Protease-activated receptor-2 stimulates intestinal epithelial chloride transport through activation of PLC and selective PKC isoforms

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van der Merwe JQ, Moreau F, MacNaughton WK. Protease-activated receptor-2 stimulates intestinal epithelial chloride transport through activation of PLC and selective PKC isoforms. Am J Physiol Gastrointest Liver Physiol 296: G1258–G1266, 2009. First published April 9, 2009; doi:10.1152/ajpgi.90425.2008.—Serine proteases play important physiological roles through their activity at G protein-coupled protease-activated receptors (PARs). We examined the roles that specific phospholipase (PL) C and protein kinase (PK) C (PKC) isoforms play in the regulation of PAR2-stimulated chloride secretion in intestinal epithelial cells. Confluent SCBN epithelial monolayers were grown on Snapwell supports and mounted in modified Ussing chambers. Short-circuit current (Isc) responses to basolateral application of the selective PAR2 activating peptide, SLIGRL-NH2, were monitored as a measure of net electrogenic ion transport caused by PAR2 activation. SLIGRL-NH2 induced a transient Isc response that was significantly reduced by inhibitors of PLC (U73122), phosphoinositol-PLC (ET-18), phosphatidylcholine-PLC (D609), and phosphatidylinositol 3-kinase (PI3K; LY294002). Immunoblot analysis revealed the phosphorylation of both PLCβ and PLCγ following PAR2 activation. Pretreatment of the cells with inhibitors of PKC (GF 109203X), PKCε/βI (Go 6976), and PKCβ (rottlerin), but not PKCζ (selective pseudosubstrate inhibitor), also attenuated this response. Cellular fractionation and immunoblot analysis, as well as confocal immunocytochemistry, revealed increases of PKCβ1, PKCδ, and PKCε, but not PKCα or PKCζ, in membrane fractions following PAR2 activation. Pretreatment of the cells with U73122, ET-18, or D609 inhibited PKC activation. Inhibition of PI3K activity only prevented PKCδ translocation. Immunoblots revealed that PAR2 activation induced phosphorylation of both cRaf and ERK1/2 via PKCδ. Inhibition of PKCβ1 and PI3K had only a partial effect on this response. We conclude that basolateral PAR2-induced chloride secretion involves activation of PKCβ1 and PKCδ via a PLC-dependent mechanism resulting in the stimulation of cRaf and ERK1/2 signaling.

ion transport; proteases; signal transduction; epidermal growth factor receptor; cRaf

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The intestinal epithelium provides a variety of important functions including the absorption of nutrients and electrolytes and the secretion of water (22). Along with its absorptive and secretory functions, the intestinal epithelium also provides an important defensive barrier to potentially harmful luminal constituents. The secretion of chloride ions by a subpopulation of epithelial cells provides the driving force for the apically directed movement of water across the epithelium, thereby contributing to epithelial barrier function by flushing the lumen of unwanted pathogens (5, 26). Impaired barrier function, including hyporesponsiveness to secretagogues, can lead to the translocation of bacteria from the lumen to the underlying mucosa and is thought to contribute to the development of inflammatory bowel disease (3, 18, 31, 42). It is, therefore, important to understand the mechanisms governing epithelial chloride transport in both health and disease.

Serine proteases can be activated by a number of different serine proteinases including thrombin, trypsin, mast cell tryptase, kallikreins, and the tissue factor/VIIa/Xa complex (28, 44, 45). Synthetic activating peptides that correspond to the tethered ligand sequence can be used experimentally to activate the receptor in the absence of receptor cleavage. These include SLIGRL-NH2 and 2-furoyl-LIGRLO-NH2, which selectively activate PAR2 (30, 41).

PAR2 has a wide distribution throughout the gastrointestinal tract, including on enterocytes (6, 15, 43). We previously reported that basolateral PAR2 activation in intestinal epithelial cells induces a chloride secretory response that is dependent on epidermal growth factor receptor (EGFr) transactivation, MAP kinase signaling, calcium, and protein kinase A (PKA) (53). Our preliminary experiments also demonstrated a potential role for protein kinase C (PKC) in this response (53). PKC is a family of serine-threonine kinases that can be subdivided into conventional PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC) isoforms on the basis of specific structural features and requirements for activation (17). Previous studies have shown that PAR2 can signal via PKCε to sensitize TRPV1 channels in HEK293 cells and rat DRG neurons (1). Furthermore, PAR2-induced chloride secretion in Calu-3 cells involved PKC and phosphatidylinositol-phospholipase C (PC-PLC) signaling (48). Interestingly, a number of studies have demonstrated opposing roles for PKC in terms of intestinal epithelial ion transport. Some reports have suggested that PKC can enhance ion transport via the phosphorylation of CFTR, thereby enhancing its activation to PKA, or by transiently increasing the activity of the basolateral NKCC1 (11, 12, 21). Conversely, others reported that PKC may in fact downregulate chloride secretory responses by promoting the internalization of NKCC1 and the inhibition of apical membrane targeting of CFTR (7, 16). The discrepancies appear to lie in the cell types or tissues used, the specific PKC isoforms involved, and the time course of the experiments.

The roles of specific PKC isoforms in PAR2-induced intestinal epithelial chloride transport are not known. We explored this issue in the chloride-secreting SCBN epithelial cell line
and demonstrated that basolateral PAR$_2$-induced chloride secretion involves PI-PLC, PC-PLC, PKCβI, and PKCδ signaling, leading to subsequent activation of MAP kinase activity.

**MATERIALS AND METHODS**

**Cell culture.** The nontransformed canine intestinal epithelial cell line SCBN (10) was used for ion transport studies as previously described (8, 9). Cells between passages 20 and 30 were grown to confluence either in flasks (75 cm$^2$) or on semipermeable Snapwell supports (Costar, Nepean, ON, Canada). Cells grown in flasks were passaged using 1.5X trypsin and fed every second day with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1-glutamine, streptomycin, Plasmocin, and tylosin. Similarly, cells grown on either Snapwell or Transwell supports were fed every second day and confluence was determined by light microscopy or an electrophotometer (EVOM, World Precision Instruments, Sarasota, FL). Only monolayers having a resistance of >500 Ω/cm$^2$ were used for Ussing chamber experiments.

**Ion transport.** SCBN cells were grown to confluence on Snapwell supports and mounted in modified Ussing chambers. The cells were bathed with modified Krebs buffer (115 mM NaCl, 2.0 mM KH$_2$PO$_4$, 2.4 mM MgCl$_2$, 1.3 mM CaCl$_2$, 25.0 mM NaHCO$_3$, and 8.0 mM KCl) on both the apical and basolateral sides of the chamber. Krebs buffer was maintained at 37°C and pH 7.4 and contained 10 mM mannitol on the apical side and 10 mM glucose on the basolateral side. Buffers were maintained at 37°C and pH 7.4 and were continuously gassed with 5% CO$_2$ and 95% O$_2$. The transepithelial potential difference was clamped to zero volts by use of a voltage clamp apparatus (VCC MC8, Physiologic Instruments, San Diego, CA), and changes in short-circuit current ($I_s$) were monitored as an indicator of net electrogenic ion transport. The $I_s$ was recorded with a digital data acquisition system (MP100, BioPac, Goleta, CA) and analyzed via AcqKnowledge software (version 3.5.7, BioPac).

To assess the role of PLC and phosphoinositide 3-kinase (PI3K) in the PAR$_2$-induced chloride secretory response, SCBN monolayers were pretreated basolaterally with inhibitors that block both phosphoinositol (PI)- and phosphatidylcholine (PC)-PLC (U73122, 10 µM), or each isoform specifically (PC-PLC, D609; PI-PLC, ET-18; both at 10 µM), or with an inhibitor of PI3K (LY294002, 50 µM). Each monolayer was exposed to only one inhibitor. Following a 15-min incubation with inhibitor, monolayers were exposed basolaterally to SLIGRL-NH$_2$ (50 µM) and the change in $I_s$ was measured. The vehicle, DMSO (1:1,000), was used as a control.

To determine a role for specific PKC isoforms in PAR$_2$-induced $I_s$ changes, confluent SCBN monolayers were mounted in Ussing chambers and pretreated basolaterally for 15 min with various selective PKC inhibitors prior to basolateral exposure to 50 µM SLIGRL-NH$_2$. The following inhibitors were used to determine the involvement of the different PKC isoforms: pan-PKC, GF109203X (GFX, 1 µM); PKCa/βI inhibitor Gö6976 (0.2 µM); PKCδ inhibitor rottlerin (4 µM); and the PKCζ-pseudosubstrate inhibitor (Myr-SIYRRGARRWRKL-OH, 10 µM). The vehicle, DMSO (1:1,000), was used as a control.

In addition experiments, SCBN monolayers were exposed to the cRaf inhibitor GW5074 (100 nM) or the Akt activation inhibitor triciribine (10 µM) prior to exposure to SLIGRL-NH$_2$ (50 µM).

**Cellular fractionation and immunoblotting.** To confirm the physiological results obtained with the various pharmacological inhibitors, cellular fractionation and immunoblot analysis were conducted to determine the activation and cellular localization of specific PKC isoforms (PKCα, βII, δ, ε, ζ). SCBN cells were grown to confluence in 75-cm$^2$ flasks, serum starved for 2 h, and then resuspended in serum-free media. Cells were then exposed to SLIGRL-NH$_2$ (50 µM) or vehicle for 45 s, placed on ice, and rinsed with ice cold PBS to stop the reaction. Cell lysis buffer (50 mM NaCl, 500 mM sucrose, 10 mM HEPES, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, protease inhibitor cocktail) was added. Following a 20-min incubation in lysis buffer, cells were homogenized by use of a Dounce homogenizer and transferred to microcentrifuge tubes. The cells were centrifuged at 2,700 g for 2 min and the supernatant was collected. This supernatant was further centrifuged at 20,800 g for 30 min. The resulting supernatant collected as the cytosolic fraction and the pellet collected as the membrane fraction. The proteins were resolved by a 10% SDS-PAGE gradient gel and transferred to a nitrocellulose membrane. Following overnight exposure to the primary antibody at 4°C, blots were treated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. Bands were then visualized with enhanced chemiluminescence reagent (ECL, Amersham Pharmacia, Baie d’Urfé, QC, Canada) and quantified by densitometry using Quantity One software (Bio-Rad).

Activation of specific PKC isoforms was determined by immunoblot analysis of the cytosolic and membrane fractionation, immunoblot for the Na$^+-$K$^+$-ATPase (membrane control), rabbit polyclonal antibody (1:2,000, Cell Signaling, Danvers, MA), and GAPDH (cytosolic control), rabbit polyclonal antibody (1:1,000, Cell Signaling), and a HRP-conjugated goat anti-rabbit secondary antibody (1:12,500, Jackson ImmunoResearch Laboratories, West Grove, PA) were conducted. To confirm effective cytosolic and membrane fractionation, immunoblots for the Na$^+-$K$^+$-ATPase, rabbit polyclonal antibody (1:2,000, Cell Signaling) and a HRP-conjugated goat anti-rabbit secondary antibody (1:12,500, Jackson ImmunoResearch Laboratories) were used for all fractionation experiments.

To assess phosphorylation of PLCβ and PLCγ, whole cell lysates were collected following a 5-min exposure to SLIGRL-NH$_2$ (50 µM) or vehicle. Immunoblot analysis was conducted using a rabbit polyclonal antibody specific to phospho-PLCβ (1:1,000, Cell Signaling) and a HRP-conjugated goat anti-rabbit secondary antibody (1:10,000, Jackson ImmunoResearch Laboratories). Similarly for PLCγ, immunoblotting was conducted using a rabbit polyclonal antibody specific to phospho-PLCγ (1:1,000, Cell Signaling) and a HRP-conjugated secondary antibody (1:10,000, Jackson ImmunoResearch Laboratories).

To assess phosphorylation of PI3K, whole cell lysates were collected following a 5-min exposure to SLIGRL-NH$_2$ (50 µM) in the presence or absence of a selective EGFr inhibitor (PD153035, 1 µM, 15-min exposure). Immunoblot analysis was conducted using a rabbit polyclonal antibody specific to phospho-EGFr (1:1,000, Cell Signaling) and a HRP-conjugated goat anti-rabbit secondary antibody (1:10,000, Jackson ImmunoResearch Laboratories).

To determine the role of specific PKC isoforms in the phosphorylation of cRaf and ERK1/2, cells were pretreated for 15 min with various PKC inhibitors (GFX, rottlerin, or Gö6976) prior to SLIGRL-NH$_2$ (50 µM) exposure. To determine cRaf activation a rabbit monoclonal antibody specific to phospho-cRaf (Ser338, 1:500, Cell Signaling) and a HRP-conjugated goat anti-rabbit secondary antibody (1:1,000, Jackson ImmunoResearch Laboratories) were used. For detection of activated ERK1/2, a mouse monoclonal antibody specific to phospho-ERK1/2 (Thr202/Tyr204, 1:2,000, Cell Signaling) and a HRP-conjugated rabbit anti-mouse secondary antibody (1:10,000, Jackson ImmunoResearch Laboratories) were used. To verify protein loading, all membranes were then reprobed for total amounts of each protein using a rabbit polyclonal antibody to GAPDH (1:5,000, Jackson ImmunoResearch Laboratories).

**Immunofluorescence.** SCBN cells were grown to confluence on Transwell filters and serum-starved for 2 h. Cells were then pretreated with either vehicle or inhibitors of PLC (U73122, 10 µM), PC-PLC (D609, 10 µM), PI-PLC (ET-18, 10 µM), PI3K (LY294002, 50 µM), or EGFr (PD153035 1 µM) followed by basolateral exposure to SLIGRL-NH$_2$ (50 µM) for 40, 60, or 90 s. The cells were then rinsed with ice-cold PBS and fixed by use of −20°C methanol for 30 min. The cells were washed with PBS and blocked with 10% BSA for 2 h. Following an overnight incubation at 4°C with rabbit polyclonal antibody to phosphorylated cRaf (Ser338) (1:500, Cell Signaling) and a HRP-conjugated donkey anti-rabbit secondary antibody (1:5,000, Jackson ImmunoResearch Laboratories) the monolayers were incubated in Hoechst 33342 (1:1,000, Invitrogen) for 10 min at room temperature. The monolayers were washed three times with PBS and rinsed once in HEPES buffer (10 mM HEPES, 1 mM MgCl$_2$, 10 mM KCl). Images were obtained using a confocal microscope (Zeiss). To confirm specific PKC isoforms were present in vivo, immunofluorescence microscopy was conducted on the proximal tubule of the rat kidney. Immunofluorescence images were obtained from the renal cortex of five rats, and the results were averaged.

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**Statistical analysis.** Data are expressed as means ± SE. Statistical analyses were conducted using GraphPad Instat version 3.0 (GraphPad Software, San Diego, CA). Comparisons of two groups were made by Student’s t-test for unpaired data. Comparisons of more than two groups were made by a two-way analysis of variance with a post hoc Tukey test. An associated probability (P) value of 0.05 was considered significant.

**RESULTS**

PAR2 activation of ion transport: PLC and PKC. Activation of basolateral PAR2 in SCBN monolayers by 50 μM SLIGRL-NH2 resulted in a rapid (within 1 min) increase in I_e, indicative of chloride transport as previously described (53). Treatment of the cells with HEPES, the vehicle for SLIGRL-NH2, or with the reverse peptide LRGILS-NH2 had no effect on the I_e (data not shown).

Exposure of the cells to the pan-PLC inhibitor U73122 (10 μM) or selective inhibitors of PI-PLC and PC-PLC (ET-18, 10 μM, D609, 10 μM, respectively) all resulted in significantly reduced PAR2-mediated chloride secretion in response to SLIGRL-NH2 (Fig. 1A). To further substantiate these findings, immunoblots for phospho-PLCβ and phospho-PC-PLCγ were conducted. Activation of PAR2 by SLIGRL-NH2 resulted in significant increases in both PLCβ and PLCγ phosphorylation (Fig. 1B). These results suggest an important role for both PI-PLC and PC-PLC, specifically PLCβ and PLCγ signaling, during PAR2-mediated ion transport. Furthermore, pretreatment of the cells with a selective PI3K inhibitor Ly294002 (50 μM) resulted in significant inhibition of PAR2-mediated ion transport (Fig. 1A). In addition, an immunoblot for phospho-P13K was conducted (Fig. 1B). Interestingly, activation of PAR2 by 50 μM SLIGRL-NH2 resulted in a significant increase in P13K phosphorylation that was EGFr dependent, since P13K phosphorylation was significantly reduced by the EGFr tyrosine kinase inhibitor PD153035 (Fig. 1C).

Pretreatment of the cells with the pan-PKC inhibitor GFX 1 μM, the PKCa/β inhibitor rottlerin (4 μM), or the PKCa/β inhibitor Go6976 (0.2 μM), but not the PKCa/β pseudosubstrate inhibitor (10 μM), resulted in a significant reduction in PAR2-mediated chloride secretion (Fig. 2). These results suggest an important role for specific PKC isoforms in PAR2-mediated ion transport, including PKCδ, PKCa, and/or...
PKCβI. The role of PKCε could not be determined because of the lack of a cell-permeable PKCε inhibitor and the difficulty of transfecting with small interfering RNA in this particular cell line. None of the inhibitors used caused any change on $I_{sc}$ on their own (data not shown).

PAR$_2$-induced PKC trafficking. To further assess the role of specific PKC isozymes, cellular fractionation was conducted following PAR$_2$ activation by 50 $\mu$M SLIGRL-NH$_2$ or 2 $\mu$M PMA. Stimulation with SLIGRL-NH$_2$ or PMA for 45 s resulted in increased PKCβI, PKCδ, and PKCε detection in the membrane fractions compared with cells pretreated with the vehicle control (Fig. 3). PKCβII was not detected by immunofluorescence in the SCBN cell line (data not shown). Following cellular fractionation, immunoblots for Na$^+$/K$^+$-ATPase (membrane control) and GAPDH (cytosolic control) were conducted to demonstrate both equal protein loading and effective cytosolic and membrane separation (Fig. 3). Furthermore, PKCα and PKCζ appeared to be constitutively expressed at the membrane, and activation by SLIGRL-NH$_2$ did not further enhance this membrane localization (Fig. 3).

To further demonstrate specific PKC isoform trafficking following stimulation with SLIGRL-NH$_2$, immunofluorescence confocal microscopy was conducted. Immunofluorescence revealed that, following SLIGRL-NH$_2$ stimulation for either 40 or 60 s, PKCβI, PKCδ, and PKCε all translocated to the plasma membrane, indicating their activation (Fig. 4). The effect was transient since PKCβI, PKCδ, and PKCε appeared to be returning to baseline levels 90 s after exposure to SLIGRL-NH$_2$, although some membrane fluorescence was still evident at this time point (Fig. 4). Analysis of z-stack images from confocal microscopy showed that PKCβI seemed to translocate primarily to the apical membrane, whereas PKCδ and PKCε translocated to both the apical and basolateral membranes (Fig. 5). These results are indicative of the rapid and transient nature of PKC signaling in PAR$_2$-mediated ion transport. PKCα and PKCζ were not affected by stimulation with SLIGRL-NH$_2$, although, in concordance with the immunoblot studies, there did appear to be some staining at the cell membrane in control monolayers (Fig. 4).

To further determine the role of PLC and PI3K in PAR$_2$-induced PKC activation, immunofluorescence of PKC isoforms was conducted in the presence of the pan-PLC inhibitor U73122 (10 $\mu$M), the selective PI-PLC inhibitor ET-18 (10 $\mu$M), the selective PC-PLC inhibitor D609 (10 $\mu$M), the PI3K...
inhibitor LY294002 (50 μM), and the EGFr tyrosine kinase inhibitor PD153035 (1 μM). Inhibition of either PI-PLC or PC-PLC resulted in complete abrogation of PAR2-induced PKCα, PKCβ1, and PKCε translocation following SLIGRL-NH2 treatment (Fig. 6). Interestingly, PI3K inhibition only significantly inhibited PKCδ translocation and did not affect the SLIGRL-NH2-induced trafficking of either PKCβ1 or PKCε (Fig. 6). Furthermore, selective inhibition of the EGFr completely abolished PKCδ trafficking and only partially inhibited PKCβ1 and PKCε trafficking (Fig. 6). These results suggest that PAR2-induced PKCβ1 and PKCε activation primarily involves PI-PLC and PC-PLC activity, whereas the activation of PKCδ involves PI-PLC, PC-PLC, PI3K, and EGFr activity.

Role of cRaf and Akt activation. cRaf participates in the signaling pathway involving EGFr transactivation and MAP kinase activation. Pretreatment of SCBN cells with the cRaf inhibitor GW5074 (100 nM) significantly (P < 0.01) reduced the I_sc response to SLIGRL-NH2 (Fig. 7). In addition, since Akt has been implicated in epithelial responses to PAR2 activation (50), we assessed the effect of Akt in PAR2-induced chloride secretion. Pretreatment of SCBN cells with the Akt activation inhibitor triciribine (10 μM) did not affect the I_sc response to SLIGRL-NH2 (Fig. 7). Neither GW5074 nor triciribine affected I_sc on their own (data not shown).

MAP kinase activation. We have previously shown that PAR2-induced chloride secretion was dependent on the activation of ERK1/2 MAP kinase (53). To determine a role for cRaf and specific PKC isoforms in PAR2-induced MAP kinase activation, immunoblots for both phospho-cRaf and phospho-ERK1/2 were conducted. Pretreatment of the cells with inhibitors of PKCα, PKCβ1, PKCδ, PI3K, or cRaf activation re-
sulted in a significant reduction in PAR₂-induced cRaf phosphorylation (Fig. 8A). Interestingly, however, only inhibitors of PKCζ or cRaf activation significantly reduced PAR₂-mediated increases in ERK1/2 phosphorylation (Fig. 8B). These results suggest that PAR₂-mediated PKCζ activation is playing an important role in ERK1/2 and cRaf phosphorylation. Importantly, both PKCβ and PI3K increase cRaf phosphorylation but do not appear to have any effect on ERK1/2 activity, suggesting the differential signaling of cRaf on the basis of its activation (Fig. 8B).

**DISCUSSION**

We have shown that basolateral PAR₂ activation in intestinal epithelial cell monolayers induces vectorial chloride transport via a PI-PLC- and PC-PLC-mediated mechanism that is dependent on PI3K, specific isoforms of PKC, cRaf, and subsequent activation of MAP kinase activity. PAR₂ has a wide distribution throughout the gastrointestinal tract and is expressed on both the apical and basolateral membranes of enterocytes (6, 15, 43). In a previous study, we demonstrated that basolateral PAR₂ activation in SCBN cells induces a chloride secretory response that is dependent on EGFr trans-activation, increases in intracellular calcium, and cAMP, PKA, and MAP kinase signaling (53). The PAR₂-induced chloride secretion is not peculiar to SCBN cells, since we showed that it is also observed in the human T84 colonic epithelial cell line (53). Preliminary experiments from our earlier study also revealed a potential role for PKC regulation of the PAR₂-induced secretory response (53). However, the specific roles of PLC, PI3K, and PKC isoforms regulating PAR₂-mediated chloride secretion remained unknown. The present study provides insight into the roles these kinases play in regulating PAR₂-induced chloride secretion in intestinal epithelial cells. The diverse signaling pathways involved are depicted in Fig. 9.

PLC consists of 13 isoforms divided into six main families based on structural and activation characteristics. They are activated by Gₐ or Gₐ/b subunits that result in the catalysis of the membrane substrate phosphatidyl inositol 4,5-bisphosphate (PIP₂) to generate inositol 1,4,5-trisphosphate and diacylglycerol (DAG). The PI-PLC family members PLCβ and PLCγ are important in the generation of an intracellular calcium response and production of DAG (46), both of which are important in the activation of PKCβ (Ca²⁺ and DAG) and PKCζ (DAG) (17). The activation of PC-PLC on the other hand primarily generates a DAG response (19). We demonstrated that both PI-PLC and PC-PLC are required for basolateral PAR₂-induced chloride secretory responses. Furthermore, we demonstrated a role for EGFr-mediated PI3K regulation of PAR₂-mediated chloride secretion since inhibition of EGFr reduced PI3K phosphorylation to a level seen in the controls. PI3K activity results in the generation of PIP3 from membrane lipids and the subsequent activation of a number of signaling complexes, some of which include Akt/PKB, PLCγ, PDK1, and PKC (25).

PKC is a family of serine-threonine kinases that are activated by calcium, DAG, or protein-protein interactions, depending on the given PKC isoform (17). The PKC family is divided into three main subfamilies, cPKC, nPKC, and aPKC isoforms, based on their structural and activation characteristics. For our studies, we chose to determine a role for PKCs α, βI/II, δ, ε, and ζ since these have been demonstrated to play a role in PAR₂-induced signaling events, ion transport, and epithelial barrier function (1, 11–13, 20, 24, 48). We demonstrated using pharmacological inhibitors, membrane fractionation, and confocal immunofluorescence mi-

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**Fig. 6.** PAR₂-induced PKC translocation is mediated by PLC, PI3K, and EGFr dependent. Immunofluorescence for specific PKC isoforms (PKCζ, βI, βII, δ, ε, ζ) was conducted following pretreatment of the SCBN cells with inhibitors of PLC (U73122), PI-PLC (ET-18), PC-PLC (D609), PI3K (LY294002), or EGFr (PD153035) and brief basolateral PAR₂ activation (60 s) by 50 μM SLIGRL-NH₂. Representative of n = 3–4 per group.

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**Fig. 7.** PAR₂-induced change in is dependent on cRaf but not Akt. SCNB monolayers were mounted in Ussing chambers and pretreated with the cRaf inhibitor GW5074 (100 nM) or the Akt activation inhibitor triciribine (10 μM) prior to exposure to the PAR₂-activating peptide, SLIGRL-NH₂ (50 μM). **p < 0.01 vs. control group exposed to SLIGRL-NH₂; n = 4 per group.
crosscopy that PKC activity regulates PAR2-induced chloride secretion and that PKCβ1 and PKCδ are the dominant isoforms regulating this response. Activation of these isoforms of PKC was assessed by determining the cytosolic to membrane translocation of immunoreactive PKC. Effective cell fractionation was demonstrated by clear separation of the cytosolic marker, GAPDH, and the membrane marker, Na⁺/H⁺-ATPase. Interestingly, membrane fractionation showed a PAR2-induced increase in membrane PKCδ and PKCβ1, without a significant concomitant decrease in cytosolic amounts of these isoforms. In contrast, confocal microscopy revealed a decrease in cytosolic PKC when membrane PKC immunoreactivity was increased. The explanation for these apparently discrepant results could lie in technical differences in the two experiments. First, cell fractionation was conducted on cells grown on plastic, whereas confocal microscopy was conducted on cells grown on Transwell filters. Second, even though there was more total protein in the cytosolic than membrane fractions, equal amounts of protein from each fraction were loaded onto gels for Western blot. Thus changes in cytosolic concentrations of PKC might have been underestimated because of the relatively large amount of protein present compared with the membrane fractions. Nevertheless, both techniques show increased PKC translocation to the membrane, indicative of increased activity, following PAR2 activation.

Our PKC results concur with previous studies that have demonstrated a role for PKC in epithelial chloride secretion (11, 12, 21). However, conflicting reports have suggested that PKC may either enhance or reduce chloride secretory responses (7, 11, 12, 16, 21). In the lung epithelial Calu-3 cell line, PKC was demonstrated to play a role in the desensitization of PC-PLC signaling involved in PAR2-induced chloride secretion (48). In this same cell line, however, it has also been demonstrated that PKCδ can directly associate with actin that can then serve as a scaffold to upregulate the activity of NKCC1 (37-39), which is required for epithelial chloride secretion (5). PKCε on the other hand has been demonstrated to reduce NKCC1 activity and induce its internalization following exposure to phorbol esters (16). In human gall bladder epithelium, bile salt-induced chloride secretion involves a PKC-
mediated potentiation of adenylyl cyclase activity and cAMP responses (13). Similarly, in the adult rabbit distal colon, the bile acid taurodeoxycholate was demonstrated to induce a chloride secretory response dependent on PKC-ε (29).

In support of these findings, we have shown that both PKC-ε translocation and signaling are required for PAR2-mediated chloride secretion in SCBN cell monolayers. We further demonstrated that PAR2-induced PKC-ε translocation involves EGFr-dependent activation of PI3K. The EGFr, however, appears to play a minor role in the activation of PKC-β1 and PKC-ε since inhibition of EGFr tyrosine kinase activity had a minimal effect on PAR2-induced PKC-β1 and PKC-ε trafficking. It has been previously reported that PI3K activation of PDK1 can result in the phosphorylation and, hence, increased activity of PKC-ε in human embryonic kidney cells (34). Therefore, PKC-ε could potentially regulate PKC-ε via PDK1 signaling in SCBN cells following PAR2 activation. In the murine colon it has been demonstrated that PKC-β1 can upregulate cell surface expression of CFTR, thereby enhancing chloride secretion (51, 52). Interestingly, in Calu-3 epithelial cells downregulation or inhibition of PKC-ε attenuated cAMP-induced CFTR activation (36). The same group has more recently demonstrated that in Calu-3 cells PKC-ε interacts with CFTR via a RACK1-NHERF1 protein complex to regulate its activity and expression (4). We have demonstrated that PAR2 activation results in PKC-ε translocation, indicative of its activation. However, we were unable to ascertain its direct role in PAR2-mediated chloride secretion because of experimental constraints as described in MATERIALS AND METHODS. Therefore, given our own studies demonstrating a role for cAMP, PKA, and CFTR in PAR2-mediated chloride secretion (53), and those by others, PKC-ε could potentially regulate PAR2-mediated chloride secretion via interactions with CFTR or NKKC1. Further studies are required to determine the role for PKC in the regulation of CFTR and NKKC1 activity following PAR2 stimulation in intestinal epithelial cells.

In our previous studies in SCBN cells, we demonstrated that PAR2-induced chloride secretion involved ERK 1/2 MAP kinase activity, in addition to increases in intracellular calcium and PKC activity (53). Therefore, we sought to determine a role for select PKC isoforms in the activation of MAP kinase signaling induced by PAR2 activation. In this study, we have demonstrated that the activation of PKC-ε involves EGFr-dependent PI3K activation. Furthermore, we have shown that PAR2 activation results in both cRaf and ERK1/2 activation involving PKC-ε. A number of studies have demonstrated similar results, suggesting a role for PKC-ε in the activation of Raf-MAPK signaling events (2, 23, 32, 33, 49). However, few studies have examined PKC in terms of MAP kinase signaling in intestinal epithelia, and to our knowledge this is the first study to demonstrate the role for specific PKC isoform activation of cRaf-ERK1/2 signaling in the regulation of chloride secretion in intestinal epithelial cells. Interestingly, PKC-β1 and PI3K appeared to be involved in cRaf phosphorylation but not in the activation of ERK1/2, suggesting the potential for the differential signaling of cRaf on the basis of its activation by different kinases and protein interactions. Indeed, previous studies have demonstrated multiple phosphorylation sites on cRaf that are important in its activation and inactivation. Therefore, the kinase-dependent and independent effects of cRaf could potentially regulate multiple signaling pathways (35, 54). It is, therefore, possible that PKC-β1 and PI3K regulate cRaf activity resulting in ERK-independent signaling events, whereas PKC-ε regulation of cRaf kinase activity results in the stimulation of ERK1/2 activity. Previous studies have demonstrated that PI3K activation of Akt can result in a Akt-Raf inhibitory complex formation and the prevention of ERK1/2 activation (47). This is unlikely to be the case in our studies, since cRaf appeared to be involved in stimulation, rather than inhibition, of ERK1/2 activation, and the Akt activation inhibitor triciribine failed to affect PAR2-induced changes in I_sc.

In considering our previous studies (53) together with the present data, we have uncovered an interesting relationship among cRaf, EGFr activation, and ERK1/2 activity. We previously showed that PAR2 activated cRaf in an apparently EGFr-independent manner, but we showed no link between cRaf activation and chloride secretion in that study (53). In the present study, we showed that cRaf activation does, indeed, play a role in PAR2-induced ERK activation and chloride secretion. It may be, given the data from these two studies, that there are EGFr-dependent and independent mechanisms underlying ERK activation, with the ERK-independent mechanism involving cRaf. These pathways may be related, since inhibition of EGFr with PD153035 and of cRaf with GW5074 both almost completely block ERK activation and chloride secretion.

Taken together, these results suggest that a complex network of signaling pathways, regulated by several specific PKC isoforms, is important in PAR2-mediated chloride secretory responses in intestinal epithelia. Given the role of epithelial barrier dysfunction in inflammatory bowel disease, and the important contribution that chloride and water transport make to overall barrier function, an understanding of the mechanisms of serine proteinase-induced ion transport is of clinical relevance.

GRANTS

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