Role of PKC-δ on substance P-induced chemokine synthesis in pancreatic acinar cells

Raina Devi Ramnath, Jia Sun, Sharmila Adhikari, Liang Zhi, and Madhav Bhatia

Department of Pharmacology, National University of Singapore, Singapore

Submitted 13 August 2007; accepted in final form 17 December 2007

Role of PKC-δ on substance P-induced chemokine synthesis in pancreatic acinar cells. Am J Physiol Cell Physiol 294: C683–C692, 2008. First published December 26, 2007; doi:10.1152/ajpcell.00360.2007.—Interaction of the neuropeptide substance P (SP) with its high-affinity neurokinin-1 receptor (NK1R) plays an important role in the pathophysiology of acute pancreatitis. SP is known to stimulate the production of chemokines monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, and MIP-2 in pancreatic acinar cells via the activation of NF-κB. However, the signaling mechanisms by which the SP-NK1R interaction induces NF-κB activation and chemokine production remain unclear. To that end, in the present study, we investigated the participation of PKC in SP-induced chemokine production in pancreatic acinar cells. In this study, we showed that SP stimulated an early phosphorylation of PKC isoform PKC-δ followed by increased activation of MAPKKK MEKK1 and MAPK-ERK and JNK as well as transcription factor NF-κB and activator protein-1 driven chemokine production. Depletion of PKC-δ with its inhibitor rottlerin or the specific PKC-δ translocation inhibitor peptide dose dependently decreased SP-induced PKC-δ, MEKK1, ERK, JNK, NF-κB, and AP-1 activation. Moreover, rottlerin as well as PKC-δ translocation inhibitor inhibited SP-induced chemokine production in a concentration-dependent manner. We also demonstrated that PKC-δ activation was attenuated by CP96345, a selective NK1R antagonist, thus showing that PKC-δ activation was indeed mediated by SP in pancreatic acinar cells. These results show that PKC-δ is an important proinflammatory signal transducer for SP-NK1R-induced chemokine production in pancreatic acinar cells.

protein kinase C-δ; mitogen-activated protein kinase kinase kinase-1; extracellular signal-regulated kinase; c-Jun NH2-terminal kinase; nuclear factor-κB; activator protein-1

ACUTE PANCREATITIS is an inflammatory disorder. The activation of digestive enzymes within pancreatic acinar cells plays a crucial initiating event in acute pancreatitis, resulting in acinar cells damage and a localized inflammatory response (20, 43, 54). Secreted bioactive molecules from infiltrating leukocytes contribute to local damage and subsequently to the systemic inflammatory response, which may result in multiple organ dysfunction and ultimately in death (4). Several inflammatory mediators have been implicated in the recruitment of leukocytes into the pancreas (4, 19). Inflammatory mediators such as substance P (SP) and chemokines are known to play significant roles in the pathogenesis of acute pancreatitis (3, 6, 7).

SP is an 11-amino acid neuropeptide that is released from nerve endings in many tissues. It has been shown to play an important role in asthma, inflammatory bowel disease, arthritis, and other inflammatory processes (11, 52). Subsequent to its release from nerve endings, SP binds to its G protein-coupled receptor, the neurokinin-1 receptor (NK1R), on effector cells, increases microvascular permeability, and promotes plasma extravasation from the intravascular to extravascular space. Pancreatic acinar cells are known to express NK1R, and SP has been detected within the pancreas (40, 48). A study (7) has found that pancreatic levels of SP and the expression of NK1R on pancreatic acinar cells are increased during experimental acute pancreatitis. It has also been shown that genetic deletion of NK1R reduces the severity of pancreatitis and pancreatitis-associated lung injury (9). Furthermore, knockout mice deficient in the preprotachykinin-A gene, which codes for SP, are protected against acute pancreatitis and associated lung injury (9). These results suggest an important proinflammatory role for SP and NK1R in acute pancreatitis and associated lung injury. These results were further substantiated by the observation that knockout mice deficient in neutral endopeptidase, the enzyme that hydrolizes SP, thereby terminating its action, are more susceptible to acute pancreatitis and associated lung injury (8, 35). The exact mechanism by which SP contributes to the proinflammatory signaling in acute pancreatitis is not completely understood. In the present study, we used isolated pancreatic acinar cells, in which critical initiating events in acute pancreatitis take place, as our model to try to address the above concern.

The PKC family of proteins consists of 12 members of phospholipid-dependent serine/threonine kinases (15, 21, 22, 33, 44) that are involved in the regulation of cellular processes including growth, migration, and inflammatory responses (40). The PKC superfamily is classified into three subfamilies based on their domain structure and their ability to respond to Ca2+ and diacylglycerol (37). The three subfamilies are Ca2+-dependent conventional PKCs (α, β1, β2, and γ) as well as Ca2+-independent subgroups: novel PKCs (δ, ε, η, and θ) and atypical PKCs (ζ, λ, τ, and μ) (22, 33, 44). Phorbol ester activates all subgroups except the atypical subgroup. It has been demonstrated previously that PKC-δ may modulate inflammatory responses, as evidenced by its capability to induce matrix metalloproteinase and chemokine expression in vitro (23, 32).

However, little is known of the ability of the SP-NK1R interaction to activate PKC-δ and its effects on proinflammatory mediator chemokines in pancreatic acinar cells. In this study, we sought to investigate whether SP activates PKC-δ and also to determine the underlying signaling mechanism in the involvement of SP-NK1R-PKCδ in chemokine production in pancreatic acinar cells.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
**MATERIALS AND METHODS**

Preparation of mouse pancreatic acini. All animal experiments were approved by the Animal Ethics Committee of the National University of Singapore and carried out in accordance with established International Guiding Principles for Animal Research.

Pancreatic acini were obtained from the mouse pancreas by collagenase treatment as previously described (3). Briefly, pancreases from three Swiss mice (20–25 g) were removed, infused with buffer A [containing (in mM) 140 NaCl, 4.7 KCl, 1.13 MgCl2, 1 CaCl2, 10 glucose, and 10 HEPES, pH 7.2] containing 200 IU/ml collagenase and 0.5 mg/ml soybean trypsin inhibitor, incubated in a shaking water bath for 10 min at 37°C. The digested tissue was passed through 50 mg/ml BSA and washed twice with buffer A for further experiments.

Viability of mouse pancreatic acinar cells. The viability of the pancreatic acinar cells was determined by trypsin blue dye exclusion assay. One drop of 0.4% trypsin blue dye was added to one drop of the isolated acinar cells, and the viability was checked under a light microscope. In all experiments, cell viability was >95%.

Cell signaling experiments. Pancreatic acini were treated with SP (Sigma) at a dose of 10−6 M (1 µM) for 0, 3, 5, 10, 15, 30, 45, 60, and 120 min at 37°C, after which cells were lysed to detect for PKC-δ or MEKK1 activation by Western blot analysis. In some experiments, cells were also pretreated with the selective PKC-δ inhibitor rottlerin at 1, 5, and 10 µM (Calbiochem) for 1 h and then stimulated with 1 µM SP or vehicle for 10 or 45 min at 37°C. A specific PKC-δ translocation inhibitor (6β1-1: S-F-N-S-Y-E-L-G-S-L) (13) was synthesized (Sigma Genosys). The peptide was conjugated to a Drosophila melanogaster antennapedia peptide (R-Q-I-K-I-W-F-Q-N-R-R-M-K-W-K-K) to make it cell permeable. This peptide corresponds to specific sequences in the V1 regions that are responsible for anchoring PKC-δ to its translocation site. Thus, the peptide competitively inhibits the binding of PKC-δ to its anchoring protein. Hence, this prevents the activation of this particular PKC isoform (45, 46). Pancreatic acinar cells were pretreated with 5 and 10 µM of the specific PKC-δ translocation inhibitor for 3 h followed by stimulation with 1 µM SP or vehicle for 10 or 45 min at 37°C.

In other experiments, cells were preincubated with the selective NK1R antagonist CP69365 at 1 µM (Pfizer Diagnostics) for 30 min followed by treatment with 1 µM SP or vehicle for 10 min at 37°C. Subsequently, the supernatant was used for chemokine detection, and the pellet was used for either nuclear extraction to detect NF-κB or MEKK1 activation by Western blot analysis. In one experiment, cells were preincubated with the selective NK1R antagonist CP69365 at 1 µM (Pfizer Diagnostics) for 30 min followed by treatment with 1 µM SP or vehicle for 10 min at 37°C. Subsequently, the supernatant was used for chemokine detection, and the pellet was used for either nuclear extraction to detect NF-κB or MEKK1 activation by Western blot analysis to detect PKC-δ, MEKK1, ERK, and JNK.

Immunofluorescence. After being treated with SP, pancreatic acinar cells were fixed in 3.7% formaldehyde and placed on microscope slides using a CytoFuge 2 cytocentrifuge (StatSpin, Westwood, MA, USA). Fixed cells were then blocked for nonspecific binding with 1% BSA for 30 min at room temperature. Fluorescent labeling was performed by incubating the cells with a 1:50 dilution of PKC-δ mAb [phosphorylated (p-)PKC δ Tyr3243 (27), Santa Cruz Biotechnology, Santa Cruz, CA] for 90 min at room temperature followed by secondary detection for 40 min in the dark with a 1:200 dilution of FITC-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology). Staining was observed with a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) and captured by a digital camera (Carl Zeiss). Permeabilization and fluorescent labeling was followed by Western blot analysis. After treatment, pancreatic acinar cells were homogenized on ice in RIPA buffer supplemented with 1 mM PMSF and a protease inhibitor cocktail containing pepstatin, leupeptin, chymostatin, antipain, and aprotinin (5 µg/ml of each) and centrifuged at 4°C for 15 min at 14,000 g. Supernatants were collected and stored at −80°C until use. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Western blot analysis. Cell lysates (50 µg) were separated on a 12% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes. Nonspecific binding was blocked by 1 h of incubation of the membranes in 5% nonfat dry milk in 0.05% Tween 20 in PBS (PBST). Blots were then incubated overnight with the following primary antibodies: p-PKC-δ Thr505 (30, 38, 51), p-ERK1/2, p-SAPK/JNK (Cell Signaling Technology), MEKK1, and hypoxanthine-guanine phosphoribosyl transferase (HPRT) (Santa Cruz Biotechnology). HPRT was used as the housekeeping protein. The above antibodies were used at 1:1,000 dilutions in buffer containing 2.5% nonfat dry milk in PBST. They were then washed four times with PBST and finally incubated for 1 h with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at 1:2,000 dilutions in buffer containing 2.5% nonfat dry milk in PBST. Blots were developed for visualization using an ECL detection kit (Pierce, Rockford, IL).

Nuclear cell extract preparation. Nuclear cell extracts were prepared by employing a kit from Active Motif. In brief, cells were washed, collected in ice-cold PBS in the presence of phosphatase inhibitors to limit further protein modifications, and then centrifuged at 24 g for 5 min. Pellets were resuspended in a hypotonic buffer, treated with detergent, and centrifuged at 14,000 g for 30 s. After the cytoplasmic fraction had been collected, nuclei were lysed, and nuclear proteins solubilized in lysis buffer containing proteasome inhibitors. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories).

NF-κB DNA-binding activity. The binding of NF-κB to DNA was measured in nuclear extracts with a fast, user-friendly ELISA-based TransAM NFκB p65 assay kit (Active Motif). This assay uses multiwell plates coated with an unlabeled oligonucleotide containing the consensus binding site for NF-κB (5′-GGGACTTCC-3′) (39). Nuclear proteins (5 µg) were added to each well and incubated for 1 h to allow NF-κB DNA binding. Subsequently, using an antibody directed against the NF-κB p65 subunit, the NF-κB complex bound to the oligonucleotide was detected. The addition of the secondary antibody conjugated to horseradish peroxidase provided a sensitive colorimetric readout that was easily quantified by spectrophotometry.

AP-1 DNA-binding activity. TransAM AP-1 kits are designed specifically to detect and quantify AP-1 activation. Similar to TransAM NF-κB p65, TransAM AP-1 kits contain a 96-well plate, on which was immobilized an oligonucleotide that contains a 12-O-tetradecanoylphorbol 13-acetate-responsive element (5′-TGAGTCGTCA-3′). AP-1 dimers in nuclear extracts (5 µg) specifically bound to this oligonucleotide. The primary antibodies used recognized accessible epitopes on c-Jun proteins upon DNA binding. Secondary antibody conjugated to horseradish peroxidase gave the colorimetric reaction. Absorbance was read at 450 nm within 5 min.

Total RNA isolation. Total RNA was extracted from pancreatic acinar cells using TRIzol reagent (Invitrogen) following the manufacturer’s instructions with some modifications. Briefly, isolated cells were homogenized in TRIzol reagent. Chloroform was then added to the homogenates, and samples were shaken, incubated for 5 min at 4°C, and centrifuged for 15 min at 12,000 g at 4°C. The aqueous phase was separated, and RNA was precipitated by the addition of isopropyl alcohol. After RNA had been pelleted by centrifugation (12,000 g for 10 min at 4°C), the pellet was washed twice in 70% ethanol, air dried, and dissolved in RNase-free water. RNA was quantitated spectrophotometrically by absorbance at 260 nm. The purity of RNA was assessed by a 260-to-280-nm ratio between 1.6 and 2.0. The integrity of RNA was verified by the presence of distinct 28S and 18S rRNA bands on a denaturing agarose gel.

Semiquantitative RT-PCR. Semiquantitative RT-PCR was performed to analyze mRNA expression levels of monocye chemotactrant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, and MIP-2 in acini. Total RNA (1 µg) was reverse transcribed with use of the iScript cDNA synthesis kit (Bio-Rad). The cDNA synthesized was used as the template for PCR amplification by IQ Supermix (Bio-Rad). The PCR protocol consisted of optimized 36 cycles of denaturation at 95°C for 30 s, annealing for 30 s (at 55, 58, and 55°C for MCP-1, MIP-1α, and MIP-2, respectively), and exten-
sion at 72°C for 30 s performed in MyCycler (Bio-Rad). The following specific primer pairs (Promega, Singapore) of chemokines were used: MCP-1 sense 5'-GGAAATGGATCCACACCTTCGTC-3' and antisense 5'-TCTTCTCTTCCACCACTGAGC-3', resulting in a 582-bp product; MIP-1α sense 5'-ACTGCCCTTGCTGTTCTT-3' and antisense 5'-GCATTCAGTCCAGTGCAGTA-3', resulting in a 261-bp product; MIP-2 sense 5'-TGCCGAAAGCCTGCACAAAGG-3' and antisense 5'-GTTGGCTTGCCTTTGTTT-3', resulting in an 189-bp product; and 18S sense 5'-CAGGCATCCAATCGG-3' and antisense 5'-GGAAAAATGGATCCACACCTTGC-3', resulting in a 150-bp product. All PCR products were normalized to 18S internal controls.

### RESULTS

**Chemokine detection.** Pancreatic acinar cell supernatants were assayed for MCP-1, MIP-1α, and MIP-2 using a sandwich ELISA according to the manufacturer’s instructions (Duoset kit; R&D Systems, Minneapolis, MN). For MCP-1, for example, in brief, anti-MCP-1 primary antibody was aliquoted onto ELISA plates and incubated at 4°C overnight. Samples and standards were incubated for 2 h, plates were washed, and a biotinylated anti-MCP-1 antibody was added for 2 h. Plates were washed, and streptavidin bound to horseradish peroxidase was added for 20 min. After a further wash, tetramethylbenzidine was added for color development, and the reaction was terminated with 2 M H2SO4. Absorbance was measured at 450 nm. The same procedure was followed for the detection of the remaining chemokines (MIP-1α and MIP-2).

**Statistical analysis.** Results are presented as means ± SE with six replicates for each condition. Each experiment was repeated at least three times. The significance of changes was evaluated using ANOVA, and Tukey’s method was used as a post hoc test for the difference between groups. A P value of ≤0.05 was taken as the level of significance.

### RESULTS

**SP induces phosphorylation of PKC-δ in a time-dependent manner.** To investigate if SP causes phosphorylation of PKC-δ, pancreatic acinar cells were stimulated with 1 μM SP for 0, 3, 5, 10, 15, 30, and 45 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis using antibodies against p-PKC-δ and HPRT. As shown in Fig. 1, A and B, SP induced phosphorylation of PKC-δ in pancreatic acini, which was evident from 3 min and increased in a time-dependent manner up to 10 min, followed by a time-dependent decrease until 45 min. Densitometric analysis of Western blot experiments revealed a significant increase in the phosphorylation of PKC-δ at all the above-mentioned time points compared with the 0-min control.

The specific effect of SP on PKC-δ activation in pancreatic acinar cells was further confirmed by immunostaining. As shown in Fig. 1C, in unstimulated pancreatic acini, staining of PKC-δ was present in the cytosolic area of pancreatic acinar cells. Treatment of the cells with 1 μM SP decreased the presence of PKC-δ in the cytosol and increased its presence near the membrane, indicating translocation of PKC-δ from the cytosol to the cell membrane as a measure of PKC-δ activation.

**SP stimulates activation of MEKK1 in a time-dependent manner.** To find out if MEKK1 is activated upon SP stimulation, pancreatic acinar cells were stimulated with 1 μM SP for 0, 3, 5, 10, 30, 45, 60, and 120 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis using antibodies against MEKK1 and HPRT. As shown in Fig. 2A, SP induced activation of MEKK1 in pancreatic acini, which was evident from 3 min and increased in a time-dependent manner up to 45 min, followed by a time-dependent decrease until 120 min. Densitometric analysis of Western blot experiments, as shown in Fig. 2B, revealed a significant increase in MEKK1 at 10, 30, and 45 min compared with the 0-min control.

**SP-induced PKC-δ is involved in the activation of MEKK1, ERK, and JNK.** To determine if PKC-δ is involved in the activation of MEKK1, ERK, and JNK, pancreatic acinar cells were pretreated with either rottlerin (a selective PKC-δ inhibitor), ranging from 1 to 10 μM, or with 5 and 10 μM of the specific PKC-δ translocation inhibitor peptide (δV1-1) fol-
with either rottlerin, ranging from 1 to 10 μM, and MIP-2. Pancreatic acinar cells were pretreated against MEKK1 and HPRT.  

A

![Fig. 2. SP stimulates the activation of MEKK1 in a time-dependent manner.](image)

B

![Fig. 2. SP stimulates the activation of MEKK1 in a time-dependent manner.](image)

**Figure 2.** SP stimulates the activation of MEKK1 in a time-dependent manner. Freshly isolated pancreatic acini, obtained from 3 mice, were incubated with 1 μM SP for 0, 3, 5, 10, 30, 45, 60, and 120 min at 37°C. A: cells were lysed, and cell proteins were subjected to Western blot analysis using antibodies against MEKK1 and HPRT. B: densitometric analysis of Western blot experiments from pancreatic acini. Results are means ± SE. *P ≤ 0.05 compared with the 0-min control.

allowed by stimulation with 1 μM SP. Cells were then lysed, and cell proteins were subjected to Western blot analysis. Both rottlerin and δV1-1 dose dependently decreased the phosphorylation of PKC-δ, as shown in Fig. 3, confirming their inhibitory effect on SP-induced activation of PKC-δ. Rottlerin as well as δV1-1 attenuated SP-induced activation of MEKK1 and phosphorylation of ERK and JNK in a concentration-dependent manner.

**PKC-δ is involved in SP-induced NF-κB and AP-1 activation.** To determine the role of PKC-δ in SP-induced NF-κB and AP-1 activation, pancreatic acinar cells were preincubated with either rottlerin, ranging from 1 to 10 μM, or with 5 and 10 μM of the specific PKCδ translocation inhibitor peptide (δV1-1) followed by stimulation with 1 μM SP. Nuclear fractions from the cells were then extracted, NF-κB and AP-1 DNA-binding assays revealed that treatment with SP led to a notable increase in the activity of NF-κB p65 and AP-1 c-Jun, indicating that NF-κB and AP-1 play an important role in the SP-triggered signaling pathway in acinar cells. As shown in Fig. 4, A and B, pretreatment with either rottlerin or δV1-1 attenuated the SP-induced DNA-binding activity of NF-κB p65 and AP-1 c-Jun in a dose-dependent manner.

**Effect of PKC-δ inhibitors on gene expression and secretion of several proinflammatory chemokines in pancreatic acinar cells.** We also determined the role of PKC-δ on SP-induced chemokine production both at the mRNA and protein levels. Treatment of pancreatic acini with 1 μM SP resulted in the enhanced expression of proinflammatory chemokines MCP-1, MIP-1α, and MIP-2. Pancreatic acinar cells were pretreated with either rottlerin, ranging from 1 to 10 μM, or with 5 and 10 μM of the specific PKC-δ translocation inhibitor peptide (δV1-1) followed by stimulation with 1 μM SP. Total RNA from cells was extracted, and RT-PCR for MCP-1, MIP-1α, and MIP-2 was performed. We also determined the protein levels of these chemokines by ELISA. Our results, as shown in Figs. 5 and 6, showed that pretreatment with rottlerin markedly decreased MCP-1, MIP-1α, and MIP-2 mRNA levels as well as their protein levels in a dose-dependent manner. Similarly, pretreatment with δV1-1 significantly attenuated SP-induced MCP-1, MIP-1α, and MIP-2 production in a concentration-dependent manner in pancreatic acinar cells.

**SP-NK1R interaction is involved in PKC-δ and MEKK1 activation.** To show that SP-induced PKC-δ and MEKK1 activation was indeed mediated by SP, we pretreated pancreatic acini with 1 μM CP96345, a selective NK1R antagonist, followed by stimulation with 1 μM SP. Cells were then lysed, and cell proteins were subjected to Western blot analysis. Our results, as shown in Fig. 7, Aa and Ab, demonstrated that CP96345 significantly reduced SP-induced PKC-δ activation in pancreatic acinar cells compared with SP only-treated cells. Moreover, SP-induced MEKK1 activation was significantly downregulated in the presence of CP96345 compared with SP only-treated cells.

**DISCUSSION**

Although the mechanism of inflammation in acute pancreatitis is still not fully understood, a substantial body of evidence suggests that inflammatory mediators such as SP and chemokines play a critical role in the pathogenesis of acute pancreatitis (3, 4, 6, 7, 9). Chemokines are a family of small cytokines, such as MCP-1 and MIP-1α, that are believed to principally affect monocytes, whereas CXC chemokines, such as MIP-2, are believed to act on neutrophils (11). Recent work by us as well as other investigators (5, 10, 18) has, however, shown that these narrow definitions are no longer valid.

We (11) have previously shown that SP stimulated the production of chemokines MCP-1, MIP-1α, and MIP-2 in pancreatic acinar cells via the activation of NF-κB. However, the signaling mechanisms by which the interaction between SP and the G protein-coupled receptor NK1R mediates NF-κB driven chemokine production remain unclear. Previous studies have demonstrated that SP induced the phosphorylation of PKC-δ in rat parotid acinar cells. Moreover, it has been reported that PKC-δ plays an important role in SP-induced proinflammatory signaling in human colonocytes (30, 50); however, to our knowledge, no studies have looked into the involvement of PKC-δ in SP-NK1R-induced chemokine production in pancreatic acinar cells.

Pancreatic acini have previously been used as a model cell type to study the mechanisms of protein secretion, hormone action, and stimulus-secretion coupling (53). We used primary cultures of acinar cells isolated from mouse pancreatic tissue because there are no cell lines that authentically reproduce the
Fig. 3. SP-induced PKC-δ is involved in the activation of MEKK1, ERK, and JNK. Freshly isolated pancreatic acini, obtained from 3 mice, were preincubated with either rottlerin (A) at different doses of 1, 5, and 10 μM for 1 h at 37°C or with 5 and 10 μM of the specific PKC-δ translocation inhibitor peptide (δV1-1; C) for 3 h at 37°C followed by stimulation with 1 μM SP for 10 min at 37°C. Cells were subsequently lysed, and cell proteins were subjected to Western blot analysis using antibodies against p-PKC-δ (a), MEKK1 (b), p-JNK (c), p-ERK (d), and HPRT (e). The corresponding densitometric analyses of Western blot experiments from pancreatic acini are shown (B). Results are representative of 3 independent experiments. ND, not detected. Results are means ± SE. *P ≤ 0.05 compared with the control; +P ≤ 0.05 compared with SP.
function of the acinar cell. In the present study, we assessed the functional consequences of treating isolated pancreatic acinar cells, which are known to express NK1R (24, 40, 48), to the neuropeptide SP and determined the role of PKC-\(\delta\)/H9254 in the SP-triggered signaling pathway. We report here that SP induced a rapid increase in the phosphorylation of PKC-\(\delta\)/H9254. SP also caused the translocation of PKC-\(\delta\)/H9254 from the cytosol to the cell membrane, as a measure of PKC-\(\delta\)/H9254 activation, which was confirmed by immunofluorescence analysis. We determined the role of PKC-\(\delta\)/H9254 in SP-induced MCP-1, MIP-1\(\alpha\)/H9251, and MIP-2 production by demonstrating that rottlerin, a selective PKC-\(\delta\)/H9254 inhibitor, decreased the gene expression and secretion of these proinflammatory chemokines in pancreatic acinar cells. To further confirm the critical role of PKC-\(\delta\)/H9254 in chemokine production, we have shown that pretreatment of cells with the specific PKC-\(\delta\)/H9254 translocation inhibitor peptide attenuated SP-induced chemokine production. Satoh et al. (45, 46) have previously shown that PKC-\(\delta\)/H9254 translocation inhibitor...

Fig. 4. PKC-\(\delta\) is involved in SP-induced NF-\(\kappa\)B and activator protein (AP)-1 activation. In experiment 1, freshly isolated pancreatic acini, obtained from 3 mice, were preincubated with rottlerin at different doses of 1, 5, and 10 \(\mu\)M for 1 h followed by stimulation with 1 \(\mu\)M SP for 45 min. In experiment 2, pancreatic acinar cells from 3 mice were preincubated with 5 and 10 \(\mu\)M of the specific PKC-\(\delta\)/H9254 translocation inhibitor peptide (\(\delta\)V1-1) for 3 h followed by stimulation with 1 \(\mu\)M SP for 45 min. Acini were separated from the incubation medium by centrifugation. The pellet (acini) was used for NF-\(\kappa\)B (\(\alpha\)) and AP-1 (\(\beta\)) nuclear extraction, and NF-\(\kappa\)B (p65) and AP-1 (c-Jun) DNA-binding assays were carried out. Results are representative of 3 independent experiments. Results are means \(\pm\) SE. \(\ast\) \(P < 0.05\) compared with the control; \(\pm\) \(P < 0.05\) compared with SP.

Fig. 5. PKC-\(\delta\) is involved in SP-induced monocyte chemoattractant protein-1 (MCP-1), monocyte inflammatory protein (MIP)-1\(\alpha\), and MIP-2 mRNA expression. Freshly isolated pancreatic acini, obtained from 3 mice, were preincubated with rottlerin at different doses of 1, 5, and 10 \(\mu\)M for 1 h followed by stimulation with 1 \(\mu\)M SP for 45 min. Acini were separated from the incubation medium by centrifugation to carry out total RNA extraction followed by mRNA expression for MCP-1 (\(A\)), MIP-1\(\alpha\) (\(B\)), and MIP-2 (\(C\)) by RT-PCR. 18S was used as a loading control. Results are representative of 3 independent experiments. Results are means \(\pm\) SE. \(\ast\) \(P < 0.05\) compared with the control; \(\pm\) \(P < 0.05\) compared with SP.
peptide has a high potency and specificity in pancreatic acini.

It is generally known that NF-κB plays a key role in the regulation of inflammation because of its ability to control the expression of numerous inflammatory mediators. However, despite its crucial role in inflammation, it is unlikely that the mere activation of NF-κB is sufficient to trigger any single target gene that is involved in the initiation of inflammatory responses (25). Therefore, NF-κB requires the support from other transcription factors, for instance, AP-1. Our group as well as others (14, 41, 42) have shown that SP stimulates the activation of not only NF-κB but AP-1 as well. We (42) have also previously shown that SP activates NF-κB and AP-1 in a time-dependent manner and that both transcription factors are required for SP-induced chemokine production in pancreatic acinar cells. In the present study, rottlerin as well as PKC-δ translocation inhibitor attenuated SP-induced NF-κB and AP-1 activation in a dose-dependent manner, consequently leading to a concentration-dependent decrease in chemokine production in pancreatic acinar cells. A study conducted by Satoh et al. (45) has shown that both PKC-δ and ε are required to activate NF-κB when induced by cholecystokinin in pancreatic acinar cells. However, in our study, we found that supraphysiological concentrations of SP significantly increased PKC-δ activation and that its activation alone was sufficient to significantly upregulate the activation of not only NF-κB but also AP-1 in pancreatic acinar cells. Both PKC-δ translocation inhibitor and rottlerin attenuated SP-induced activation of PKC-δ as well as NF-κB and AP-1 activation.

We (42) have recently shown that SP stimulates ERK and JNK phosphorylation in a time-dependent manner and that these MAPKs mediate NFκB and AP-1 signaling pathways in mouse pancreatic acini. Similarly, various studies have implicated SP in acute inflammation and that it activates a number of intracellular signaling molecules such as MAPK members (12, 29, 31, 34, 55). A typical MAPK cascade is composed of MAPK (e.g., ERK and JNK), the kinase that activates the MAPK through phosphorylation on serine and tyrosine residues [MAPKK (e.g., MEK)], and the kinase that activates MAPKK [MAPKKK (e.g., MEKK1)] (25). It is known that once activated, MEKK1 can activate at least three known downstream pathways, thus leading to ERK, JNK, and NF-κB activation (17). In the present study, SP activated MEKK1 in a time-dependent manner and induced the phosphorylation of ERK and JNK in pancreatic acinar cells. The activation of these kinases was significantly inhibited by PKC-δ translocation inhibitor as well as by rottlerin, a specific PKC-δ inhibitor, but not MAPK inhibitor (26, 30, 49). These results indicate that the activation of the above kinases is mediated through PKC-δ. Moreover, 10 μM PD-98059 [a selective MEK inhibitor (1, 16)] or 10 μM SP-600125 [selective JNK inhibitor (2, 47)] had no significant effect on SP-NK1R-induced phosphorylation of PKC-δ in pancreatic acinar cells (data not shown), suggesting that PKC-δ is upstream of MAPK ERK and JNK in SP-induced chemokine synthesis. Also, maximum PKC-δ phosphorylation and activation were

Fig. 6. PKC-δ is involved in SP-induced MCP-1, MIP-1α, and MIP-2 secretion. In experiment 1, freshly isolated pancreatic acini, obtained from 3 mice, were preincubated with rottlerin at different doses of 1, 5, and 10 μM for 1 h followed by stimulation with 1 μM SP for 45 min. In experiment 2, pancreatic acinar cells from 3 mice were preincubated with 5 and 10 μM of the specific PKC-δ translocation inhibitor peptide (δV1-1) for 3 h followed by stimulation with 1 μM SP for 45 min. The supernatant was used to measure MCP-1 (A), MIP-1α (B), and MIP-2 (C) levels by ELISA. The negative control, in which pancreatic acini were pretreated with 10 μM rottlerin for 1 h followed by stimulation with placebo for 45 min, had no significant effect on chemokine production compared with unstimulated controls (data not shown). Results are representative of 3 independent experiments. Results are means ± SE. *P ≤ 0.05 compared with the control; +P ≤ 0.05 compared with SP.
obtained at 10 min, whereas those of MEKK1 were activated up to 45 min. This supports the flow chart shown in Fig. 8. Zhou et al. (56) have shown that the activation of MEKK1 and NF-κB was mediated by PKC-β in LPS-stimulated rat peritoneal macrophages. It is possible that SP is activating conventional as well as other novel PKC isoforms in addition to PKC-β, thus contributing to the activation of NF-κB and ultimately to chemokine production. However, further experiments need to be carried out to confirm these possibilities.

To further understand the molecular mechanism and to show that the activation of PKC-δ was indeed mediated by SP, and not by some nonspecific effects upon acinar cells isolation, we pretreated cells with the selective NK1R antagonist CP96345. In the present study, CP96345 decreased SP-induced PKC-δ activation. Moreover, CP96345 attenuated SP-induced MEKK1 activation in pancreatic acinar cells. We (42) have previously shown that SP-induced activation of ERK, JNK, NF-κB, and AP-1 driven chemokine production was attenuated by CP96345 in pancreatic acinar cells, thus showing that SP-induced PKC-δ activation and its downstream signaling pathway are dependent on NK1R. Taken together, we proposed the mechanism by which SP induces chemokine production in pancreatic acinar cells (Fig. 8).

Our findings indicate that PKC-δ acts as an important proinflammatory signal transducer in SP-NK1R-induced production of proinflammatory mediators MCP-1, MIP-1α, and MIP-2 in pancreatic acinar cells. The secretion of these proinflammatory mediators is mediated through the signaling cascade of PKC-δ-MEKK1-(ERK and JNK)-(NF-κB and AP-1), thereby contributing to local inflammation and consequently leading to systemic inflammation in acute pancreatitis. Thus, the reason for understanding the mechanisms by which SP-NK1R modulates its downstream functions is in establishing the feasibility of selectively targeting mechanisms so as to prevent disease progression such as acute pancreatitis or improve treatment efficacy.
ACKNOWLEDGMENTS

The authors thank Mei Leng Shoon for technical assistance.

GRANTS

This work was supported by Grant R-184-000-069-305 and R-184-000-139-101.

REFERENCES


C692 ROLE OF PKC-δ IN CHEMOKINE SYNTHESIS