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Ethanol sensitizes NF-κB activation in pancreatic acinar cells through effects on protein kinase C-ϵ

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Satoh, Akihiko, Anna S. Gukovskaya, Joseph R. Reeve Jr, Tooru Shimosegawa, and Stephen J. Pandol. Ethanol sensitizes NF-κB activation in pancreatic acinar cells through effects on protein kinase C-ϵ. *Am J Physiol Gastrointest Liver Physiol* 291: G432–G438, 2006. First published April 6, 2006; doi:10.1152/ajpgi.00579.2005.—Although ethanol abuse is the most common cause of pancreatitis, the mechanism of alcohol's effect on the pancreas is not well understood. Previously, we demonstrated that in vitro ethanol treatment of pancreatic acinar cells augmented the CCK-8-induced activation of NF-κB, a key signaling system involved in the inflammatory response of pancreatitis. In the present study, we determine the role for individual PKC isoforms in the sensitizing effect of ethanol on NF-κB activation. Dispersed rat pancreatic acini were treated with and without ethanol and then stimulated with CCK-8; 100 nM CCK-8 caused both NF-κB and PKC-δ, -ϵ, and -ζ activation, whereas 0.1 nM CCK-8 did not increase PKC-ϵ, PKC-ζ, or NF-κB activity. CCK-8 (0.1 nM) did activate PKC-δ. PKC-ϵ activator alone did not cause NF-κB activation; however, together with 0.1 nM CCK-8, it caused NF-κB activation. Ethanol activated PKC-ϵ without affecting other PKC isoforms or NF-κB activity. Of note, stimulation of acini with ethanol and 0.1 nM CCK-8 resulted in the activation of PKC-δ, PKC-ϵ, and NF-κB. The NF-κB activation to 0.1 nM CCK-8 in ethanol-pretreated acini was inhibited by both PKC-δ inhibitor and PKC-ϵ inhibitor. Taken together, these results demonstrate the different modes of activation of PKC isoforms and NF-κB in acini stimulated with ethanol, high-dose CCK-8, and low-dose CCK-8, and furthermore suggest that activation of both PKC-ϵ and -δ is required for NF-κB activation. These results suggest that ethanol enhances the CCK-8-induced NF-κB activation at least in part through its effects on PKC-ϵ.

alcoholic pancreatitis; cholecystokinin; inflammatory response; protein kinase C-ϵ translocation activator

ETHANOL ABUSE IS THE MOST common cause of acute pancreatitis; however, the mechanism of ethanol's effect on the pancreas remain unknown (1, 32). The fact that ethanol feeding to rats resulted in little or no pancreatic damage (18, 33) suggests that the effect of ethanol may be due to its ability to sensitize the pancreas to injurious actions of other agents. Previously, Pandol et al. (24) reported that ethanol feeding sensitized rats to pancreatitis in response to a low-dose CCK-8 that did not induce pancreatitis in control rats through, at least in part, its ability to augment NF-κB activation in the pancreas. Also, Gukovskaya et al. (13) recently reported that the in vitro ethanol treatment had a similar effect to enhance the CCK-8-induced activation of NF-κB activity in acinar cells through the activation of PKC.

PKCs are a family of serine/threonine kinases comprising 10 isoforms that differ in their structures and regulations (7, 26). These isoforms are subdivided into three classes on the basis of their molecular structure and mode of activation, namely, conventional PKC isoforms (α, βI, βII, and γ), novel PKC isoforms (δ, ϵ, η, and θ), and atypical PKC isoforms (ζ and λ/ι). Each PKC isoform has a different pattern of cell distribution, can be activated independently by specific stimuli, and mediates distinct biological functions. In general, the activation of PKCs is associated with their translocation to distinct intracellular compartments. Specific anchoring proteins target individual PKCs to different intracellular components and confer specificity for different substrates (7, 22, 26). In pancreatic acinar cells, four PKC isoforms, α, δ, ϵ, and ζ, have been detected (2, 19, 27). We identified that PKC-δ and -ϵ are responsible for NF-κB activation induced by both TNF-α and high-dose CCK-8 in pancreatic acinar cells (31).

Thus in the present study we addressed the possibility that the different effects of a low dose and high dose of CCK on NF-κB activation might be caused by differences in the patterns of PKC isoform activation and that ethanol activates a specific PKC isoform, which, in turn, mediates the effects of ethanol observed in acute pancreatitis.

We found that high-dose CCK-8 caused both NF-κB and PKC-δ, -ϵ, and -ζ activation, whereas low-dose CCK-8 activated only PKC-δ. Furthermore, ethanol activated PKC-ϵ. Together with both PKC-ϵ activator or ethanol, low-dose CCK-8 caused NF-κB activation. The NF-κB activation to 0.1 nM CCK-8 in ethanol-pretreated acini was inhibited by both PKC-δ inhibitor and PKC-ϵ inhibitor. Thus our data indicate the different modes of activation of PKC isoforms and NF-κB in acini stimulated with ethanol, high-dose CCK-8, and low-dose CCK-8. Furthermore, our data suggest that activation of both PKC-ϵ and -δ is required for NF-κB activation. The signal pathways that ethanol uses to enhance the CCK-8-induced NF-κB activation is due to its effect on PKC-ϵ.

MATERIALS AND METHODS

Reagents. CCK-8 was from American Peptide (Sunnyvale, CA); medium 199, from GIBCO (Grand Island, NY); [γ-^{32}P]ATP, from ICN Biomedicals (Costa Mesa, CA); GF109203X, PKC-δ peptide substrate, and PKC-ϵ peptide substrate, from Calbiochem (La Jolla,

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CA); PKC-ζ substrate, from Biosource International (Camarillo, CA); antibodies against PKC-α, PKC-ε, PKC-δ, and PKC-ζ, from Santa Cruz Biotechnology (Santa Cruz, CA); conventional PKC substrate, from Upstate Biotechnology (Charlottesville, VA); T4 polynucleotide kinase, from New England BioLabs (Beverly, MA); and poly(dI-dC), from Boehringer-Mannheim (Indianapolis, IN). All other chemicals were from Sigma Chemical (St. Louis, MO). PKC inhibitors were synthesized by BioSource (Lewisville, TX) using standard 9-fluorenylmethoxycarbonyl (FMOC) coupling strategies and unblocking in trifluoroacetic acid containing the appropriate scavengers. The PKC peptides were synthesized with the antennopedia peptide as a continuous solid phase synthesis using standard protocols for FMOC peptide synthesis. Crude peptides were purified by reverse-phase HPLC and lyophilized to dryness. All peptides were above 95% purity by analytical reverse-phase HPLC.

Preparation of dispersed pancreatic acini. Pancreatic acini were prepared from Sprague-Dawley rats (75–100 g) using a collagenase digestion method as described previously (12–15) and then incubated in 199 medium supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml) for 3.5 h at 37°C in a 5% CO_2-humidified atmosphere.

Preparation of nuclear extracts and electrophoretic mobility shift assay. Preparation of nuclear and cytosolic protein extracts and the EMSA have been described in detail (14, 15). In pancreatic acinar cells, the NF-κB band has two components. It has been previously shown (14, 24) that the upper component corresponds to the p50/p65 heterodimer and the lower component to the p50/p50 homodimer. For the present study, we quantified the total (combined) intensity of the NF-κB band.

Immunoprecipitation. Pancreatic acini were suspended in 1 ml of ice-cold homogenization buffer, sonicated 5 times for 10 s on ice, and incubated for 45 min at 4°C. The samples were centrifuged for 15 min at 15,000 *g*, and a specific antibody against an individual PKC isoform (1:100 dilution) was added to the lysate. The samples were then rotated overnight at 4°C. Protein A-Sepharose beads (50% slurry) were added, and the samples were rotated for another 2 h at 4°C. The beads were washed twice in the lysis buffer followed by an additional three washes with the kinase buffer [in nM: 20 MOPS (pH 7.2), 25 β-glycerophosphate, 5 EGTA, 1 Na_3VO_4, and 1 DTT]. The samples were then resuspended in a final 50-μl volume of kinase buffer.

Isoform specific PKC kinase assay. The kinase assay was performed with the PKC isoform-specific immunoprecipitate as described previously (31). Measurements were performed in duplication.

Subcellular fractionation and Western blot analysis. Cytosolic and membrane fractions from pancreatic acinar cells were prepared as described in detail (31) and used as samples for Western blot analysis. After samples were adjusted for protein concentration, equal amounts of protein were fractionated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked by overnight incubation in Tris-buffered saline (TBS), supplemented with 5% nonfat dry milk, and probed with an antibody against either PKC-δ or PKC-ε (1:200 dilution each) for 2 h at room temperature. The membranes were incubated with secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. Blots were developed using the enhanced chemiluminescence detection kit (Pierce, Rockford, IL). When reprobing was necessary, the membrane was stripped of bound antibody by incubating in stripping buffer at room temperature for 20 min.

Immunohistochemistry. Pancreatic acini were suspended in PBS and plated onto polylysine-coated glass coverslips. They were allowed to attach for 10 min at room temperature and then washed with PBS to remove unattached cells. The remaining cells were fixed with 4% paraformaldehyde for 15 min. After permeabilization with 0.5% Triton X-100 for 10 min, slides were incubated with PBS containing 1% BSA and 1% whole goat serum for 30 min, followed by primary antibodies specific for either PKC-δ or PKC-ε (1:100 dilution) for 30 min at room temperature. The cells were then washed three times with PBS and incubated with fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody (Cappel, Aurora, OH) for 1 h at room temperature. Imaging was performed with fluorescence microscopy (Eslipse TE 2000-S; Nikon, Lake Forest, CA).

Pharmacological analysis. For pharmacological analysis, we used a broad-spectrum PKC inhibitor, GF109203X. We synthesized a specific PKC-δ translocation inhibitor (δV1–1, S-F-N-S-Y-E-L-G-S-L), a PKC-ε translocation inhibitor (εV1–2, E-A-V-S-L-K-P-T), a specific PKC-ε translocation activator (ε-activator, H-D-A-P-I-G-Y-D), and a scrambled peptide (L-S-E-T-K-P-A-V) according to previous descriptions (31). For each of the PKC isoforms, these peptide inhibitors correspond to specific sequences in the V1 regions, which is responsible for anchoring the individual isoform to its translocation site (5, 10). Thus the peptide inhibitors competitively inhibit the binding of a specific isoform of PKC to its anchoring protein. This inhibition of binding prevents activation of the PKC isoform. The PKC-ε translocation activator binds to receptors for activated C-kinase sites in the V1 region of PKC-ε and destabilizes the inactive conformation of PKC-ε, resulting in an activation status for PKC-ε (5, 10). Each of these peptides was conjugated to a *Drosophila antennapedia* peptide (R-Q-I-K-I-W-F-Q-N-R-R-M-K-W-K-K) to make them cell permeable. Satoh et al. (31) has previously shown high potency and specificity of PKC isoform specific inhibitors in pancreatic acini.

As previously shown (13–15), CCK-8 causes a rapid and prolonged NF-κB activation in isolated rat pancreatic acini in a dose- and time-dependent manner and that the NF-κB response to 100 nM CCK-8 reaches a maximum at 30 min after the stimulation. Based on these results, we preincubated pancreatic acini with 100 mM ethanol, each PKC inhibitor (10 μM), scrambled peptide (10 μM), or vehicle for 3 h, followed by a 30-min incubation with the indicated concentration of CCK-8 or vehicle.

Statistical analysis. Values are means ± SE. The changes in NF-κB activation and PKC activity were calculated as the difference between stimulated and unstimulated conditions. The data were compared using an ANOVA, followed by the Fisher's protected least-significant difference test. A difference with a P value of <0.05 was considered statistically significant.

RESULTS

High-dose CCK-8 activates PKC-δ, -ε, and -ζ, whereas low-dose CCK-8 activates only PKC-δ in rat pancreatic acini. We stimulated isolated rat pancreatic acini with either 0.1 nM CCK-8 (low dose) that causes maximal digestive enzyme secretion or 100 nM CCK-8 (high dose) that is supramaximal for enzyme secretion and causes inhibition of enzyme secretion. As shown in Fig. 1, 0.1 nM CCK-8 does not cause NF-κB activation, whereas the high dose does. The CCK-8-induced NF-κB activation was inhibited by the broad-spectrum PKC inhibitor GF109203X by 98% (Fig. 1, *A* and *B*), suggesting the involvement of PKC in the signaling pathway. Treatment of pancreatic acini with PMA, a general activator of PKC, resulted in the activation of NF-κB, providing evidence that activation of PKC is sufficient for the NF-κB activation in pancreatic acinar cell.

Satoh et al. previously showed (31) that activation of both PKC-δ and PKC-ε are necessary for the NF-κB activation induced by both 100 nM CCK-8 and TNF-α in isolated rat pancreatic acini. To examine whether the different effects of a low dose and high dose of CCK on NF-κB activation might be caused by differences in the patterns of PKC isoform activation, we next determined PKC isoform specific activities by kinase assays using PKC isoform-specific immunoprecipitates. To date, four PKC isoforms, conventional PKC-α, novel PKC-δ and PKC-ε, and atypical PKC-ζ, have been identified in

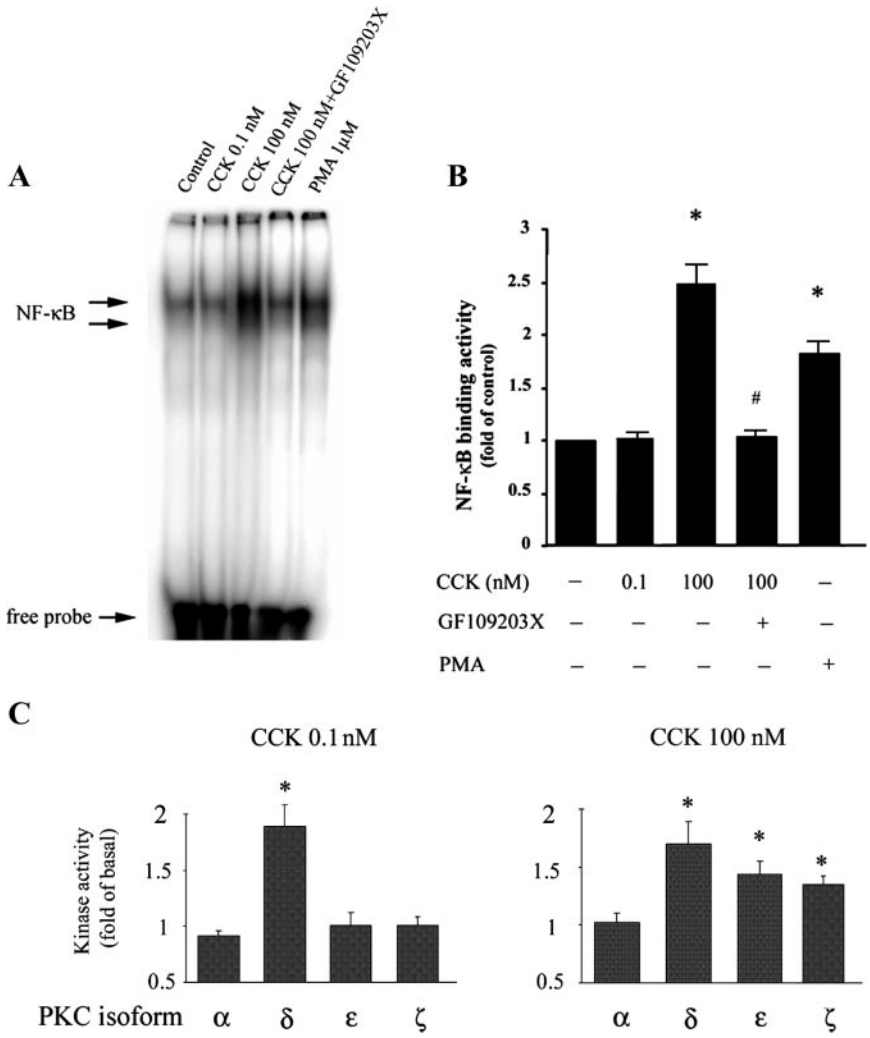

Fig. 1. Different modes of NF-κB and PKC activation in response to CCK-8 in pancreatic acini. Pancreatic acini were incubated for 3.5 h with the broad-spectrum PKC inhibitor GF109203X (10 μM) or with DMSO and then stimulated with 100 nM CCK-8 or 1 μM PMA during the last 30 min of the incubation. *A*: NF-κB binding activity was measured in nuclear extracts by EMSA. Representative of 5 independent experiments. *B*: NF-κB band intensities were quantified in the PhosphorImager and normalized on the band intensity in unstimulated control acini. Values are means ± SE of the values from 5 separate experiments. $^{*}P < 0.05$ compared with unstimulated control. $^{\#}P < 0.05$ compared with 100 nM CCK-8 alone. *C*: changes in PKC kinase activities stimulated by CCK-8. Individual PKC isoforms were immunoprecipitated from whole cell lysates, and PKC activities were measured by kinase assay using isoform-optimized substrates. For each PKC isoform, activity values were normalized on its basal activity in unstimulated control acini. Values are means ± SE from 6 to 8 separate experiments. $^{*}P < 0.05$ compared with each isoform basal activity.

the pancreatic acinar cell (2, 20, 27), each with unique modes of activation. As shown in Fig. 1*C*, 100 nM CCK-8 increased kinase activities for PKC-δ, -ε, and -ζ. PKC-α activity was not significantly altered by CCK-8. Treatment with 0.1 nM CCK-8 activated PKC-δ to about the same degree as that occurring with 100 nM CCK-8 without an activation of PKC-α, -ε, nor -ζ. Taken together, the results in Fig. 1 demonstrate differential effects of low-dose and high-dose CCK-8 on NF-κB and PKC isoform activation. Furthermore, the results along with those we previously reported suggest that activation of PKC-δ alone is not sufficient for CCK-8-induced NF-κB activation.

PKC-ε isoform-specific activator induces selective translocation of PKC-ε. We synthesized an isoform-specific PKC-ε translocation activator. The peptide activator destabilizes the inactive conformation of PKC-ε and acts as a PKC-ε selective agonist. To demonstrate the specificity of the PKC-ε activator in pancreatic acini, we examined its effects on PKC-ε and -δ. Treatment with PKC-ε translocation activator decreased the presence of PKC-ε in the cytosolic fraction and increased it in the membrane fraction, indicating translocation from cytosol to cell membranes as measure of PKC activation (Fig. 2*A*, *left*). In contrast, no changes in the subcellular localization of PKC-δ (Fig. 2*A*, *right*), -α, and -ζ (data not shown) were detected after the PKC-ε activator stimulation. The specific effect of the PKC-ε activator on PKC-ε translocation was furthermore confirmed by immunostaining. In unstimulated pancreatic acini, the staining of PKC-ε was present in the cytosolic area of the pancreatic acinar cell (Fig. 2*B*, *left*). When pancreatic acini were incubated with the PKC-ε activator, we observed a ring-like pattern of staining for PKC-ε around the nucleus and cell periphery (Fig. 2*B*, *right*), indicating the translocation of PKC-ε to the perinuclear region and plasma membrane.

Combination of PKC-ε activator and low-dose CCK-8 induces the NF-κB activation in pancreatic acini. To furthermore investigate the role for PKC-ε in the regulation of NF-κB activation in pancreatic acini, we used the PKC-ε translocation activator. As shown in Fig. 3, neither 0.1 nM CCK-8 nor PKC-ε activator alone could activate NF-κB, suggesting that the activation of either PKC-δ or PKC-ε is not sufficient to lead to NF-κB activation. When acini were incubated with 0.1 nM CCK-8 together with the PKC-ε translocation activator, increased NF-κB activation as measured by EMSA was observed. These results demonstrate that low-dose CCK-8 requires the activation of PKC-ε for NF-κB activation in pancreatic acinar cell.

Inhibition of PKC-δ and -ε prevents the ethanol-sensitized NF-κB activation to low-dose CCK-8. In the next set of experiments, pancreatic acini were incubated with 100 mM of

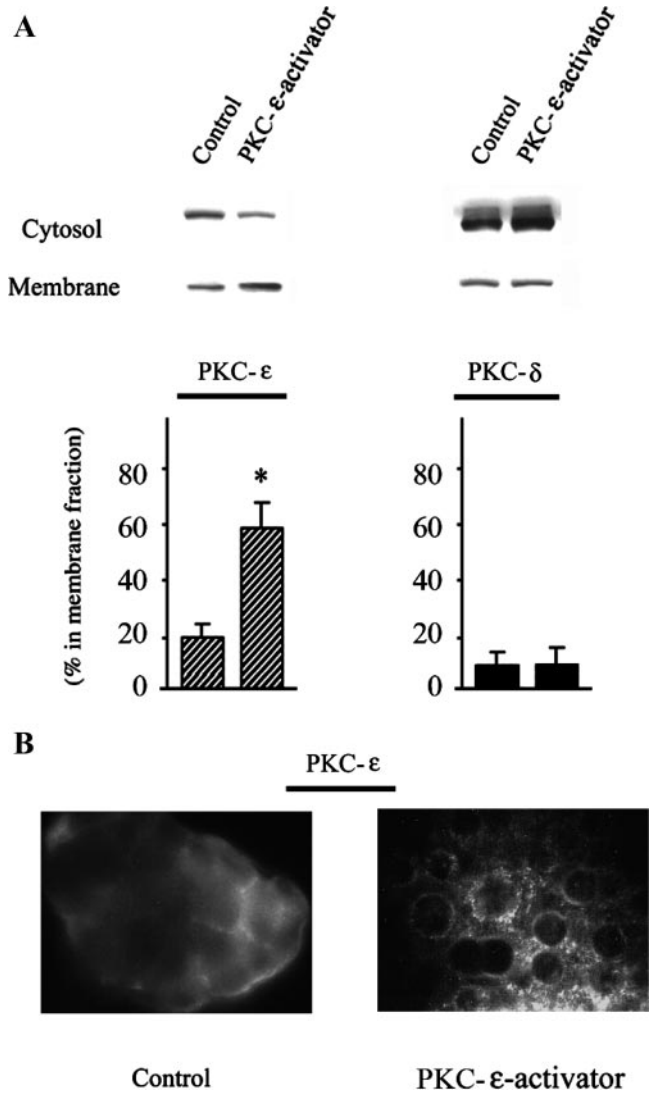

Fig. 2. The PKC-ϵ specific translocation activator induces selective translocation of PKC-ϵ in pancreatic acinar cells. Pancreatic acini were preincubated with 10 μM of PKC-ϵ translocation activator or scrambled peptide (control) for 3 h. *A*: Western blots (*top*) from independent experiments. Cytosolic and membrane fractions were subjected to SDS-PAGE and blotted using antibodies specific for PKC-ϵ or -δ. The graphs (*bottom*) represent the intensity of the band for each PKC isoform in the membrane fraction vs. the cytosolic plus membrane fraction. Values are means ± SE from 3 separate experiments for each isoform. $*P < 0.05$ compared with the value for unstimulated cells. *B*: micrographs showing immunofluorescence localizaion of PKC-ϵ in pancreatic acinar cells. Original magnification, ×100.

ethanol for 3 h and then stimulated with 0.1 nM CCK-8 for 30 min. The concentration of ethanol consists with the alcohol concentration in blood of rats continuously fed ethanol (24). Gukovskaya et al. (13) previously reported that such treatment with ethanol did not affect cell viability and CCK-induced amylase secretion in pancreatic acini. In acini pretreated with ethanol, 0.1 nM CCK-8 induced an increase in NF-κB activation as measured by EMSA (Fig. 4, *A* and *B*). To determine the role of PKC isoforms in mediating the ethanol-sensitized NF-κB activation by CCK-8, we performed pharmacological inhibitory analysis with isoform-specific PKC inhibitors. Ethanol-sensitized NF-κB activation to 0.1 nM CCK-8 was almost completely inhibited by the PKC-δ translocation inhibitor δV1–1 and the PKC-ϵ translocation inhibitor ϵV1–2 (Fig. 4, *A* and *B*). Neither δV1–1 nor ϵV1–2 alone affected the basal NF-κB activity. The conventional PKC isoform inhibitor (Gö6976) or PKC-ζ inhibitor (PKC-ζ pseudosubstrate) did not affect NF-κB activation (data not shown). These results indicate that both PKC-δ and PKC-ϵ mediate ethanol-sensitized NF-κB activation in response to low doses of CCK-8 in pancreatic acinar cells.

Ethanol activates PKC-ϵ in rat pancreatic acini. We next addressed the possibility that ethanol activates PKC-ϵ and -δ in pancreatic acinar cells and that the activation of these PKC isoforms mediates the effects of ethanol on NF-κB activation observed with low-dose CCK-8 stimulation. In acini treated with 100 mM ethanol for 3 h, the increase in kinase activity was observed only in PKC-ϵ, but not in PKC-α, -δ, or -ζ (Fig. 5*A*, *left*). The combination of ethanol and low-dose CCK-8 induced the activation of PKC-ϵ and -δ. Consistent with the results on kinase activity, translocation of PKC-ϵ from the cytosol to the membrane fraction was observed in acini treated with ethanol alone or ethanol plus low-dose CCK-8 (Fig. 5, *B* and *C*). Also illustrated in Fig. 5*C* are the effects of ethanol alone and ethanol with low-dose CCK-8 on PKC-δ. In contrast to PKC-ϵ, ethanol had no effect on PKC-δ translocation,

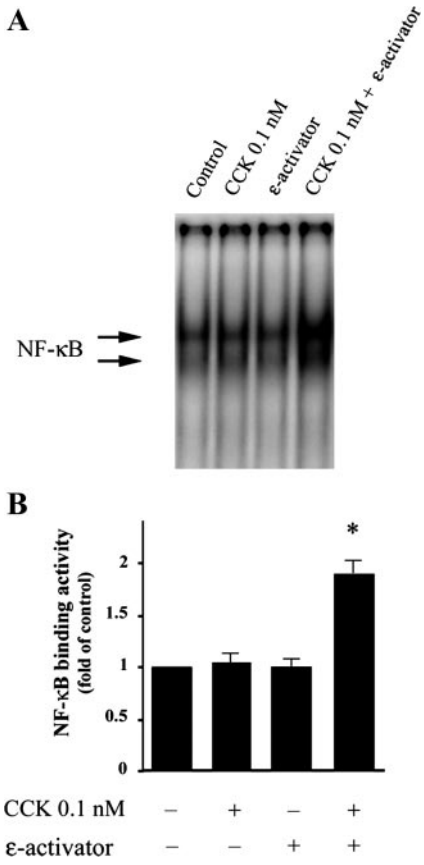

Fig. 3. Effects of the PKC-ϵ specific translocation activator on NF-κB activation in pancreatic acini. Pancreatic acini were preincubated with 10 μM of PKC-ϵ translocation activator or scrambled peptide for 3.5 h and then stimulated with 0.1 nM CCK-8 during the last 30 min of the incubation. *A*: NF-κB binding activity was measured in nuclear extracts by EMSA. *B*: NF-κB band intensities were quantified in the PhosphorImager and normalized on the band intensity in unstimulated control acini. Values are means ± SE from four separate experiments. $*P < 0.05$ compared with unstimulated control.

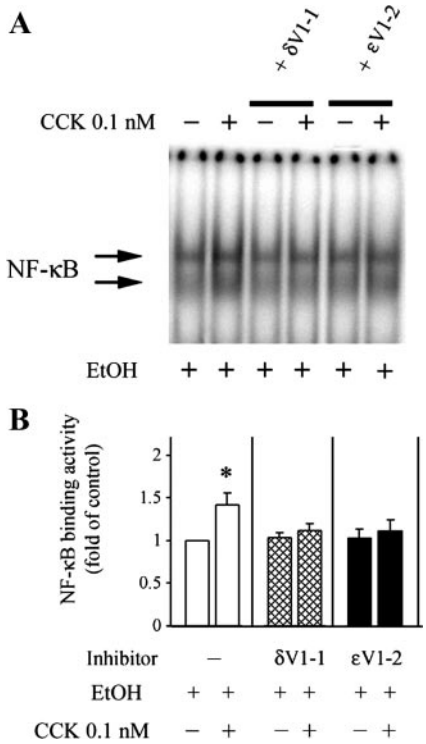

Fig. 4. Inhibition of PKC-δ or -ϵ prevents ethanol-sensitized NF-κB activation to low doses of CCK-8 in pancreatic acini. Pancreatic acini were incubated with PKC-δ translocation inhibitor (δV1–1), PKC-ϵ translocation inhibitor (ϵV1–2), or with scrambled peptide, 10 μM each for 4 h. Thirty minutes after the initiation of the incubation, 100 mM of ethanol was added; then 0.1 nM CCK-8 was added during the last 30 min of the incubation. *A*: NF-κB binding activity was measured in nuclear extracts by EMSA. Representative of 6 independent experiments. *B*: NF-κB band intensities were quantified in the PhosphorImager and normalized on the band intensity in unstimulated control acini. Values are means ± SE from 6 separate experiments. $*P < 0.05$ compared with unstimulated control.

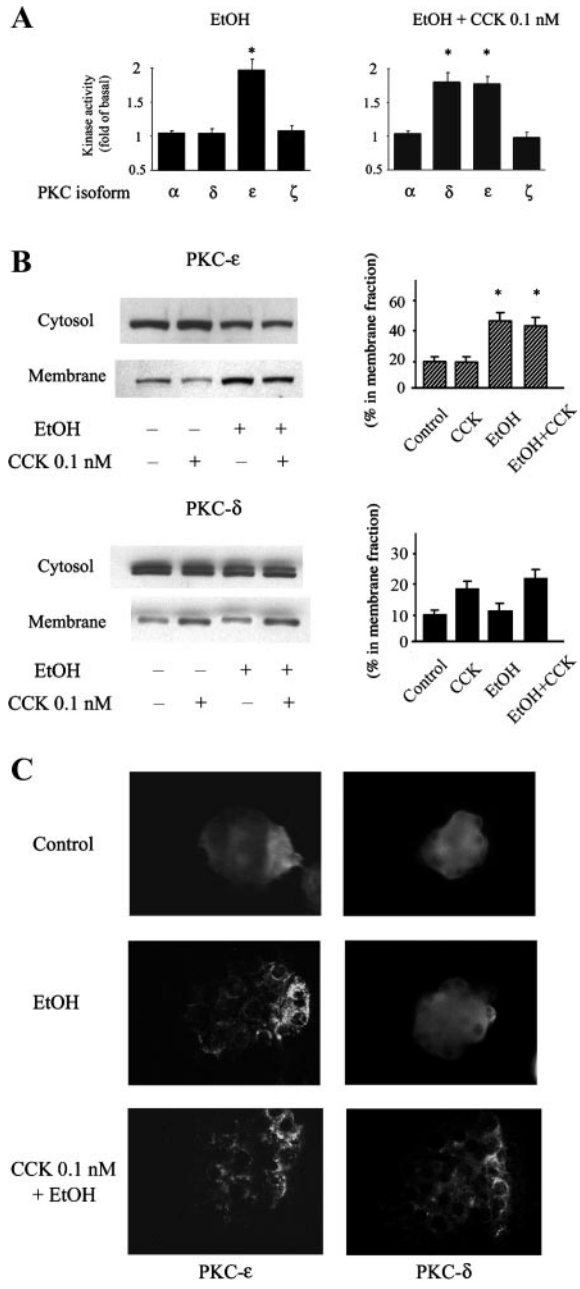

Fig. 5. Effects of ethanol on PKC activation in pancreatic acinar cells. Pancreatic acini were incubated with 100 mM of EtOH for 3.5 h and then stimulated with 0.1 nM CCK-8 during the last 30 min of the incubation. *A*: changes in PKC kinase activities stimulated by ethanol and CCK-8. Individual PKC isoforms were immunoprecipitated from whole cell lysates, and PKC activities were measured by kinase assay using isoform-optimized substrates. For each PKC isoform, activity values were normalized to its basal activity as measured in unstimulated control acini. Values are means ± SE from 6 to 8 separate experiments. $*P < 0.05$ compared with each isoform basal activity. *B*: cytosolic and membrane fractions were subjected to SDS-PAGE and blotted using antibodies specific for PKC-ϵ or -δ. The graphs (*right*) represent the intensity of the band for each PKC isoform in the membrane fraction vs. the cytosolic plus membrane fraction. Values are means ± SE from 3 separate experiments for each isoform. $*P < 0.05$ compared with the value for unstimulated cells. *C*: micrographs showing immunofluorescence localizaion of PKC-ϵ (*left*) and PKC-δ (*right*) in pancreatic acinar cells. Images are from 3 independent experiments. Original magnification, ×100.

whereas the combination of low-dose CCK-8 and ethanol did cause PKC-δ translocation. Of note, the activation of PKC-ϵ with ethanol and low-dose CCK-8 to activate PKC-δ is sufficient for the NF-κB activation in pancreatic acinar cell (see Fig. 4). The activation of PKC-ϵ by ethanol became evident after 2 h (Fig. 6), and that finding correlated with our previous observation in which the augmentation effect of ethanol on CCK-8-induced NF-κB activation requires over 2 h incubation period. Considering these findings, it seems likely that ethanol enhances the CCK-8-induced NF-κB activation, at least in part, through the activation of PKC-ϵ.

Inhibition of PLC did not prevent the ethanol-induced PKC-ϵ activation. Previous studies demonstrated that CCK-8 activates PKC through activation of PLC, which results in the hydrolysis of phosphatidylinositol and the resulting production of diacylglycerol. To investigate whether ethanol-induced PKC-ϵ activation is mediated by PLC in pancreatic acini (17, 25, 34), we applied U-73122, an inhibitor of PLC. As shown in Fig. 7, U-73122 did not affect the ethanol-induced PKC-ϵ activation. We previously showed that U-73122 at the same concentration prevented the CCK-induced PKC activation and NF-κB activation (31). Therefore, the result indicates that the activation of PKC-ϵ by ethanol is not mediated by PLC in pancreatic acini.

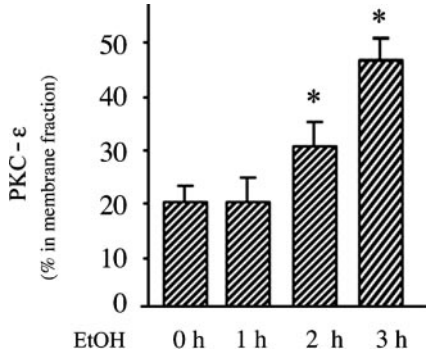

Fig. 6. Ethanol activates PKC-ϵ in pancreatic acini. Pancreatic acini were incubated with and without 100 mM of EtOH for the indicated times. Results represent the intensity of the band for each PKC isoform in the membrane fraction vs. the cytosolic plus membrane fraction. Values are means ± SE from 3 separate experiments for each isoform. *$P < 0.05$ compared with the value for unstimulated cells (0 h).

DISCUSSION

In the present study, we investigated the intracellular signaling pathways that mediate ethanol-sensitized NF-κB activation in rat pancreatic acini, especially the role for PKC isoforms. We found that high-dose CCK-8 activates both NF-κB and PKC-δ, -ϵ, and -ζ, whereas low-dose CCK-8 activated only PKC-δ. Furthermore, our results provide evidence that low-dose CCK-8 can cause NF-κB activation when PKC-ϵ was activated in pancreatic acini. Indeed, we demonstrated that ethanol exposure is capable of activating PKC-ϵ. The activation of PKC-δ and -ϵ isoforms is required for the NF-κB activation induced by the combination of ethanol and low-dose CCK-8. Our results also showed that PLC is not involved in mediating PKC-ϵ activation by ethanol.

Among four PKC isoforms, α, δ, ϵ, and ζ, that have been identified in the pancreatic acinar cell, PKC-δ and -ϵ mediate both CCK-8-induced and TNF-α-induced NF-κB activation (31). Our results indicate that activation of one of these PKC isoforms is not enough for NF-κB activation because individually low-dose CCK-8 and ethanol activated PKC-δ and PKC-ϵ, respectively. However, neither low-dose CCK-8 or ethanol activated NF-κB. On the other hand, a general PKC activator PMA stimulated the activation of NF-κB, suggesting that activation of PKC is sufficient for the NF-κB activation in the pancreatic acinar cell. It has been shown that conventional PKC-α and novel PKC-δ and -ϵ, but not atypical PKC-ζ, are PMA-responsible isoforms (7, 30), and Gukovskaya et al. (13) previously showed that PKC-α is not involved in the PMA-induced NF-κB activation. Taken together, it seems likely that activation of both PKC-δ and -ϵ are necessary and sufficient for NF-κB activation in pancreatic acinar cells.

In the present study, the PKC-ϵ translocation activator, designed to destabilize the inactive conformation of PKC-ϵ, induced NF-κB activation together with low-dose CCK-8. Of note, the PKC-ϵ translocation activator did not affect PKC-δ, indicating that low-dose CCK-8 requires the activation of PKC-ϵ for NF-κB activation in pancreatic acinar cells.

We used the *antennapedia*-conjugated cell penetrating peptide inhibitors and activator. The homeodomain of *antennapedia* has been shown to translocate through the plasma membrane in a receptor-independent manner (3, 8, 9, 28, 29) and to allow the cellular delivery of conjugated biomolecules across the cell membrane without inducing leakage. Although the mechanism of membrane penetration remains unclear, three different models, namely, inverse micelle model (8, 28), electroporation-like model (3, 35), and endocytic model (29), are currently being considered. In the previous study (31), the high potency and specificity of the translocation inhibitors δV1–1 and ϵV1–2 in pancreatic acini were demonstrated. Furthermore, each inhibitor abolished the CCK-induced increase in their target isoform's kinase activity without affecting the other isoform's activity. The effects of both δV1–1 and ϵV1–2 were evident at 0.1 μM, reached a maximum at 1 μM (31), and required at least 3 h of incubation (data not shown).

Despite the finding that ethanol exposure sensitizes acinar cells to CCK-induced NF-κB activation (13, 24), the signaling pathways through which this was mediated remained unclear. Importantly, we demonstrate that ethanol exposure leads to the activation of PKC-ϵ in pancreatic acinar cells. Furthermore, the activation of NF-κB by low-dose CCK-8 in ethanol-treated acini was almost completely inhibited by the PKC-δ inhibitor δV1–1 and the PKC-ϵ inhibitor ϵV1–2. Therefore, the effects of ethanol to sensitize NF-κB activation result from its effect on PKC-ϵ. Similar effects of ethanol by PKC-ϵ activation has been reported in a number of cells (11, 21, 36).

Because U-73122, an inhibitor of PLC, did not alter the ethanol-induced activation of PKC-ϵ, this activation signaling appears to be independent of the PLC pathway. One possibility is that ethanol interacts directly or indirectly with the membrane lipid bilayer (7, 23, 30), modulates its fine structure, and thereby activates the enzymatic activity of the PKC-ϵ. Another possibility is its effect on the phosphorylation status of PKC-ϵ (4, 7, 26). Additional studies are necessary to understand the mechanism of how ethanol selectively activates PKC-ϵ.

Pandol et al. (24) previously showed upregulation of NF-κB and inflammatory molecule expression in a model of pancreatitis in which an ethanol diet sensitizes rats to pancreatitis induced by low-dose CCK. On the other hand, activation of NF-κB with PMA does not lead to pancreatitis responses in pancreatic acini. Compared with CCK-8 stimulation, the increase in NF-κB activity by PMA is shorter and weaker (13, 16). Therefore, the different mode of NF-κB activation or

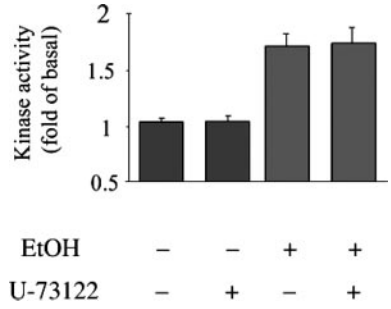

Fig. 7. Effects of PLC inhibitor on PKC-ϵ activity induced by ethanol. Pancreatic acini were incubated with PLC inhibitor U-73122 (10 μM) for 3.5 h and 100 mM of ethanol (EtOH) for the last 3 h of the incubation. Whole cell lysates were immunoprecipitated with PKC-ϵ antibody, and PKC-ϵ activity was measured by kinase assay using isoform-optimized substrate. Activity values were normalized to the basal activity in unstimulated control acini. Values are means ± SE from 3 separate experiments. *$P < 0.05$ compared with each isoform basal activity.

additional signals other than NF-κB activation (6, 20) might be involved in the injurious effect of ethanol.

In conclusion, the results of the present study provide new insights into the signaling pathways by which ethanol causes injury to the pancreatic acinar cell. Ethanol activates PKC-ϵ, which cooperates with PKC-δ activation by CCK to cause NF-κB activation. Together with the previous findings (12–15, 24, 31), the results of the present study furthermore establish the key role for the novel PKC isoforms δ and ϵ in the mechanism of acute pancreatitis.

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