 μM). Significant differences from cells stimulated with CCh alone are indicated (*P < 0.05; n = 5).
regulate Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} secretion in colonic epithelial cells. JNK inhibits basolateral K\textsuperscript{+} conductance in T84 cells. Recycling of K\textsuperscript{+} through channels in the basolateral membrane is a rate limiting step for Ca\textsuperscript{2+}-dependent secretory responses (4). We therefore examined whether JNK exerts its influence on epithelial secretory responses through modulation of basolateral K\textsuperscript{+} channels. Monolayers of T84 cells were mounted in Ussing chambers, a K\textsuperscript{+} gradient was applied in the apical to basolateral direction, and the basolateral membrane was electrically isolated by permeabilizing the apical membrane with amphotericin B (50 μM) (Fig. 6). Under these conditions, I\textsubscript{sc} responses to CCh are reflective of basolateral K\textsuperscript{+} conductance (I\textsubscript{k}). We found that pretreatment of T84 cells with SP600125 (2 μM) significantly potentiated subsequent I\textsubscript{k} responses to CCh, with maximal responses in SP600125-pretreated cells being 148 ± 13.0 μA/cm\textsuperscript{2} compared with 97.5 ± 8.0 μA/cm\textsuperscript{2} in control cells (n = 9; *P < 0.05). Furthermore, the effects of SP600125 appear to be specific for K\textsuperscript{+} channels because, in further experiments, we found that the JNK inhibitor had no effect on CCh-stimulated Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity. In similar experiments, we found that SP600125 also potentiated I\textsubscript{k} responses to TG (2 μM) by 50.7 ± 3.6% (n = 5; P < 0.05) but did not alter TG-induced Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity (data not shown).

DISCUSSION

MAPKs are emerging as critical regulators of epithelial transport function. Although we have previously demonstrated antisecretory roles for ERK and p38 MAPK in regulating responses to neuroimmune agonists of G\textsubscript{q}PCRs (21, 22), other laboratories have demonstrated that MAPKs regulate both the

Fig. 5. CCh-induced JNK activation is mediated by epidermal growth factor receptor (EGFR) transactivation in T84 cells. A: representative blot showing the time course of JNK MAPK phosphorylation at various times after treatment with forskolin (FSK) (10 μM). B: densitometric analysis of 3 similar experiments where data are expressed as means ± SE increases in JNK phosphorylation. C: bilateral pretreatment of voltage-clamped T84 cells with SP600125 (○) did not significantly alter subsequent I\textsubscript{k} responses to bilateral stimulation with FSK (10 μM) compared with controls (●). Data are expressed as means ± SE increases in I\textsubscript{k} (ΔI\textsubscript{k}) induced by FSK addition as shown (n = 6).

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DISCUSSION

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activity and expression of transport proteins in a variety of experimental settings (2, 5, 17, 32, 39, 40). Although JNK MAPK is known to be expressed in intestinal epithelial cells where it plays an important role in regulating cell survival (6, 30, 36, 41, 42), to date there is little known of its role in regulating epithelial transport. Our present data demonstrate that activation of GqPCRs rapidly stimulates JNK phosphorylation in cultured monolayers of epithelial cells with a time course that temporally correlates with the resolution of GqPCR-stimulated secretory responses. To investigate the potential role for JNK in regulating secretory responses we employed SP600125, a reversible ATP-competitive inhibitor that displayed high selectivity for JNK over other MAPKs (7). SP600125 was found to be an effective and specific inhibitor of JNK in our cell model because it potently reduced CCh-induced phosphorylation of its substrate, c-Jun, whereas it did not alter phosphorylation of JNK itself or of ERK or p38 MAPK. We found that pretreatment of voltage-clamped cells with SP600125 resulted in a potentiation of subsequent secretory responses to CCh compared with control cells. Again, this effect of SP600125 was specific because a structurally related compound, the JNK II negative control, was without effect. Together, these data indicate that agonists acting at intestinal epithelial GqPCRs have the ability to activate JNK MAPK and that this, in turn, exerts an antisecretory influence that downregulates the extent of secretory responses to such agonists.

Classically, GqPCR agonists are linked to a signaling cascade that leads to activation of phospholipase C, mobilization of IP3, and consequent release of intracellular Ca2+. Our present studies indicate that such increases in intracellular Ca2+ alone are sufficient to activate the JNK MAPK-mediated antisecretory mechanism in colonic epithelial cells. This is based on the observation that TG, an agent that specifically increases intracellular calcium by inhibiting its reuptake via the sarco-endoplasmic reticulum Ca2+-ATPase pump, mimicked the effects of CCh on JNK phosphorylation. Furthermore, we found that, similar to its effects on CCh-evoked responses, inhibition of JNK with SP600125 also significantly potentiated Cl− secretory responses to TG. Thus our data suggest that, at the same time that they induce Cl− secretion, agonist-stimulated increases in intracellular Ca2+ induce activation of the antisecretory JNK MAPK-dependent signaling mechanism.

Over the past decade the importance of receptor cross talk in regulating responses to GPCR agonists has become increasingly apparent. In many systems, transactivation of growth factor receptors, particularly the EGFR, mediates GPCR-induced activation of both ERK and p38 MAPKs (15, 31). More recent studies suggest that, at least in some cell types, transactivation of the EGFR also underlies GPCR-induced activation of JNK MAPK (29, 33). Indeed, the data presented here are consistent with previous work from our group in which GqPCR-induced transactivation of the EGFR was identified as an antisecretory signaling mechanism in colonic epithelial cells (22). Our present studies suggest that JNK MAPK functions as a downstream component of this signaling pathway. This conclusion is based on the observations that CCh-induced JNK activation was attenuated in the presence of the EGFR inhibitor, tyrphostin AG1478. Furthermore, when cells were pretreated with both SP600125 and PD 98059 to simultaneously inhibit JNK and ERK MAPKs, respectively, secretory responses to CCh were not significantly different from those observed in cells treated with either SP600125 or PD 98059 alone. Taken together, these data suggest that GqPCR-induced JNK activation occurs downstream of EGFR activation and exerts its antisecretory effects through a pathway common with ERK MAPKs.

By maintaining membrane hyperpolarization, K+ efflux across the basolateral membrane is a rate-limiting step for epithelial secretory responses to Ca2+-dependent agonists. We therefore investigated whether JNK might regulate Ca2+-dependent Cl− secretion through an action on K+ conductances. Employing a well-established technique for analyzing K+ channel conductance in polarized cells, we found that, similar to its actions on transepithelial Cl− secretion, the JNK inhibitor, SP600125, potentiated both CCh and TG-induced K+ conductances. This suggests that JNK limits Ca2+-dependent Cl− secretion by inhibiting K+ recycling through basolateral channels. Although there is little information in the literature regarding regulation of K+ channels by JNK, it is worth noting that some studies have found that ERK MAPK can downregu-
late K+ channel activity in some cell types through direct phosphorylation of the channel (35). We aim in future work to determine whether JNK exerts its antisecretory effects by direct phosphorylation of KCNN4, the Ca2+-dependent K+ channel that supports Cl− secretion in intestinal epithelia (13), or whether it is an indirect effect mediated by downstream signaling intermediates.

Neuroimmune agonists that act at GqPCRs are also important promoters of intestinal epithelial secretory responses. In our previous work, we have shown that such agonists, which act through elevations in intracellular cAMP, also induce transactivation of the EGFR in epithelial cells. However, in contrast to its role in limiting Ca2+-dependent signaling mechanisms in regulation of intestinal epithelial secretory responses, is a topic of ongoing investigation in our laboratory.

In summary, the present study identifies a novel role for JNK MAPK in regulation of intestinal epithelial secretory responses. Agonists acting through GqPCRs to elevate intracellular Ca2+ rapidly stimulate JNK activation, which, in turn, downregulates Cl− secretory responses to such agonists; rather this effect is mediated by phosphatidylinositol 3-kinase. In the present study, we determined whether JNK MAPK might also have a role to play in regulating cAMP-dependent secretory responses. However, we found that elevating levels of intracellular cAMP did not induce phosphorylation of JNK nor did JNK inhibition alter cAMP-mediated Isc responses. Thus our data suggest that JNK specifically regulates Ca2+ but not cAMP-dependent Cl− secretion in T84 colonic epithelial cells. How transactivation of the EGFR by cAMP- and Ca2+-dependent agonists leads to differential regulation of downstream signaling pathways, and ultimately Cl− secretory responses, is a topic of ongoing investigation in our laboratory.

DISCLOSURES

No conflicts of interest are declared by the author(s).

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