THE NOVEL PROTEIN KINASE C ISOFORMS -δ AND -ε MODULATE CAERULEIN-INDUCED ZYMGEN ACTIVATION IN PANCREATIC ACINAR CELLS

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Running title: Novel PKC isoforms modulate pancreatitis

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Abstract

Isoforms of protein kinase C (PKC) have been shown to modulate some cellular responses such as pathologic secretion and generation of inflammatory mediators during acute pancreatitis (AP). We propose that PKC also participates in premature zymogen activation within the pancreatic acinar cell, a key event in the initiation of AP. This hypothesis was examined in in vivo and cellular models of caerulein-induced acute pancreatitis using PKC activators and inhibitors. Phorbol ester, 12O-tetradecanoylphorbol-13-acetate (TPA; 200 nM), a known activator of PKC, enhanced zymogen activation at both 0.1 nM and 100 nM caerulein, concentrations which mimic physiologic and supraphysiologic effects of the hormone cholecystokinin (CCK) respectively, in preparations of pancreatic acinar cells. Isoform-specific PKC inhibitors for PKC δ and PKC ε reduced supraphysiologic caerulein-induced zymogen activation. Using a cell-free reconstitution system we showed that inhibition of PKC δ and ε, reduced zymogen activation in both zymogen granule-enriched and microsomal fractions. In dispersed acinar cells 100 nM caerulein stimulation caused PKC δ and ε isoform translocation to microsomal membranes using cell fractionation and immunoblot analysis. PKC translocation was confirmed with in-vivo studies and immunofluorescence microscopy in pancreatic tissues from rats treated with or without 100 nM caerulein. PKC ε redistributed from an apical to a supranuclear region following caerulein administration. The signal for PKC ε overlapped with GRAMP 92, an endosomal/lysosomal marker, in a supranuclear region where zymogen activation takes place. These results indicate that PKC δ and ε isoforms translocate to specific acinar cell compartments and modulate zymogen activation.
**Key words:** Protein kinase C (PKC); zymogen activation; translocation; PKC activator phorbol ester (TPA); PKC inhibitor
Introduction

The pathophysiology of acute pancreatitis (AP) is determined by many sequential events in the exocrine pancreas. These include activation of zymogens in the acinar cell, decreased apical secretion of digestive zymogens, release of inflammatory cytokines, inflammation, edema, and cell death. Isoforms of the serine/threonine kinase, protein kinase C (PKC), have been shown to modulate some of these AP responses (8, 28, 29). Of the ten known PKC isoforms, four have been identified in pancreatic acinar cells (acini): conventional PKC α (DAG- and Ca^{2+}-sensitive); novel PKC δ and PKC ε (DAG-sensitive and Ca^{2+}-insensitive) and atypical PKC ζ (DAG- and Ca^{2+}-insensitive) (2, 23).

The role PKC isoforms play has largely been determined using cellular and in vivo experimental models of AP. These models often use supramaximal concentrations of the hormone cholecystokinin (CCK) or its ortholog, caerulein to induce AP (28, 29, 33). The cellular model of acute pancreatitis, using dispersed groups of pancreatic acini, has been particularly useful for studying the early events in AP. The effects of ethanol, a known factor in induction of AP, have been studied in these models (29). Pre-treatment with pharmacological inhibitors of PKC isoforms in these model systems has revealed that PKC δ alone mediates physiologic secretion whereas diverse pathologic responses, ranging from lack of exocytosis to generation of inflammatory mediators, are regulated by more than one PKC isoform. Conventional PKC α, for example, has been implicated in mediating pathologic secretory processes in experimental pancreatitis whereas the novel PKC δ and PKC ε isoforms have been shown to regulate expression of
inflammatory mediators (8, 28, 29, 33). The function of PKC ζ has yet to be determined in the pancreatic acinar cell.

In the present study we investigated a potential role for PKC isoforms in regulating premature zymogen activation, using both the in vitro caerulein model of AP, and a cell-free reconstitution system, in conjunction with PKC activators and inhibitors. Two concentrations of caerulein were used to stimulate acinar cells: 0.1 nM and 100 nM to mimic physiologic and supraphysiologic effects of CCK, respectively (25, 27). Inhibition of PKC δ and ε decreased caerulein-stimulated zymogen activation. Further, immunoblot studies showed that both isoforms translocated from the cytosol to microsomal membranes upon supraphysiologic caerulein stimulation. With in-vivo studies, using immunofluorescence, we observed a redistribution of PKC ε from the apical region of the cell to a supranuclear region. PKC ε distribution, post-stimulation, overlapped with cellular compartments positive for the lysozomal/endosomal marker, GRAMP 92. Our laboratory has previously shown that a subset of GRAMP 92-containing compartments overlap with compartments positive for the trypsinogen activation peptide or TAP (19), a marker for zymogen activation. Our study suggested that relevant PKC isoforms were present in areas of the cell where zymogen activation took place. These studies demonstrated that PKC δ and ε modulated caerulein-stimulated protease activation in the pancreatic acinar cell.
Materials and Methods

Preparation of isolated pancreatic acini. Acini were isolated as previously described (7). Briefly, fasted male Sprague-Dawley rats 50–150 g (Charles River Laboratories, Wilmington, MA) were euthanized by CO₂ using a protocol approved by the Yale University and the Veterans Administration Animal Care and Use Committees. Acinar media was prepared as follows (in mM): 10 HEPES (pH 7.4), 95 NaCl, 4.7 KCl, 0.6 MgCl₂, 1 NaH₂PO₄, 10 glucose, 2 glutamine, plus 0.1% BSA, 1x MEM-amino acids (GIBCO-BRL, San Jose, CA) and 1.3mM CaCl₂. The pancreas was collected in 15ml of calcium-free acinar media. The pancreas was then minced in a minimal volume of calcium free medium for 5 min, washed 3 times with calcium free medium. The minced tissue was then placed into a 50-ml flask with 12 ml of acinar medium containing 50 U/ml of type-4 collagenase (Worthington, Freehold, NJ) for 60 min at 37°C with shaking (120 rpm). The digest was filtered through a 300-400-μm mesh (Sefar American, Depew, NY) and washed with acinar medium. Isolated acini (groups of 20-100 acinar cells) were distributed among the 24 wells (0.5 ml suspension/well) of a 24-well Falcon tissue culture plate (Becton Dickinson, Franklin Lakes, NJ). All reagents were purchased from Sigma, St. Louis, MO, unless otherwise noted.

Acinar experimental protocol. Acini were recovered for 120 min at 37°C under constant O₂ with shaking (90 rpm). Medium was changed at 60 min. At 120 min acini were treated without changing medium. Acini were treated with caerulein (0.1 nM and 100 nM) for 60 min (unless otherwise noted). Samples were collected, placed in 1.5-ml centrifuge tubes (USA Scientific, Waltham, MA) and centrifuged for 1 min at 30 g. 50μl
of the resulting cell-free supernatant was removed to a 0.5-ml microcentrifuge tube to assay for secreted amylase. The remaining 450µl of cells + media was retained for zymogen activation assays and determination of total amylase. All samples were stored at -80°C.

**PKC Activator.** Phorbol ester 12O-tetradecanoylphorbol-13-acetate (TPA; 200 nM) or the biologically-inactive form of TPA, TPA4α (200 nM) was added 15 min prior to caerulein stimulation. In some treatment groups the broad spectrum PKC inhibitor GF109203X (10 µM) was added 120 min prior to addition of TPA. During the recovery period GF109203X was re-added after the media change.

**PKC Inhibitors.** Acinar cells were treated with 10 µM of each of the following: GF109203X; isoform-specific PKC δ translocation inhibitor (δV1-1: S-F-N-S-Y-E-L-G-S-L); isoform-specific PKC ε translocation inhibitor (εV1-2: E-A-V-S-L-K-P-T); and a control scrambled peptide (L-S-E-T-K-P-A-V) for 120 min prior to caerulein stimulation. Each of these peptides was synthesized as an amino terminal extension to a *Drosophila antennapedia* peptide (R-QI-K-I-W-F-Q-N-R-R-M-K-W-K-K) to make it cell permeable (28, 29).

**Enzymatic activity assays.** Enzyme activities were carried out as previously described (7). Briefly, samples were thawed, homogenized and centrifuged. To each well of a 24-well plate (Greiner Bio-one *Cellstar* TC-Plate) was added the following: 100 µl of post-nuclear supernatant, 350 µl of trypsin assay buffer [50 mM Tris (pH 8.1), 150 mM NaCl, 1 mM CaCl2, 0.01% BSA]. The assay was initiated by the addition of 50 µl of 400 µM enzyme substrate (fluorometric trypsin substrate; Peptides International, Louisville, KY and fluorometric chymotrypsin substrate; Calbiochem, San Diego, CA) diluted in trypsin
assay buffer (40 μM final). The plate was read by using a fluorometric microtiter plate reader (model HTS 7000; Perkin-Elmer Analytical Instruments, Shelton, CT. 380-nm excitation; 440-nm emission; 20 reads /10 min.)

Amylase assay. Amylase activity was determined by using a commercial kit (Phaebadas kit; Pharmacia Diagnostic, Rochester, NY) as described (7). Amylase secretion was calculated as the percent total release [medium/(medium + cells)].

Reconstitution experiments. Pancreatic fractionation was carried out as described (34). Briefly, the pancreas was divided in two and each half homogenized in 10 volumes (approximately 5 ml) of 300 mM sucrose, 1 mM DTT. Homogenates were centrifuged at 500 g for 5 min to generate a post nuclear supernatant (PNS). All preparations and storage were at 4°C unless otherwise stated. The PNS fraction was centrifuged to generate various membrane fractions as previously described (34). Briefly, PNS was serially centrifuged at 3000 g (10min); 15000 g (10min) and 180000 g (60min) over a 2 M sucrose cushion. The interface of each centrifugation was collected i.e. zymogen granule, mitochondrial and microsomal fractions respectively. Only the zymogen granule and microsomal fractions were retained for further study. All particulate fractions were washed and diluted as described (34).

Cytosol was prepared from PNS as described (34). Briefly, PNS was centrifuged at 180000 g for 60 min on a 2 M sucrose cushion. The supernatant was removed and dialyzed in cytosol buffer: 300 mM sucrose, 25 mM Tris pH 7.2, 100 mM KCl for 60 min using a dialysis cassette (Slide-A-Lyzer Dialysis Cassette, 3500 MWCO, 0.5-3ml capacity, Pierce Biotech). The dialyzed fraction was centrifuged in tubes precoated with
cytosol buffer at 288000g for 15 min to pellet any small vesicles. The resultant supernatant (cytosol) was collected.

Enzyme activity in cell fractions was assayed as previously described (34). To each well of a 24-well plate the following was added: 350 µl of assay buffer (50 mM Tris, pH 7.6; 150 mM KCl), 50 µl of zymogen granule-enriched or microsomal fractions and 50 µl of 400 µM enzyme substrate (Peptides International, Louisville, KY). After 15 min incubation at room temperature, 50 µl of buffer (control) or cytosol were added to each well and incubated for a further 15 min. ATP (5 mM) was then added and fluorescence emissions were recorded (excitation wavelength 380 nm; emission 440 nm; 20 recordings over 10 min with a HTS 7000 fluorimeter (Perkin-Elmer Analytical Instruments, Shelton, CT)). Enzyme activity was normalized to amylase content and expressed as fold activation versus the zymogen granule/microsomal fraction plus cytosol plus ATP condition.

In previous studies, PKC was found to be predominantly in the cytosol and thought to be in an active or semi-active form prior to stimulation (14, 16, 28). Thus, cytosol was pre-incubated for 15 min with isoform-specific PKC inhibitors before addition to the well as described earlier in this section for the assay of zymogen activation in cellular fractions. The PKC inhibitors used in the cell-free system were not conjugated to Drosophila antennapedia peptide since cell-permeability was not required.

Preparation of cells for immunoblot. Cells were prepared as described in preparation of isolated pancreatic acini, with the following modifications. After 30 min supraphysiologic caerulein treatment, cells and medium (500 µl) were collected. Samples were allowed to sediment by gravity and the cell free medium was removed. An equal
volume (500 µl) of homogenization buffer (25 mM Hepes pH 7.4; 300 mM sucrose; 1 mM benzamidine; Complete Protease Inhibitor cocktail, EDTA-free (1 tablet per 25 ml stock solution; Roche, Mannheim, Germany)) was added and cells homogenized in a conical 1.5-ml Eppendorf tube with a pestle, and centrifuged at 1,000 g for 1 min. The post-nuclear supernatant was removed and further centrifuged at 3000 g for 5 min; the pellet was collected as the zymogen granule fraction. This fraction was washed with homogenization buffer (500 µl) and spun two more times. The supernatant from the 3000 g step was centrifuged at 233 000 g and the supernatant collected as the cytosolic fraction; the pellet as the microsomal fraction. To solubilize proteins, the zymogen granule and microsomal pellets were resuspended in Laemmli buffer (125 µl of 1x). 50 µl of 6x Laemmli buffer was added to 300 µl of cytosol. Samples were heated to 95°C for 5 min and then stored at -80°C.

**Immunoblot analysis.** Western blot analysis was performed to detect PKC isoforms. Briefly, samples were separated on 10% SDS-PAGE gels (Bio-Rad, Hercules, CA) and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Membranes were blocked for 60 min at room temperature with Blotto (TBS, 5% nonfat dry milk, 0.05% Tween-20). Membranes were then probed with primary antibody (rabbit anti-PKC δ or ε, 1:200; Santa Cruz Biotechnology, CA) in Blotto for 120 min at room temperature, washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) for 60 min at room temperature. Membranes were washed in TBS, and autoradiography was performed by using a SuperSignal West Pico chemiluminescence kit (Pierce, Rockford, IL).
Immunofluorescence microscopy. Sprague Dawley rats were anesthetized and a supraphysiologic dose of cerulein (40 ug/kg) was administered IP. After 60 min rat tissues were fixed by transcardiac perfusion of 2% paraformaldehyde and 10mM benzamidine to optimize tissue morphology. Rat pancreata were fixed for an additional 30 min at RT, sucrose infiltrated overnight at 4C, and frozen in Tissue Tek OCT compound (Sakura, Torrance, CA). Cut sections (5 microns thick) were permeabilized in 0.05% saponin and quenched in 500 mM NH₄Cl and 3% normal goat serum. Primary antibodies (rabbit anti-PKC δ or ε, 1:100; mouse anti-GRAMP 92, 1:20; Santa Cruz Biotechnology, CA) were incubated with tissue sections for 120 min at room temperature in a humidified chamber. Primary antibodies were detected with AlexaFluor488-labeled goat anti-rabbit (1:1000) or AlexaFluor555-labeled goat anti-mouse (1:1000) secondary antibodies and sections were mounted in Prolong Gold with DAPI (Molecular Probes). Labeled tissues were examined by epifluorescence using an Axioplan microscope (Carl Zeiss Inc., Thornwood, NY) and digital images were collected with a Spot camera and analyzed with software version 4.0.9 (Diagnostic Instruments, Inc., Sterling Heights, MI). Digital images were assembled and labeled in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA). Further images were obtained by use of a Zeiss LSM510 laser scanning confocal microscope and analyzed using LSM510 image browser software.

Statistical analysis. Data represent means ± SE of at least 3 individual experiments unless otherwise noted, with each experiment being performed in at least duplicate. A Student’s t-test analysis was used to determine statistical significance and P values of < 0.05 were assigned significance.
Results

Phorbol ester, TPA, enhances caerulein-induced zymogen activation in pancreatic acini. Our initial study investigated whether PKC stimulation could affect basal or caerulein stimulated zymogen activation. Pancreatic rat acini were pre-treated with the phorbol ester 12O-tetradecanoylphorbol-13-acetate (TPA; 200 nM), a known activator of PKC isoforms (33). Both trypsin and chymotrypsin activities, normalized to amylase content and expressed as fold vs. maximal, were used as measures of zymogen activation using specific fluorogenic substrates. TPA pre-treatment alone had no effect, but TPA pre-treatment followed by caerulein stimulation led to increased zymogen activation both at 0.1nM and 100 nM caerulein (Fig. 1a and 1 b). The enhancement of caerulein stimulated zymogen activation was not seen with the biologically inactive form of TPA, TPA 4-α (200 nM), or TPA in the presence of GF109203X (10 µM), a broad spectrum PKC inhibitor.

Although low concentrations of caerulein (0.1 nM) stimulation alone caused little, if any, zymogen activation, when combined with TPA zymogen activation was comparable to that seen with supraphysiologic caerulein alone (Fig 1a and b). Supraphysiologic caerulein-induced zymogen activation itself almost doubled with TPA-pretreatment. The PKC inhibitor, GF109203X, reduced activation, but not to basal levels. The incomplete response could indicate that a component of zymogen activation may be PKC-insensitive or that higher concentrations of GF109203X might be required to completely inhibit PKC in this condition.
TPA appeared to have little effect on caerulein-stimulated amylase secretion (Fig 1 c). A slight increase in amylase secretion in low dose (0.1 nM) caerulein-stimulated cells was seen but this was not statistically significant.

Protein Kinase C inhibitors reveal a role for PKC in caerulein-induced zymogen activation. In previous studies PKC isoforms were shown to be activated by CCK and caerulein in pancreatic acinar cells (16, 28, 29). Further, as shown in this study, TPA enhanced 0.1 nM caerulein-induced zymogen activation to levels comparable to that seen with 100 nM caerulein alone, indicating that PKC sensitized the cell to caerulein treatment. To examine whether endogenously-activated PKC mediated supraphysiologic caerulein-induced zymogen activation, dispersed acinar cells were pre-treated with increasing concentrations of GF109203X followed by supraphysiologic caerulein stimulation (Fig. 2). Caerulein-induced trypsin and chymotrypsin activity was reduced in the presence of 1µM and 10µM GF109203X, being significant at 10µM, a concentration used routinely in the literature (16, 28, 29), indicating that PKC was involved in caerulein stimulated zymogen activation.

To address which PKC isoforms mediated zymogen activation, acini were pre-treated with isoform-specific PKC inhibitors and then stimulated with supraphysiologic concentrations of caerulein. Inhibition of the PKC α isoform with the conventional PKC inhibitor Gö 6976 did not affect zymogen activation (data not shown). This observation was consistent with the report that PKC α was not activated by supraphysiologic caerulein or CCK (28). Preliminary studies showed that inhibition of PKC ζ with a specific pseudosubstrate did not affect zymogen activation either (data not shown). Thus, PKC α and ζ isoforms were not studied further in the context of zymogen activation.
Inhibition of PKC δ or ε, however, significantly reduced zymogen activation (Fig. 3a and b) but did not affect stimulated amylase secretion (not shown). When cells were incubated with a combination of inhibitors (either δ/ε or δ/ε/GF109203X), zymogen activation was significantly reduced but the effect was similar to that seen with δ, ε or GF inhibition alone, suggesting that the effects of inhibiting PKC isoforms was not additive (Fig. 3c). Finally, pre-incubation of cells with a scrambled peptide similar to the isoform-specific PKC translocation inhibitors did not affect zymogen activation, indicating that the reduction in zymogen activation due to PKC inhibition was specific.

**PKC δ and ε isoforms mediate zymogen activation in both zymogen granule-enriched and microsomal fractions in a cell-free system.** PKC has been shown to phosphorylate the CCK receptor (9, 31). To determine whether the PKC effects we observed were downstream of the receptor, we examined the effects of kinase isoform inhibition in a cell-free reconstitution system. Using this preparation we have shown that both zymogen granule-enriched and microsomal fractions from the pancreas could support cytosol-dependent zymogen activation (34). Furthermore, with chymotrypsin, there was an ATP-dependent component to activation that was sensitive to protein kinase inhibition.

To determine whether the unidentified kinase was an isoform of PKC, zymogen activation was measured in both zymogen granule-enriched and microsomal fractions reconstituted with dialyzed cytosol and ATP, as described (34). Although there is no ATP-dependent increase in trypsin activation, a reduction in cytosol-dependent trypsin activity was observed in the presence of PKC δ or ε inhibitor (Fig 4 a and b), agreeing with our acinar cell data which demonstrated that trypsin activation was PKC-sensitive. A similar effect was seen for cytosol stimulated chymotrypsin activation in both
membrane fractions. In addition, the enhanced activation seen in the presence of both cytosol and ATP was also inhibited (Fig 4 c and d). A greater effect was seen for chymotrypsin than trypsin with up to a 50% reduction from maximal in chymotrypsin activation in both zymogen granule-enriched and microsomal fractions (Fig 4 c and d). Finally, the lack of any effect with the scrambled peptide further validated the specificity of the PKC inhibitors.

Thus, results from the cell-free system demonstrated that effects of PKC were downstream from the CCK receptor and that PKC-sensitive zymogen activation could take place in more than one type of acinar cell compartment.

Translocation and cellular distribution of PKC isoforms. Translocation of PKC isoforms from cytosol to membranes is a characteristic of activated PKCs. In previous studies, activated PKC δ and ε have been shown to translocate to intracellular membranes, whereas activated PKC ζ did not translocate (28). Since PKC δ and ε affected zymogen activation in diverse cellular compartments in a cell-free system, we investigated whether they translocated from cytosol to a zymogen granule enriched or microsomal fraction in cells treated with supraphysiologic caerulein for 30 min followed by organelle fractionation.

Using immunoblot analysis, we observed that this treatment lead to reduced PKC δ content in the cytosol and increased in microsomes (Fig 5a). When PKC δ levels were normalized to protein content in each fraction, the average increase of PKC δ signal in the microsomal fraction after 30 min was 2.0- to 2.5-fold (Fig 5 b). PKC δ inhibitor reduced caerulein-stimulated translocation of PKC δ to microsomal membranes (Fig 5c). Similar results were seen for PKC ε, with a 2.6-3.4-fold increase of PKC ε signal in
microsomal membranes (Fig 6a, b). PKC ε translocation was reduced by PKC ε inhibitor (Fig 6c). There was relatively little change in PKC δ or ε content in zymogen granules. Thus, in isolated pancreatic acinar cells, supraphysiologic caerulein stimulation resulted in PKC δ and ε translocating predominantly to microsomal membranes.

PKC translocation was confirmed with in-vivo studies and immunofluorescence microscopy (see Methods). In tissues from animals treated with or without supraphysiologic concentrations of caerulein, the distribution of PKC δ was not clearly defined, perhaps due to efficacy of the antibody. The distribution of PKC ε, however, was more obvious. In unstimulated animals, there was intense PKC ε immunoreactivity at the apical region of the acinar cell. PKC ε was also diffusely distributed throughout the cytosol, with some punctuate structures being evident (Fig 7a). Upon supraphysiologic caerulein stimulation, re-distribution of PKC ε was observed. Its apical signal decreased and PKC ε-positive punctate structures increased in the supranuclear region (Fig 7b). The redistribution of PKC ε to a supranuclear region was consistent with its translocation to microsomal membranes as observed in our cell fractionation studies (Fig 6a).

**PKC ε co-distributes with GRAMP-92 positive compartments.** The exact site(s) of zymogen activation in the acinar cell is controversial (11-13, 15, 19, 24, 30). Previous studies from our laboratory have shown that trypsinogen activation peptide (TAP), a small peptide which is cleaved upon trypsinogen activation and marker for zymogen activation, is found in compartments containing GRAMP, a marker of late endosomes/lysosomes (10, 13, 19, 20). Conflicting fixation requirements did not permit co-localization of TAP with PKC ε antibody so studies were done with GRAMP 92 as the marker for the site of zymogen activation.
Confocal immunofluorescence demonstrated a predominant localization for PKC ε in the apical region of the cell (shown in red), with a lesser distribution throughout the cytosol (Fig 8a). GRAMP 92 was distributed throughout the cell and could be seen in some cells as sharp punctuate structures (shown in green) (Fig 8a). Upon supraphysiologic caerulein stimulation there was a re-distribution of GRAMP 92 positive compartments and PKC ε to a supranuclear region (co-localization shown in yellow), although some apical signal for PKC ε remained. The overlap between PKC ε and GRAMP 92-positive compartments and this co-distribution was further verified by the z-section analysis (Fig 8) in which the tissue was scanned at different levels and the individual sections stacked to give a 3-dimensional evaluation. In a previous study we showed that not all GRAMP 92 positive compartments supported zymogen activation, but all TAP-containing compartments were GRAMP 92-positive (19). Although not directly demonstrated, the current study suggests that PKC ε distribution overlaps with compartments which may support zymogen activation following supraphysiologic caerulein stimulation.
Discussion

In this study we investigated a potential role for PKC isoforms in premature digestive zymogen activation in acute pancreatitis using PKC activators and inhibitors. Of the four PKC isoforms present in the pancreatic acinar cell, the α, δ, and ε isoforms are sensitive to phorbol esters, known activators of PKC. Pre-treatment of isolated pancreatic acinar cells with the phorbol ester TPA led to enhancement of caerulein-induced zymogen activation, both at 0.1 nM and 100 nM (representing ‘physiologic’ vs. ‘supraphysiologic’ stimulation). In another study TPA was shown to induce translocation of PKC isoforms in the acinar cell, although in our study TPA alone was insufficient by itself to cause zymogen activation; caerulein stimulation was still necessary for PKC-mediated zymogen activation (16).

Using broad spectrum and isoform-specific inhibitors, we have identified two isoforms, PKC δ and ε, as the candidates for mediating aberrant zymogen activation. Inhibition of these isoforms did not completely block zymogen activation (Fig 3). This may be a reflection of the degree and duration of the inhibitors although this is unlikely for a number of reasons. First, previous studies have demonstrated that PKC activity was completely inhibited by these isoform-specific peptide inhibitors at the same concentration used in the current study. Further, these inhibitors have been shown to be effective for at least four hours, comparable to the time-frame of the present investigation, although their efficacy beyond this time point has not been determined. Finally, the inhibitors given in our current study are at concentrations 10-100 fold higher than their Ki to ensure that an adequate final concentration of inhibitor is present in the cell. Thus, the incomplete inhibition of zymogen activation we observed suggested that
there could be a PKC-independent component to the process. Each inhibitor reduced zymogen activation by a similar degree, an effect also observed for NFκB activation (28). When a combination of inhibitors was applied (δ/ε/GF109203X) the reduction in zymogen activation was similar to that seen for δ, ε or GF inhibition alone indicating that the effect was not additive but maybe sequential.

In a previous study using a cell-free reconstitution system we were able to induce zymogen activation in both zymogen-granule enriched and microsomal fractions by adding back cytosol and ATP (34). Further, we found that cytosol/ATP-dependent activation could be reduced by broad spectrum protein kinase inhibitors. In this study our evidence strongly supported PKC δ and ε as candidate kinases for mediating zymogen activation. Both trypsin and chymotrypsin activity were significantly reduced in the presence of PKC δ or ε inhibitors. A lower concentration of inhibitor was used in the cell-free system compared to the intact cells studies (1 µM vs. 10 µM) as cell permeability of the inhibitors was not an issue in the cell-free system. Preliminary studies showed that the same degree of inhibition was seen for either concentration of PKC inhibitor in the cell-free environment (data not shown); hence the lower concentration was used. Reduction from maximal activity for trypsin was much less than that seen for chymotrypsin (10-20% for trypsin vs 40-60% for chymotrypsin). In the reconstituted cell system, we did not observe a significant ATP-dependent increase in trypsin activity, but did find that ATP had a prominent effect on the activation of chymotrypsinogen. Additional data from our previous reconstitution studies also suggested that some trypsinogen and chymotrypsinogen activation may be mediated by independent mechanisms (34). This sensitivity of chymotrypsinogen activation to PKC seen in the
reconstituted cell system (Fig. 4) was further supported by our acinar cell studies which showed that chymotrypsinogen activation was much more sensitive to PKC inhibition than trypsinogen activation. At 1 µM GF109203X, for example, we saw a reduction in chymotrypsin but not trypsin activity (Fig 2). The mechanism responsible for the differences in trypsinogen and chymotrypsinogen activation however, remains unclear.

The reconstituted cell method used unstimulated tissue, therefore one might expect PKC to be largely in an inactive form located predominantly in the cytosol. However, we and others found that even in unstimulated tissues, there was a basal level of zymogen activation. Similarly, some activation of PKC was observed in unstimulated acini (S. Pandol, unpublished data). Finally, we found that about 10% of total PKC δ and ε were associated with membranes in unstimulated cells (Figs 5 and 6). These observations suggested that a fraction of PKC was active in unstimulated tissues.

A further observation from the reconstituted cell studies was that cytosol-induced zymogen activation could be reduced in the zymogen granule-enriched fraction by PKC inhibitors, even when ATP was not present (Fig 4a and c). The cytosol used in these assays had been dialyzed to remove endogenous ATP. Therefore, one would not expect protein kinases to be active in the cytosol. However, it was possible that the zymogen granules, which contained ATP, could release endogenous ATP during the incubation with cytosol (32). This might provide sufficient ATP for activation of PKC isoforms, particularly those which may be anchored to the surface of the granule (Fig 5a and 6a).

From our reconstituted cell studies we have shown that PKC δ and ε can modulate zymogen activation in diverse cellular compartments such as zymogen granules and microsomes. However, in intact cells, PKC translocates preferentially to microsomal
membranes upon stimulation. What can account for this difference? Firstly, anchoring sites for PKC isoforms are present on both zymogen granules and microsomes as is evident from their localization in resting cells i.e. some PKC is endogenously bound to these membranes. Thus PKC has the capacity to bind to target sites on either organelle and mediate zymogen activation, but selectively translocate to microsomal compartments in intact cells. The mechanism by which PKC translocates is unknown, but it is possible that the cellular machinery responsible for this phenomenon may not be intact in the cell-free preparation.

Whether the zymogen activation compartments observed in reconstituted cell studies reflect the site of PKC-sensitive zymogen activation in the stimulated intact acinar cell is unclear. In previous studies, PKC δ and ε have been shown to translocate to intracellular membrane fractions from the cytosol following caerulein or CCK stimulation (28). We explored this further and showed that these isoforms translocated predominantly to microsomal membranes as opposed to zymogen granule membranes upon supraphysiologic caerulein stimulation (Figs 5 and 6). Studies from a number of laboratories, including ours, indicated that early intracellular zymogen activation was occurring in a non-zymogen granule compartment (19, 30). The fact that PKC ε and δ mediated zymogen activation and were translocating to microsomal membranes in subcellular-fractionation studies supported this idea. Using immunofluorescence, we observed that PKC ε redistributed in the acinar cell, following stimulation, to a supranuclear region (Fig 7) in agreement with that observed in previous studies (29). Our data further showed that it co-distributed with GRAMP 92, an endosomal lysosomal marker which has been associated with zymogen activation compartments (Fig 8) (19, 21,
26, 35). Although this does not directly demonstrate that PKC translocates to zymogen activation compartments it demonstrates that PKC ε is in the correct location to participate in zymogen activation.

This study provides strong evidence for a role of PKC δ and ε in zymogen activation, but the targets of the kinases remain unclear. Two candidate processes that mediate zymogen activation are changes in (i) intracellular calcium and (ii) intraluminal pH of zymogen-containing compartments (21, 26, 35). Several lines of evidence suggest that PKC can regulate these responses. First, PKC can phosphorylate calcium release channels, such as the inositol 1,4,5-trisphosphate receptor, altering the calcium dynamics of the cell (1, 5, 6, 17, 22). Some of these studies show an increase in calcium, a known effector of premature zymogen activation, but others show a decrease in calcium levels. None of these studies, however, demonstrate which PKC isoform is involved in these processes. Second, vacuolar ATPase (vATPase) is an ATP-dependent proton pump which acidifies zymogen-containing compartments, optimizing conditions for zymogen activation (35). Little is known about the mechanism of vATPase regulation. It is likely that phosphorylation of various vATPase subunits by kinases including PKC may play a role in its assembly or activation. This would lead to acidification of intracellular compartments providing a suitable environment for premature zymogen activation (4, 18).

An additional target for PKC isoforms in the pancreatic acinar cell is protein kinase D (PKD). PKD is an enzyme involved in a number of cellular processes including protein secretion, apoptosis and cell proliferation. Recent evidence suggests that PKD 1 is a
downstream target of PKC δ and it is possible that PKD may contribute to zymogen activation (3).

In conclusion, the results of the current study provide further insights into the role that the novel PKC isoforms δ and ε play in the mechanism of acute pancreatitis. In addition to activating transcription factors such as NFκB, they also mediate aberrant zymogen activation. Given their profound impact on the early stages of acute pancreatitis, novel PKC isoforms present themselves as attractive therapeutic targets. As future studies reveal more about the dynamics of the novel PKC family in the pancreatic acinar cell, strategies that block PKC-dependent mechanisms may reduce initiation and perpetuation of the disease.
Acknowledgements

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References

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Figure Legends

Figure 1. PKC activator phorbol ester (TPA) enhances caerulein-induced zymogen activation. (a) Trypsin and (b) chymotrypsin activities and (c) amylase secretion were measured. Data for trypsin and chymotrypsin were normalized to total amylase content and expressed as fold vs. maximal; amylase secretion was expressed as % total. Dispersed pancreatic acinar cells were treated 15 mins prior to caerulein stimulation with phorbol ester (TPA; 200 nM), its biologically-inactive form TPA-4α, or TPA following 120 min pre-treatment with broad-spectrum PKC inhibitor GF109203X (10 µM); All treatments were added in DMSO. DMSO was added to non-TPA or GF109203X treated cells to control for non-specific DMSO effects. Following pre-treatment, cells were either unstimulated or stimulated with caerulein (0.1 nM or 100 nM) for 60min. *p<0.05 vs. corresponding caerulein alone condition. (N=3).

Figure 2. Broad-spectrum PKC inhibitor GF109203X reduces supraphysiologic caerulein-induced trypsinogen and chymotrypsinogen activation. (a) Trypsin and (b) chymotrypsin activities were measured and data normalized to amylase content and expressed as fold vs. caerulein. Dispersed pancreatic acinar cells were stimulated with supraphysiologic caerulein for 60 min following 120 min pre-treatment with broad-spectrum PKC inhibitor GF109203X (1 and 10 µM); DMSO was added to non-inhibitor treated controls. *p<0.05 vs. caerulein (N=3).
Figure 3. PKC δ and ε isoform-specific inhibitors reduce supraphysiologic caerulein-induced trypsinogen and chymotrypsinogen activation. (a) Trypsin and (b) chymotrypsin activities were measured and data normalized to amylase content and expressed as fold vs. caerulein. Dispersed pancreatic acinar cells were stimulated with supraphysiologic caerulein for 60 min following 120 min pre-treatment with isoform-specific PKC inhibitors: PKC translocation inhibitors δV1-1 or εV1-2 (10 µM); scrambled peptide (10 µM). (c) Combined effects of PKC translocation inhibitors δV1-1 and εV1-2 with GF 109203X (10 µM) on trypsin and chymotrypsin activities. DMSO was added to non-inhibitor treated controls *p<0.05 vs. caerulein (N=5).

Figure 4. PKC δ and ε isoform-specific inhibitors reduce trypsin and chymotrypsin activation in zymogen granule-enriched and microsomal pancreatic fractions in a reconstituted system. Trypsin (a and b) and chymotrypsin (c and d) activity were enhanced by the addition of a cytosolic fraction, and, in the case of chymotrypsin enhanced further by 5 mM ATP (experimental conditions described in Materials and Methods). Data were normalized to amylase content and expressed as fold vs. ZG/MIC + Cyt + ATP. Cytosol was pre-incubated for 15 mins with relevant PKC inhibitors (1µM), Scrambled peptide (SP; 1µM), or DMSO in controls (see Methods for details). * p<0.05 vs. ZG/MIC + Cyt + ATP. ZG: zymogen granule-enriched fraction; Mic: microsomal fraction; Cyt: cytosolic fraction. (n=5).
Figure 5. Sub-cellular distribution of PKC δ isoform in pancreatic acinar cells stimulated with supraphysiologic caerulein. Dispersed rat pancreatic acinar cells were pre-incubated for 120 min and then stimulated for 30 min with supraphysiologic caerulein (100 nM). Sub-cellular distribution of PKC δ was determined in cytosol and two membrane fractions (zymogen granule and microsomal membranes) using a PKC δ antibody. (a) Representative Western blots from 3 independent experiments. (b) Pooled data on the increase of PKC δ in membrane fractions; data was normalized to total protein content in each fraction and expressed as fold vs. unstimulated condition. *p< 0.05 vs. unstimulated (N=3). (c) Representative Western blots from 3 independent experiments showing that PKC translocation inhibitor δV1-1 (10 µM) reduces translocation of PKC δ to microsomal membranes.

Figure 6. Sub-cellular distribution of PKC ε isoform in pancreatic acinar cells stimulated with supraphysiologic caerulein. Dispersed rat pancreatic acinar cells were pre-incubated for 120 min and then stimulated for 30 min with supraphysiologic caerulein (100 nM). Sub-cellular distribution of PKC ε was determined in cytosol and two membrane fractions (zymogen granule and microsomal membranes) using a PKC ε antibody. (a) Representative Western blots from 3 independent experiments. (b) Pooled data on the increase of PKC ε in membrane fractions; data was normalized to total protein content in each fraction and expressed as fold vs. unstimulated condition. *p< 0.05 vs. unstimulated (N=3). (c) Representative Western blots from 3 independent experiments showing that PKC translocation inhibitor εV1-2 (10 µM) reduces translocation of PKC ε to microsomal membranes.
**Figure 7. PKC ε redistributes from an apical region to a supranuclear region of the acinar cell following supraphysiologic caerulein stimulation in vivo.** Male Sprague-Dawley rats were treated with a supraphysiologic concentration of caerulein (given by i.p injection). After 60 min, the animals were sacrificed and pancreatic tissue harvested. The distribution of PKC ε was detected using isoform-specific antibody (shown in red) in unstimulated (Control) and supraphysiologic caerulein-stimulated (CER). Note the redistribution of PKC ε to the supranuclear region after stimulation. (Apical region: arrowhead and circle; supranuclear region: arrow). Nuclei are DAPI stained (blue).

**Figure 8. PKC ε and lysosomal/endosomal marker GRAMP 92 co-distribution after supraphysiologic caerulein stimulation in vivo.** Male Sprague-Dawley rats were treated with supraphysiologic concentrations of caerulein (given by i.p injection) for 60 min. The distribution of PKC ε and GRAMP 92 were detected using specific antibodies (PKC ε in red; GRAMP 92 in green). In tissue from unstimulated animals PKC ε had a predominantly apical signal and GRAMP 92 was diffusely distributed throughout the cytosol with some punctate structures visible. Following supraphysiologic caerulein stimulation (CER) PKC ε and GRAMP 92 redistributed to the supranuclear region. Co-distribution be seen in yellow and this is verified by the z-section through the cell shown in the side-bar of the figure. (Apical region: arrowhead and circle; supranuclear region: arrow).
Figure 2

![Graph showing enzyme activity comparison between Trypsin and Chymotrypsin.](image)

<table>
<thead>
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<th>CER (100 nM)</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
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</table>
Figure 3

A

Trypsin Activity (fold vs. caerulein)

- CER (100 nM)
- PKC-I (10 µM)

B

Chymotrypsin Activity (fold vs. caerulein)

- CER (100 nM)
- PKC-I (10 µM)
FIGURE 4

C

PKC-I (1 µM)

ZG
ZG/CYT
ZG/CYT/ATP

Chymotrypsin Activity (fold vs ZG/CYT/ATP)

D

PKC-I (1 µM)

MIC
MIC/CYT
MIC/CYT/ATP

Chymotrypsin Activity (fold vs MIC/CYT/ATP)
Figure 5

A

CER (100 nM)  -  +

CYTOSOL

ZYMOGEN GRANULES

MICROSOMES

B

PKC-δ translocation (fold increase)

<table>
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<td>Zymogen granule membranes</td>
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<td>1.0</td>
</tr>
<tr>
<td>Microsomal membranes</td>
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<td>2.5</td>
</tr>
</tbody>
</table>

* indicates a significant difference.
Figure 5

Control  CER  CER + δ-Inhibitor
Figure 6

A

CER (100 nM)  -  +

CYTOSOL

ZYMOGEN GRANULES

MICROSOMES

B

PKC-ε translocation (fold increase)

Unstimulated  CER 100 nM

Zymogen granule membranes  Microsomal membranes
Figure 6

Control    CER    CER + ε-Inhibitor