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Raf-1 Kinase Inhibitory Protein (RKIP) Mediates Ethanol-induced Sensitization of Secretagogue Signaling in Pancreatic Acinar Cells*

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Background: The molecular mechanism by which ethanol (EtOH) sensitizes pancreatic acinar cells to secretagogue stimulation is largely undefined.
Results: Ethanol stimulates PKC-dependent RKIP phosphorylation, and RKIP ablation prevents EtOH-induced sensitization of secretagogue Ca^{2+} signaling and aberrant chymotrypsin activation.
Conclusion: RKIP mediates the cytotoxic effects of EtOH on pancreatic acinar cells.
Significance: Modulation of RKIP expression may have therapeutic utility in the prevention or treatment of alcohol-associated pancreatitis.

Excessive alcohol consumption is associated with most cases of chronic pancreatitis, a progressive necrotizing inflammatory disease that can result in pancreatic insufficiency due to acinar atrophy and fibrosis and an increased risk of pancreatic cancer. At a cellular level acute alcohol exposure can sensitize pancreatic acinar cells to secretagogue stimulation, resulting in dysregulation of intracellular Ca^{2+} homeostasis and premature digestive enzyme activation; however, the molecular mechanisms by which ethanol exerts these toxic effects have remained undefined. In this study we identify Raf-1 kinase inhibitory protein as an essential mediator of ethanol-induced sensitization of cholecystokinin- and carbachol-regulated Ca^{2+} signaling in pancreatic acinar cells. We show that exposure of rodent acinar cells to ethanol induces protein kinase C-dependent Raf-1 kinase inhibitory protein phosphorylation, sensitization of cholecystokinin-stimulated Ca^{2+} signaling, and potentiation of both basal and cholecystokinin-stimulated extracellular signal-regulated kinase activation. Furthermore, we show that either suppression of Raf-1 kinase inhibitory protein expression using short hairpin RNA or gene ablation prevented the sensitizing effects of ethanol on cholecystokinin- and carbachol-stimulated Ca^{2+} signaling and intracellular chymotrypsin activation in pancreatic acinar cells, suggesting that the modulation of Raf-1 inhibitory protein expression may have future therapeutic utility in the prevention or treatment of alcohol-associated pancreatitis.

Excessive consumption of ethanol (EtOH) (≥2–5 drinks/day) is a major risk factor for pancreatitis (1, 2), a necro-inflammatory disease of varying severity that in the United States results in ~200,000 hospitalizations annually at a cost of over $2 billion (3–5). Although EtOH and its metabolites exert numerous effects on all cell types of the pancreas, the sensitization of pancreatic acinar cells to secretagogue signaling, premature intracellular zymogen activation, and deregulation of intracellular Ca^{2+} homeostasis have been identified as critical early events in the pathogenesis of the disease (6–8).

Under normal physiological conditions, the cytoplasmic concentration of free Ca^{2+} ($[Ca^{2+}]_{cyto}$) in pancreatic acinar cells is tightly regulated. The activation of G protein-coupled receptors (GPCRs)[2] by pancreatic secretagogues, such as cholecystokinin (CCK) and acetylcholine, leads to the opening of second-messenger-gated Ca^{2+} channels located on the endoplasmic reticulum (ER) and various apical acidic vesicles (9–14). The released Ca^{2+} from these internal stores, in turn, stimulates the Ca^{2+}-induced Ca^{2+} influx through plasma membrane Ca^{2+} channels (15–18) resulting in a transient and localized rise in $[Ca^{2+}]_{cyto}$, mainly in the apical region of the cell.

Limiting the secretagogue-induced increase in $[Ca^{2+}]_{cyto}$ are ATP-dependent Ca^{2+} pumps that return the $[Ca^{2+}]_{cyto}$ to resting levels by both extruding Ca^{2+} against its concentration gradient back into the extracellular space and by re-sequestering Ca^{2+} into the intracellular stores. Together, the coordinated regulation of the various ligand-gated Ca^{2+} channels, ATP-dependent pumps, and proteinaceous Ca^{2+} buffers produce the transient, localized, oscillatory increases in $[Ca^{2+}]_{cyto}$ that are responsible for the normal meal-induced secretion of fluids and zymogens into pancreatic ducts and, eventually, into the small intestine where they are activated to facilitate nutrient digestion (19–21).

Exposing either experimental animals or isolated pancreatic acinar cells to EtOH sensitizes the cells to physiologic secretagogue stimulation (22–26) and deregulates normal agonist-in-

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[2] The abbreviations used are: GPCR, G protein-coupled receptor; CCK, cholecystokinin; GRK, G protein receptor kinase; RKIP, Raf-1 kinase inhibitory protein; NT, non-targeting; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

duced Ca^{2+} responses and zymogen activation (27, 28). Specifically, it has been shown that after treatment of isolated pancreatic acinar cells either with EtOH or its non-oxidative metabolites, physiological concentrations of secretagogue induces sustained increases in $[Ca^{2+}]_{cyto}$ that are no longer limited to the apical pole but spread throughout the acinar cell (28, 29). Associated with the prolonged aberrant Ca^{2+} signal is an inhibition of apical zymogen secretion (30), impaired mitochondrial function (*i.e.* reduced ATP generation) (31), the generation of reactive oxygen species (27, 32) Ca^{2+}-dependent intracellular zymogen activation (28, 33–35), and Ca^{2+}-dependent necrotic cell death (36–38).

Despite progress in understanding the critical role of aberrant Ca^{2+} signaling in alcohol-induced acinar cell dysfunction, the underlying molecular mechanism(s) by which EtOH sensitizes acinar cells to secretagogue stimulation remains largely undefined. In this study we identify Raf-1 kinase inhibitory protein (RKIP) as an essential mediator of EtOH-induced sensitization of CCK- and carbachol-regulated Ca^{2+} signaling and intracellular chymotrypsin activation in pancreatic acinar cells, suggesting that the modulation of RKIP expression may have a future therapeutic utility in the prevention or treatment of alcohol-associated pancreatitis.

EXPERIMENTAL PROCEDURES

Materials—Antibodies to phosphorylated ERK1 and ERK2 (catalog no. 4376) and total ERK1,2 (catalog no. 9102) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies to RKIP and Ser(P)-153-RKIP (catalog no. sc-28837 and sc-32623, respectively), β-actin (catalog no. sc-1616), and PKCε (catalog no. sc-214) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Fura-2 acetoxymethyl ester was purchased from eBioscience, Inc. (San Diego, CA). Breeding pairs of RKIP null (−/−) mice were generously provided by Drs. Jan Klysik and John Sedivy through an inter-institutional materials transfer agreement between Brown University (Providence, RI) and the University of Texas Medical Branch (Galveston, TX).

AR42J Cell Line—The rat AR42J cell line was selected for these studies because it exhibits a variety of features common to normal pancreatic acinar cells, including expression of secretagogue receptors (*e.g.* cholecystokinin 1 and muscarinic acetylcholine receptors), zymogen granules, and components of the Ca^{2+}-dependent-regulated secretory pathway (39). AR42J cell cultures were maintained in Ham's F12K medium supplemented with L-glutamine (2 mM) and 20% fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Mouse Primary Acinar Cells—Mouse pancreatic acinar cells were isolated using a modification of the enzymatic dissociation method previously described (40). Briefly, the pancreata from 2–3 mice were removed, perfused with saline, washed, and minced with fine scissors in a cell isolation solution containing phosphate-buffered saline with Ca^{2+} and Mg^{2+}, pH 7.4, supplemented with 0.1% bovine serum albumin (BSA) and 0.01% soybean trypsin inhibitor. The minced tissue was digested for 15 min in 3 ml of 37 °C isolation buffer containing 0.3 mg/ml collagenase type IV (Sigma). The enzyme digestion was facilitated mechanically by continuous pipetting the minced tissue suspension with a 10-ml pipette for 15 min. The digested tissue (*i.e.* clusters of acini) were washed with 6 ml of cold isolation buffer and collected by centrifugation (1000 rpm for 2 min). The washing step was repeated twice to remove small debris and blood cells. The smaller acinar cell clusters were further separated from the larger acini by filtering through a sterile 860-μm stainless steel mesh. After centrifugation (1000 rpm for 2 min), the supernatant was removed, and the cell pellets were resuspended in 5 ml of oxygenated Dulbecco's modified Eagle's medium supplemented with 10% FBS, 1% antibiotic/antimycotic, and 1% soybean trypsin inhibitor and then plated on either laminin-coated culture plates for biochemical experiments or coverslips for intracellular Ca^{2+} imaging (see "Ratiometic Intracellular Ca^{2+} Imaging Microscopy" below). All cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C and used for experimentation within 24 h of isolation. For quality control, acinar cell viability was assessed by trypan blue staining with >95% viability obtained for each experiment presented below.

Isolation of Phosphorylated Protein—Phosphorylated proteins where isolated using the phosphoprotein enrichment kit (Pierce Protein Research Products, catalog no. 90003), Thermo Fisher Scientific, Inc. Rockford, IL). The affinity columns in the Pierce Phosphoprotein Enrichment kit contain a proprietary metal that interacts with negative charges from phosphate groups. The optimized buffer conditions enable specific capture of phosphoproteins from complex biological samples. Briefly, cells were first washed twice with a non-phosphate-based HEPES (50 mM) buffer, pH 7.4, scraped from the culture plate in 1 ml of lysis solution supplemented with CHAPS detergent (0.25%), EDTA-free Halt Protease Inhibitors, and Halt Phosphatase Inhibitors (Thermo Fisher Scientific catalog nos. 78425 and 78420, respectively), incubated on ice for 45 min with periodic vortexing, and spun in a centrifuge at 10,000 × *g* for 20 min (4 °C) to pellet cellular debris. The protein concentration of the supernatants were determined using the Bio-Rad protein assay reagent, and 2.5 mg of total protein from each assay condition was applied to a phosphoprotein affinity column equilibrated with 5 ml of cell lysis solution containing 0.25% CHAPS. The protein samples were loaded onto the columns by centrifugation at 1000 × *g* for 1 min at 4 °C. Each column was then washed 3 times to remove unbound proteins by adding 5 ml of lysis solution with 0.25% CHAPS and spinning the column in a 50-ml conical tube at 1000 × *g* for 1 min at 4 °C. The bound phosphoproteins were eluted in a high salt elution buffer containing 75 mM sodium phosphate buffer, pH 7.5, 500 mM NaCl, and 0.25% CHAPS. 1 ml of elution buffer was added to each column, which was then incubated at room temperature with occasional agitation for 3 min. The eluate was collected by centrifugation at 1000 × *g* for 1 min at 4 °C. The elution procedure was repeated 4 times for a total of five elution fractions (5 ml total). The phospho-RKIP content of the pooled fractions was assessed by Western blotting.

Western Blotting—Cells were treated as indicated in the figure legends, and protein extracts were prepared using either the phosphoprotein enrichment kit or a cell lysis solution containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet

P-40, 1 mM sodium orthovanadate, 5 mM β-glycerophosphate, 1 mM NaF, 1 mM PMSF, 1 protease inhibitor mixture tablet (Roche Diagnostics) per 50 ml of solution. Insoluble cellular material was removed by centrifugation at 14,000 rpm for 15 min, and the concentration of proteins in the supernatant was determined using the Bio-Rad protein assay reagent and BSA as a standard. 20–40 μg of soluble protein extract was loaded per well of a Bis-Tris·HCl-buffered polyacrylamide gel (Invitrogen), resolved by electrophoresis, and transferred onto a PVDF membrane (Millipore Corp., Bedford, MA). For immunostaining, PVDF membranes were incubated in blocking solution containing 5% (w/v) nonfat milk dissolved in Tris-buffered saline plus 0.1% Tween 20 (TBST) for 1 h at room temperature. All primary antibodies were diluted in TBST containing 1% nonfat milk. All blots were incubated with primary antibody overnight at 4 °C. After washing, the membranes were probed with a horseradish peroxidase-conjugated secondary antibody. Immunoreactive protein bands were detected by using ECL™ Western blotting detection reagent (Amersham Biosciences) and Kodak X-Omat film.

Ratiometic Intracellular Ca^{2+} Imaging Microscopy—Isolated acinar cells were loaded with 2 μM Fura-2-AM solution containing 0.025% Pluronic F-127 at 25 °C for 35 min as previously described (41). The cells were washed and resuspended in Krebs-Ringers Henseleit solution consisting of NaCl (125 mM), KCl (5 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (2 mM), glucose (6 mM), and HEPES (25 mM), pH 7.4. Fura-2-loaded cells were then transferred to either laminin or BD Cell-TAK™ (BD Biosciences)-coated glass coverslips (25-mm diameter) for 1 h or 15 min, respectively, at 37 °C. EtOH (100 mM) was added to the cells for 10 min at room temperature. Changes in the concentration of free cytosolic Ca^{2+} ($[Ca^{2+}]_{cyto}$) were monitored with a Nikon Diaphot inverted microscope (Nikon Instrument Group, Garden City, NY) equipped with a Nikon 40× (1.3 NA) Neofluor objective. The fluorescent light source was a PTI DeltaScan RD-1 ratio fluorescence spectrometer system (Photon Technology International, Inc., Birmingham, NJ) equipped with a light-path chopper and dual-excitation monochrometers. The Fura-2 was alternately excited at 340 and 380 nm, and fluorescence emission was monitored through a 510-nm bandpass filter with a Roper Coolsnap EZ digital camera. Data were obtained from single acinar cells and from single cells of doublets and triplet cell clusters. All experiments were performed at room temperature.

Suppression of RKIP Expression Using shRNA—RNA interference constructs containing four gene-specific shRNA sequences (Rat Pebp1 Gene ID 29542) were obtained from OriGene Technologies (Rockville, MD) and analyzed for their effectiveness at suppressing RKIP protein levels in AR42J cells. Of the four shRNA expression constructs, infection of AR42J cells with a retrovirus containing the RKIP-specific sequence (5′-GCCGCTGTCATTACAGGAGGTGGATGAGC-3′) resulted in the greatest reduction in RKIP protein levels (Fig. 4*A*). To control for off-target effects, control cultures were infected with a virus expressing a non-targeting 29-mer scrambled shRNA sequence (OriGene, catalog no. TR30013). Retroviruses were made by simultaneously transfecting HEK293FT cells (Invitrogen) with the shRNA expression plasmid plus plasmids encoding MLV gag-pol and vesicular stomatitis virus envelope protein. Transfection efficiency was >80% as determined by green fluorescent protein expression from the shRNA vector. Cell supernatants from the transfected HEK293FT cells were collected and filtered through a 0.45-μm filter to remove cell debris and stored at −80 °C. AR42J cells were plated in 12-well plates so that they were ~50% confluent at the time of infection (~24 h post-plating). To infect the cells, culture medium was removed, and 1 ml of room temperature HEK293FT cell supernatant containing virus was added. Infected cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C under puromycin dihydrochloride (10 μg/ml) selection.

Chymotrypsin Assay—Pancreatic acinar cells were isolated as described above ("Mouse Primary Acinar Cells"), and chymotrypsin activity was assayed using a modification of a previously described procedure (42). Briefly, acinar cells were pretreated with EtOH (100 mM) for 10 min and stimulated with different concentrations of CCK or vehicle over a time course. At the end of each incubation period, the cells and culture medium were transferred to an ice-cold 1.5-ml centrifuge tube, homogenized with a small pestle, and spun in a centrifuge at 1000 × *g* at 4 °C for 1 min. A portion of the supernatant proteins (20 μl) from each sample was added to a 96-well plate containing 70 μl of enzyme assay buffer (50 mM Tris, pH 8.1, 150 mM NaCl, I mM $CaCl_2$, 0.01% BSA) per well. The chymotrypsin activity assay was initiated by adding 10 μl of fluorogenic chymotrypsin substrate II (EMD Chemicals, Inc., Gibbstown, NJ) to each well (t = 0). The cleavage of substrate was monitored at 440 nm over a time course using a Gemini XPS Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, CA). Data points were collected at 15-min intervals for up to 60 min. The enzyme activity in each sample was normalized to the concentration of total protein (using the Bio-Rad protein assay reagent) in an equivalent volume of supernatant from the same sample.

Statistical Analysis—Statistical analysis (Student's t test) was performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). Statistical significance was assumed if $p \leq 0.05$.

RESULTS

EtOH Induces the PKC-dependent Phosphorylation of RKIP and Potentiates CCK Signaling—RKIP is a scaffold protein that inhibits multiple kinase-regulated signaling pathways including 1) mitogen-activated protein kinases (MAPKs), 2) GPCR kinases (GRKs), and 3) kinases regulating the activation of nuclear factor κB (NFκB) (for review, see Zeng, L. *et al.* (43)). RKIP-dependent regulation of these different pathways is controlled both by its phosphorylation status and level of protein expression. PKC-mediated phosphorylation of serine residue 153 (Ser-153) leads to increased Raf-1/MEK/ERK-dependent signaling by decreasing the inhibitory binding of RKIP to Raf-1 (44) while simultaneously increasing RKIP association with and inhibition of GRK-2, resulting in reduced agonist-induced GPCR desensitization (45–48) (Fig. 1*A*). Four different PKC isozymes have been identified in rodent pancreatic acinar cells, PKCα, -δ, -ζ, and -ϵ (49). Exposure of isolated acinar cells to EtOH (20–100 mM) has been shown to activate the classical isozyme PKCα (50, 51) and the novel isozyme PKCϵ (22). However, a molecular mechanism linking EtOH-induced activation

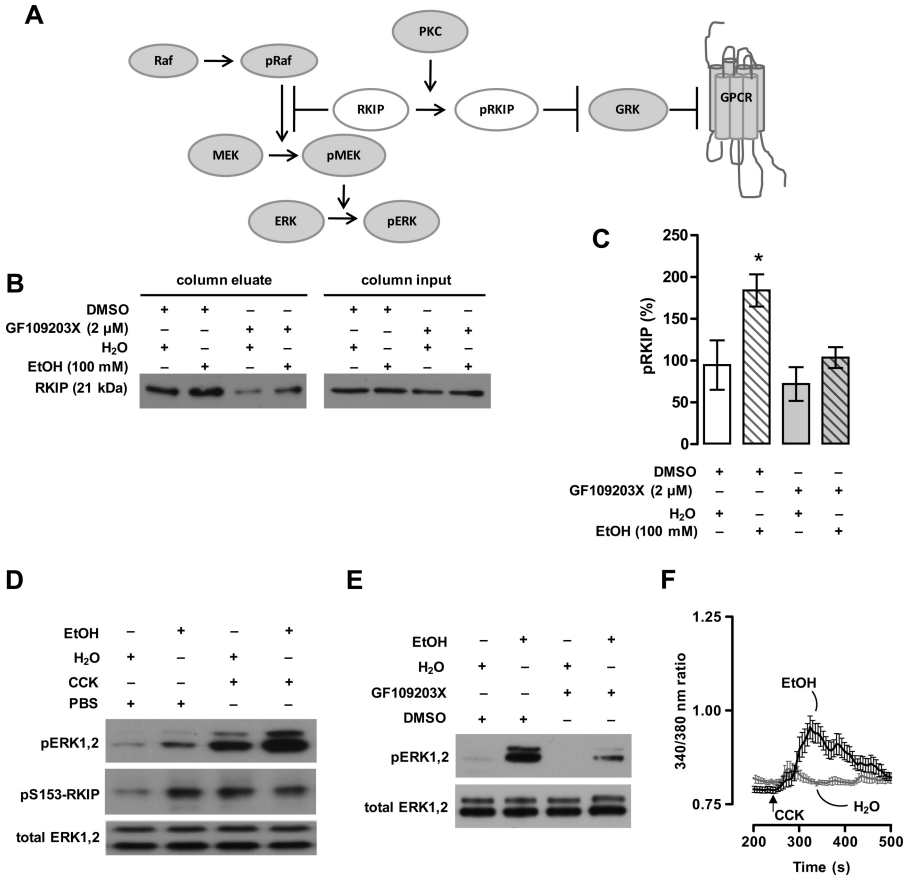

FIGURE 1. **EtOH exposure induces RKIP phosphorylation and enhances both basal and CCK-stimulated signaling.** *A*, shown is a pathway model illustrating the relationship between PKC-dependent RKIP phosphorylation and inhibition of the Raf/MEK/ERK signaling pathway and GRK. *B*, representative Western blots show levels of phosphorylated RKIP protein in column eluates after enrichment of AR42J protein extracts on phosphoprotein affinity columns (2.5 mg of protein extract was loaded on each column, and 20 μg of protein from the eluate was load per lane). AR42J cells were pretreated with 2 μM GF109203X or DMSO for 1 h and then exposed to 100 mM EtOH or H_2O for 10 min. To ensure that equivalent amount of RKIP protein was loaded on to each column, 5 μg of AR42J protein extract from each sample was resolved on a separate gel, transferred to PDVF membranes, and probed for RKIP levels (*column input*). *C*, a *bar graph* summarizes densitometry data from three independent experiments. The data represent the ratio of phosphorylated RKIP (column eluate) to the total amount of RKIP in the column input multiplied by 100. *Error bars* are S.E. Statistical analysis (Student's *t* test) was performed using GraphPad Prism 5 software. Densitometry was performed using ImageJ software (imagej.nih.gov). *D*, an immunoblot of AR42J cell protein extracts shows the effects of EtOH exposure on basal and CCK-8-stimulated phosphorylation of ERK1 and -2 (pERK1,2) and RKIP at Ser-153 (*pS153-RKIP*). Blots where reprobed with an antibody to total ERK1,2 to ensure the equal loading and transfer of proteins for each sample. *E*, an immunoblot shows the effects of GF109203X (2 μM) pretreatment (1 h) on EtOH-induced phosphorylation of ERK1 and -2 using freshly isolated mouse pancreatic acinar cells. *F*, a plot of the change in 340/380-nm ratio of Fura-2 fluorescence *versus* time (*s*) shows the effects of EtOH exposure on CCK-8-stimulated increase in $[Ca^{2+}]_{cyto}$. Each tracing is the mean data collected from 35–40 cells ± S.D.

of these PKC isozymes to the sensitization of secretagogue signaling in acinar cells has not been identified.

To begin testing the hypothesis that RKIP is a downstream target of EtOH-induced PKC activity and regulator of CCK receptor sensitization, we first pretreated AR42J cells either with the PKC inhibitor, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide (GF109203X) (2 μM), which inhibits the classical PKC isozymes, PKCα, -βI, -βII, and -γ (52), or its solvent dimethyl sulfoxide (DMSO) for 1 h and then exposed the cells to EtOH (100 mM) or an equivalent volume of its diluent (H_2O) for 10 min. After enriching for phosphorylated proteins by affinity chromatography ("Experimental Procedures"), the phosphoproteins in the column eluates were resolved by SDS-PAGE, transferred to a PVDF membrane, and probed using a rabbit polyclonal antibody to RKIP. Fig. 1*B* shows representative immunoblots depicting the levels of phosphorylated RKIP in the column eluates and the levels of total RKIP protein in an equivalent volume of each protein extract before being applied to the phosphoprotein affinity resin (*i.e.* column input). Densitometric analyses from three independent experiments showed that the level of phosphorylated RKIP increased ~2-fold in cells treated with EtOH when compared with controls (*i.e.* cells treated with either DMSO or GF109203X and H_2O) (Fig. 1*C*). Furthermore, the EtOH-induced increase in phosphorylated RKIP was significantly inhibited in cells pretreated with GF109203X (*, $p < 0.03$, EtOH alone *versus* EtOH plus GFX109203).

Having demonstrated that RKIP is a downstream target of EtOH-stimulated, GF109203X-sensitive PKC activity, we next determined the effects of EtOH treatment on ERK activation (*i.e.* ERK phosphorylation) and secretagogue-induced Ca^{2+} signaling. If RKIP is mediating the effects of EtOH on CCK-induced signal transduction, the model depicted in Fig. 1*A* predicts that phosphorylation of RKIP should reduce RKIP-mediated inhibition of the Raf/MEK/ERK pathway and enhance GPCR-mediated signaling by inhibiting GRK-dependent

receptor desensitization. Consistent with this model, immunoblots of AR42J cell extracts revealed that EtOH pretreatment stimulated an increase in the basal levels of phosphorylated (activated) ERK1 and ERK2 (*pERK1,2*) (Fig. 1*D*) that was associated with a concomitant increase in the level of Ser-153-phosphorylated RKIP (*pS153-RKIP*) (Fig. 1*D*). Combined treatment with EtOH and CCK (0.1 nM) further increased the levels of pERK1,2 when compared with either agent alone (Fig. 1*D*). CCK treatment alone also increased the level of Ser(P)-153-RKIP (Fig. 1*D*), which is consistent with previous reports showing that CCK treatment activates PKC in rodent pancreatic acinar cells (26, 51, 53, 54). However, the combined treatment with EtOH and CCK did not further increase Ser(P)-153-RKIP, suggesting that CCK can also activate ERK by mechanisms independent of the release of RKIP-mediated inhibition of Raf.

Similar to AR42J cells, exposing freshly isolated mouse pancreatic acinar cells to EtOH increased the levels of activated ERK1 and ERK2 (Fig. 1*E*). Both the basal and EtOH-stimulated levels of pERK1,2 were reduced in cells pretreated for 1 h with GF109203X (Fig. 1*E*). Finally, exposing AR42J cells to EtOH potentiated CCK-induced increases in the concentration of free cytosolic Ca^{2+} ($[Ca^{2+}]_{cyto}$) when compared with control (H_2O treated) cells (Fig. 1*F*). Collectively, these data support the hypothesis that RKIP mediates the EtOH-induced, PKC-dependent, sensitization of secretagogue (CCK) signaling in AR42J and freshly isolated pancreatic acinar cells.

PKCε Mediates EtOH-induced RKIP Phosphorylation and Sensitization of CCK-stimulated Ca^{2+} Signaling—PKCε has also been shown to be activated upon exposure of isolated rat acinar cells to EtOH (26), but it is not inhibited by GF109203X. Therefore, to specifically assess the function of PKCε in EtOH-induced RKIP phosphorylation, we inhibited its activation with a PKCε translocation inhibitor peptide (EAVSLKPT) as previously described (55, 56). This peptide corresponds to the V1 region of PKCε that is responsible for membrane translocation (activation) of the kinase. Functionally, the peptide blocks PKCε activity by competitively inhibiting the binding of the enzyme to its endogenous membrane-anchoring protein (55, 56). To ensure the effectiveness of the inhibitor peptide in blocking PKCε activation, AR42J cells were pretreated either with the PKCε translocation inhibitor peptide (10 μM) or a negative-control peptide with the sequence (LSETKPAV) (10 μM) for 30 min and then exposed to EtOH or an equivalent volume of H_2O for 10 min. After treatment, the cellular membrane and cytoplasmic protein fractions were isolated by differential centrifugation as previously described (57). Immunoblot analyses to determine PKCε levels in the two subcellular fractions showed that cells treated with the negative-control peptide exhibited relatively high levels of PKCε in the cytoplasmic fraction and little enzyme associated with cell membranes (Fig. 2*A*). Pretreatment with the PKCε inhibitor peptide caused a small increase in the level of cytoplasmic PKCε and reduced membrane quantities to below the level of antibody detection (Fig. 2*A*). Exposing the control peptide-treated cells to EtOH for 10 min decreased cytoplasmic PKCε levels and increased the amount of enzyme in the membrane fraction (Fig. 2*A*), indicating EtOH-induced enzyme activation. The stimulatory effect of EtOH on PKCε membrane translocation was blocked in cells

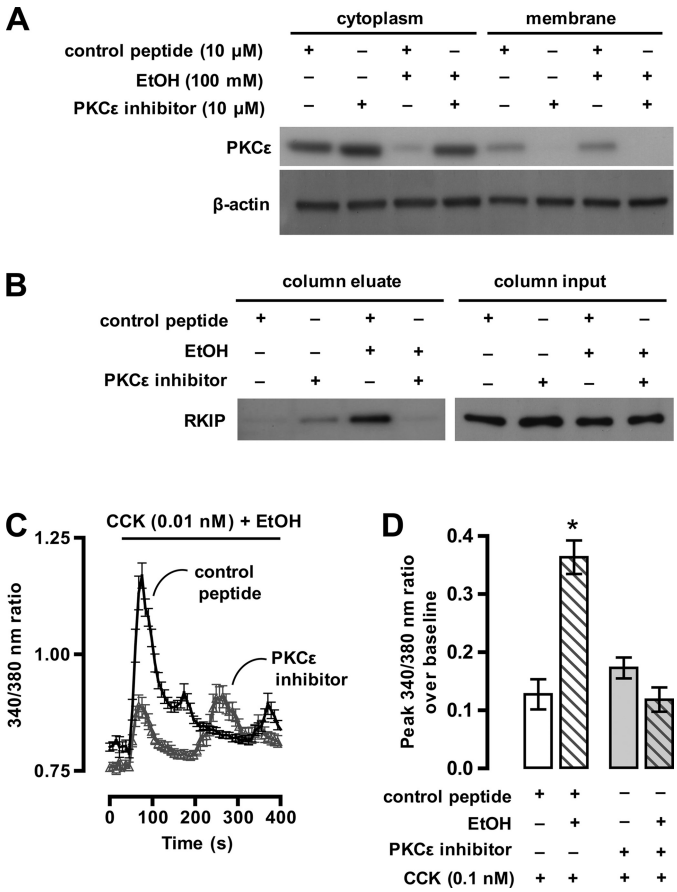

FIGURE 2. **PKCε mediates EtOH-induced RKIP phosphorylation and potentiation of CCK-stimulated Ca^{2+} signaling.** *A*, an immunoblot shows the relative expression levels of PKCε in the cytoplasm and membrane fraction of AR42J cells pretreated either with PKCε inhibitor peptide or a control peptide and exposure to EtOH (100 mM) for 10 min. The blot was reprobed with an antibody to β-actin to ensure the equal loading and transfer of proteins for each sample. *B*, an immunoblot shows the effect of PKCε inhibition on EtOH-induced RKIP phosphorylation (*column eluate*; 20 μg of eluted protein per lane). AR42J cells were treated as indicated followed by enrichment of phosphoproteins using affinity resin ("Experimental Procedures"). The level of RKIP expression in each sample was assessed to ensure that an equivalent amount of RKIP protein was loaded on to each column (column input; 2 μg of AR42J cell protein extract per lane). *C*, a plot of change in the 340/380-nm ratio of Fura-2 fluorescence *versus* time shows the effects of PKCε inhibition on EtOH-induced potentiation of CCK-8-stimulated increase in $[Ca^{2+}]_{cyto}$. Each tracing is the mean data from 30–40 cells ± S.D. Images were collected every 2–3 s. *D*, a summary plot of data from three independent experiments shows the effects of PKCε inhibition on EtOH-induced (*open bars*) potentiation of CCK-8-stimulated increase in $[Ca^{2+}]_{cyto}$. The *y* axis plots the peak change in the 340/380-nm ratio of Fura-2 fluorescence over base line ± S.E. of the initial Ca^{2+} peak after CCK-8 stimulation. Statistical analysis was performed using GraphPad Prism 5 software.

pretreated with the PKCε inhibitor peptide (Fig. 2*A*). Together these data show that, similar to previous reports using primary rat acinar cells (26), EtOH treatment of AR42J cells induces PKCε membrane translocation (*i.e.* activation). Furthermore, they demonstrate that the PKCε inhibitor peptide effectively blocks enzyme activation.

Having established the effectiveness of the PKCε inhibitor peptide in AR42J cells, we next determined the function of PKCε in EtOH-induced RKIP phosphorylation and CCK-stimulated Ca^{2+} signaling. After pretreatment either with the inhibitor or control peptides and exposure of the cells to EtOH or H_2O, the phosphorylated proteins were enriched by affinity

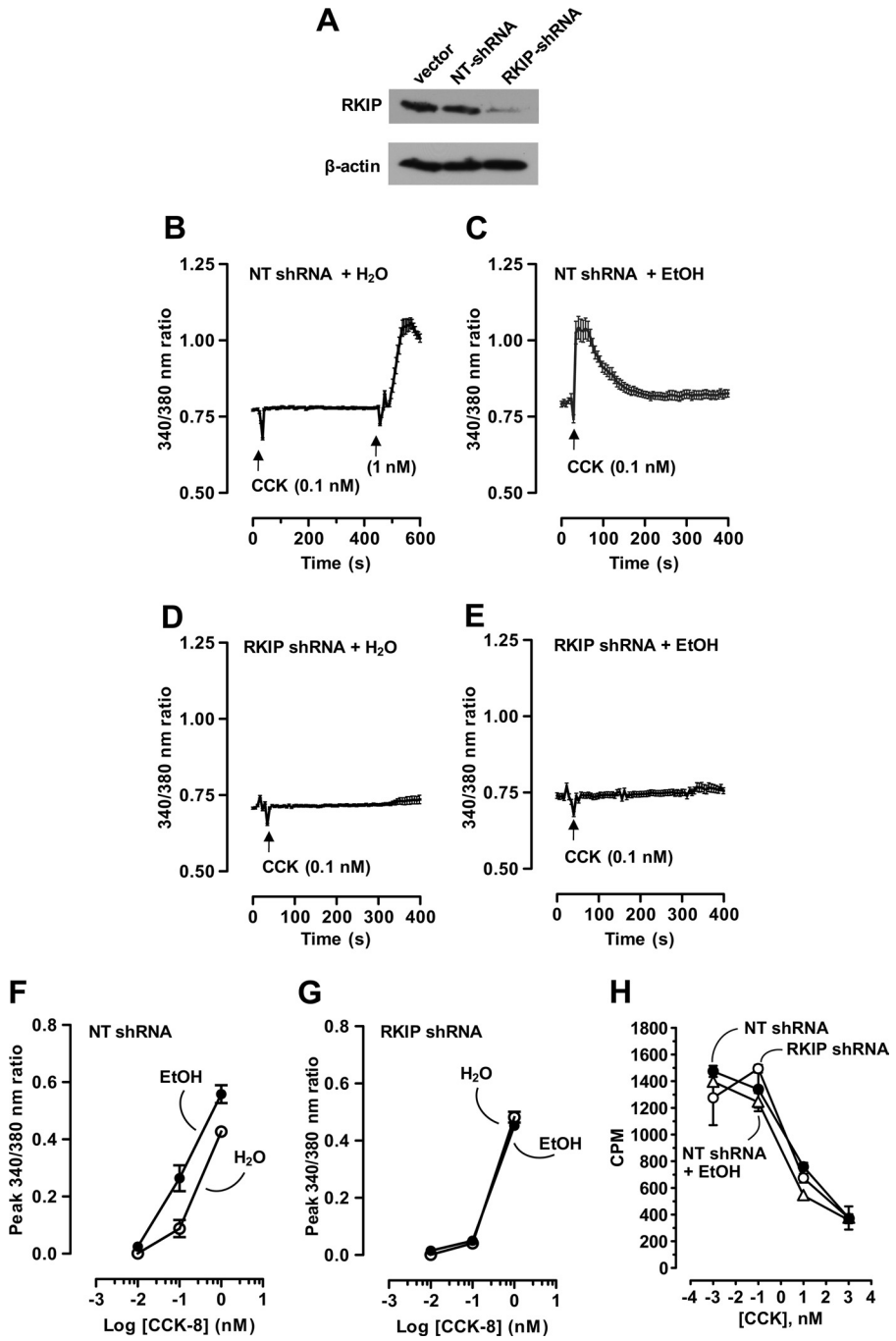

FIGURE 3. **Effect of shRNA-mediated knockdown of RKIP on EtOH-induced potentiation of CCK-stimulated Ca^{2+} signaling.** *A*, the immunoblot shows relative levels of RKIP protein expression in AR42J cell extracts after stable transduction either with a retroviral empty vector (*vector*), retrovirus contain an non-targeting control shRNA (*NT-shRNA*), or a retrovirus containing an RKIP gene-specific shRNA (*RKIP-shRNA*). The blot was reprobed with an antibody to *β*-actin to ensure the equal loading and transfer of proteins for each sample. *B* and *C*, plots of change in the 340/380-nm Fura-2 fluorescence *versus* time shows the effects of NT-shRNA expression on CCK-stimulated Ca^{2+} responses in cells exposed either to H_2O (*B*) or EtOH (100 mM) (*C*) for 10 min before agonist stimulation. *B*, cells were exposed first to a low concentration of CCK-8 (0.1 nM) which failed to induce an increase in $[Ca^{2+}]_{cyto}$ followed by a higher concentration (1.0 nM) to demonstrate that the cells were responsive to the agonist (positive control). *C*, cells exposed to EtOH showed a robust increase in $[Ca^{2+}]_{cyto}$ in response to a low concentration of CCK (0.1 nM). *D* and *E*, plots of change in the 340/380-nm ratio of Fura-2 fluorescence ± S.D. *versus* time (*s*) showed the effects of RKIP-shRNA expression on CCK-stimulated Ca^{2+} responses in cells exposed either to H_2O (*D*) or EtOH (100 mM) (*E*) for 10 min before agonist stimulation. *E*, knockdown of RKIP expression prevented EtOH-induced potentiation of CCK-stimulated increases in $[Ca^{2+}]_{cyto}$. Each tracing is the mean data collected from 30–40 cells ± S.D. Images were collected every 2–3 s. *F* and *G*, log plots of the initial peak 340/380-nm ratio of Fura-2 fluorescence ± S.D. *versus* concentration of CCK-8 (nM) compares AR42J cells transduced either with NT-shRNA (*F*) or RKIP-shRNA (*G*) and pretreated for 10 min either with EtOH (100 mM) or an equivalent volume of H_2O. *H*, competition binding assay compare the effects of EtOH exposure and RKIP expression on ^{125}I-labeled CCK binding to AR42J cells expressing either shRNA to RKIP (*RKIP shRNA*, *open circles*) or shRNA to a nonspecific nucleotide sequence (*NT shRNA*, *closed circles*).

chromatography, resolved by SDS-PAGE, and transferred to PVDF membranes. Immunoblots of the column eluates revealed an increase in the level of phosphorylated RKIP in cells treated with control peptide and exposed to EtOH, whereas the stimulatory effect of EtOH on RKIP phosphorylation was completely blocked in cells treated with the PKCε inhibitor peptide

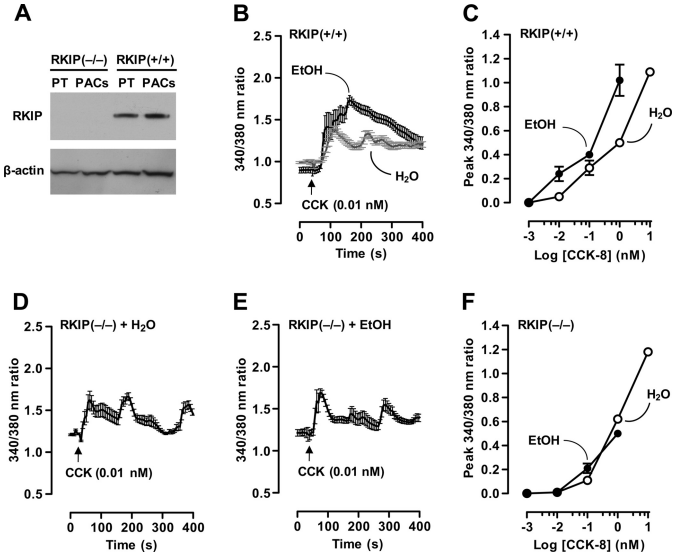

FIGURE 4. **Effect of RKIP gene ablation on EtOH-induced sensitization of the CCK-stimulated Ca^{2+} response in freshly isolated mouse pancreatic acinar cells.** *A*, the immunoblot shows levels of RKIP expression in pancreatic tissue (*PT*) and pancreatic acinar cells (*PACs*) isolated from RKIP null $RKIP^{-/-}$ and wild-type ($RKIP^{+/+}$) control mice. The blot was reprobed with an antibody to β-actin to ensure the equal loading and transfer of proteins for each sample. *B*, plots of change in the 340/380-nm ratio of Fura-2 fluorescence ±S.D. *versus* time shows the effects of EtOH exposure on CCK-stimulated (0.01 nM) increases in $[Ca^{2+}]_{cyto}$ in acinar cells from $RKIP^{+/+}$ mice. Each tracing represents the mean data ± S.D. from 30 cells. *C*, log plots of the initial peak 340/380-nm ratio of Fura-2 fluorescence ± S.D. *versus* concentration of CCK-8 (nM) compares freshly isolated acinar cells from $RKIP^{+/+}$ mice pretreated for 10 min either with EtOH (100 mM) or an equivalent volume of H_2O. *D* and *E*, plots of change in the 340/380-nm ratio of Fura-2 fluorescence ±S.D. *versus* time shows the effects of EtOH exposure on CCK-stimulated (0.01 nM) increases in $[Ca^{2+}]_{cyto}$ in acinar cells from $RKIP^{-/-}$ mice. *F*, log plots of the initial peak 340/380-nm ratio of Fura-2 fluorescence ± S.D. *versus* concentration of CCK-8 (nM) compare acinar cells for $RKIP^{-/-}$ with and without pretreatment with EtOH. All experiments were repeated 3 times using freshly isolated acinar cells with >95% viability base on trypan blue staining.

(Fig. 2*B*). Analyses of the secretagogue-stimulated Ca^{2+} responses showed that PKCε inhibition prevented the EtOH-induced potentiation of CCK-induced increases in $[Ca^{2+}]_{cyto}$ (Fig. 2*C*). A summary analysis of data from three independent experiments demonstrated an ~3-fold increase in the 340/380-nm ratio of Fura-2 fluorescence in the initial CCK-stimulated Ca^{2+} peak compared with controls when the cells were pretreated with negative-control peptide and exposed to EtOH (Fig. 2*D*). The EtOH-induced potentiation of the CCK-stimulated Ca^{2+} response was completely blocked in cells treated with the PKCε inhibitor peptide (Fig. 2*D*) (*, $p < 0.01$, control peptide + EtOH *versus* PKCε inhibitor + EtOH).

Suppression of RKIP Expression Prevents EtOH-induced Sensitization of CCK Signaling in AR42J Cells—Next, we evaluated whether suppression of RKIP expression mimicked the effects of PKC inhibition on secretagogue-stimulated increases in $[Ca^{2+}]_{cyto}$. To suppress RKIP protein expression, AR42J cells were infected with a retrovirus containing a gene-specific shRNA sequence targeting RKIP. Control cultures were infected either with a virus containing a non-targeting (NT) control shRNA or the empty retroviral expression construct. An immunoblot of protein extracted revealed almost complete suppression of RKIP expression in cells transduced with RKIP shRNA when compared with either the NT-shRNA control or empty viral vector (Fig. 3*A*). Evaluation of CCK-induced Ca^{2+} signaling showed that, compared with H_2O-treated cells (Fig. 3, *B* and *D*), EtOH exposure potentiated CCK-stimulated increases in $[Ca^{2+}]_{cyto}$ in cells infected either with NT-shRNA (Fig. 3*C*) or the empty expression vector (data not shown) but not in cells infected with the retrovirus containing RKIP shRNA (Fig. 3*E*). A comparison of the effects of EtOH exposure on CCK-stimulated increases in $[Ca^{2+}]_{cyto}$ over a range of agonist concentrations revealed that NT-shRNA-infected cells exhibited an enhanced CCK-induced Ca^{2+} responses over the range of agonist concentrations when compared with cells exposed to

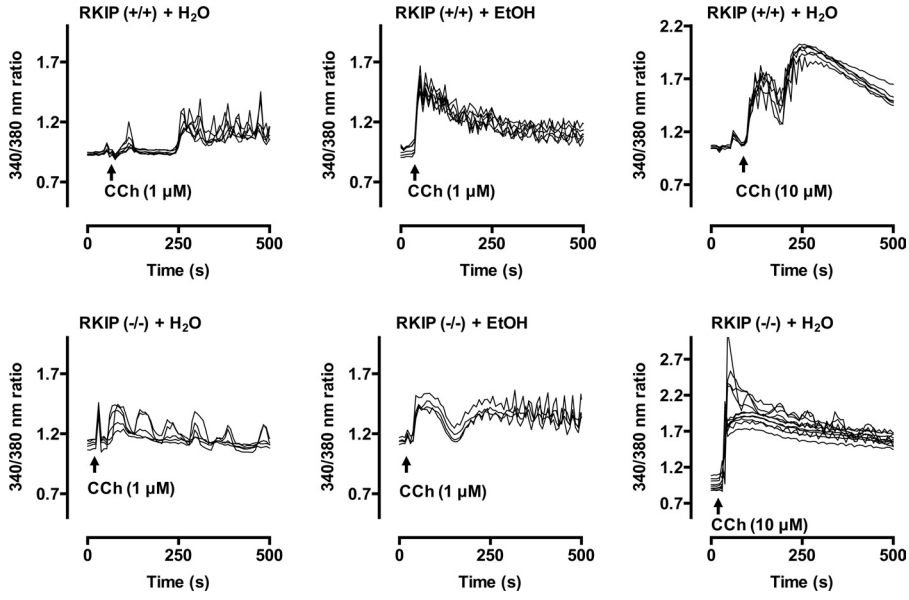

FIGURE 5. **Effects of RKIP gene ablation on EtOH-induced sensitization of carbachol (*CCh*)-stimulated Ca^{2+} responses in freshly isolated mouse pancreatic acinar cells.** Plots of change in the 340/380-nm ratio of Fura-2 fluorescence *versus* time show the effects of EtOH exposure or H_2O (control) on CCh-stimulated (1 or 10 μM) increases in $[Ca^{2+}]_{cyto}$ in acinar cells from $RKIP^{+/+}$ and $RKIP^{-/-}$ mice. All experiments used freshly isolated acinar cells with >95% viability base on trypan blue staining.

an equivalent volume of H_2O (Fig. 3*F*). In contrast, EtOH exposure failed to potentiate CCK-stimulated Ca^{2+} signaling over the same range of CCK concentrations when RKIP expression was suppressed (Fig. 3*G*). Competition binding experiments with ^{125}I-labeled CCK confirmed that neither exposure to 100 mM EtOH nor suppression to RKIP expression affected the levels or relative affinity of the CCK receptors expressed by AR42J cells (Fig. 3*H*). Together these studies demonstrate that suppression of RKIP expression is functionally equivalent to PKC inhibition (Figs. 1*F* and 2*C*) when evaluating the effects of EtOH exposure on secretagogue-stimulated Ca^{2+} signaling, suggesting the RKIP is a downstream mediator of PKC-dependent CCK receptor sensitization.

Acinar Cells from RKIP Null Mice Are Insensitive to EtOH-induced Sensitization—To confirm the function of RKIP in normal pancreatic acinar cells, we isolated cells from the pancreata of both RKIP null ($RKIP^{-/-}$) and wild-type ($RKIP^{+/+}$) control mice. Immunoblots confirmed the absence of RKIP protein expression in the pancreatic tissue (*PT*) and isolated pancreatic acinar cells (*PACs*) from $RKIP^{-/-}$ mice (Fig. 4*A*). Similar to AR42J cells, the acinar cells from $RKIP^{+/+}$ mice pretreated with EtOH (100 mM) for 10 min exhibited enhanced CCK-induced Ca^{2+} responses when compared with cell exposed to an equivalent volume of H_2O (Fig. 4*B*), and the EtOH-induced sensitization of the CCK-stimulated Ca^{2+} response was observed over a range of agonist concentrations (Fig. 4*C*). In contrast to cells from wild-type animals, EtOH had no effect on the sensitivity of the CCK-stimulated Ca^{2+} responses in acinar cells isolated from $RKIP^{-/-}$ mice (Fig. 4, *D* and *E*). A dose-response study with the $RKIP^{-/-}$ acinar cells showed no differences in the CCK-induced Ca^{2+} responses between the EtOH- and H_2O-pretreated cells over a range of agonist concentrations (Fig. 4*F*). A similar RKIP-dependent sensitization, the carbachol-stimulate Ca^{2+} response, was observed in acinar cells pretreated for 10 min with 50 mM EtOH (Fig. 5). Together these data establish that RKIP plays a critical role in EtOH-induced sensitization of secretagogue-stimulated Ca^{2+} signaling in pancreatic acinar cells.

Loss of RKIP Expression Prevents the Sensitizing Effect of EtOH on CCK-induced Chymotrypsin Activation—A hallmark of the pathogenesis of pancreatitis is the aberrant activation of the digestive enzymes within the acinar cell (for review, see Halangk and Lerch (58)). EtOH exposure has been shown to enhance secretagogue-induced intra-acinar trypsin and chymotrypsin activation in isolated rodent acinar cells (24, 28). To assess the role of RKIP in EtOH-induced chymotrypsin activation, acinar cells were isolated from $RKIP^{-/-}$ and wild-type mice, exposed to EtOH or H_2O for 10 min, and then stimulated either with CCK or vehicle. To validate the functionality of the isolated acinar cells, we assessed the cleavage of the fluorogenic chymotrypsin substrate in response to a high concentration of CCK (10 nM). The super-physiological concentration of CCK induced chymotrypsin activity in cells from both wild-type and $RKIP^{-/-}$ mice (Fig. 6, *A* and *B*, *CCK* (10 nM) *versus Control*, $t =$ 60 min), confirming previous studies which show that in the absence of a sensitizer like EtOH, secretagogue hyperstimulation is sufficient to induce intracellular digestive enzyme activation (35, 51, 59). Furthermore, pretreatment of acinar cells

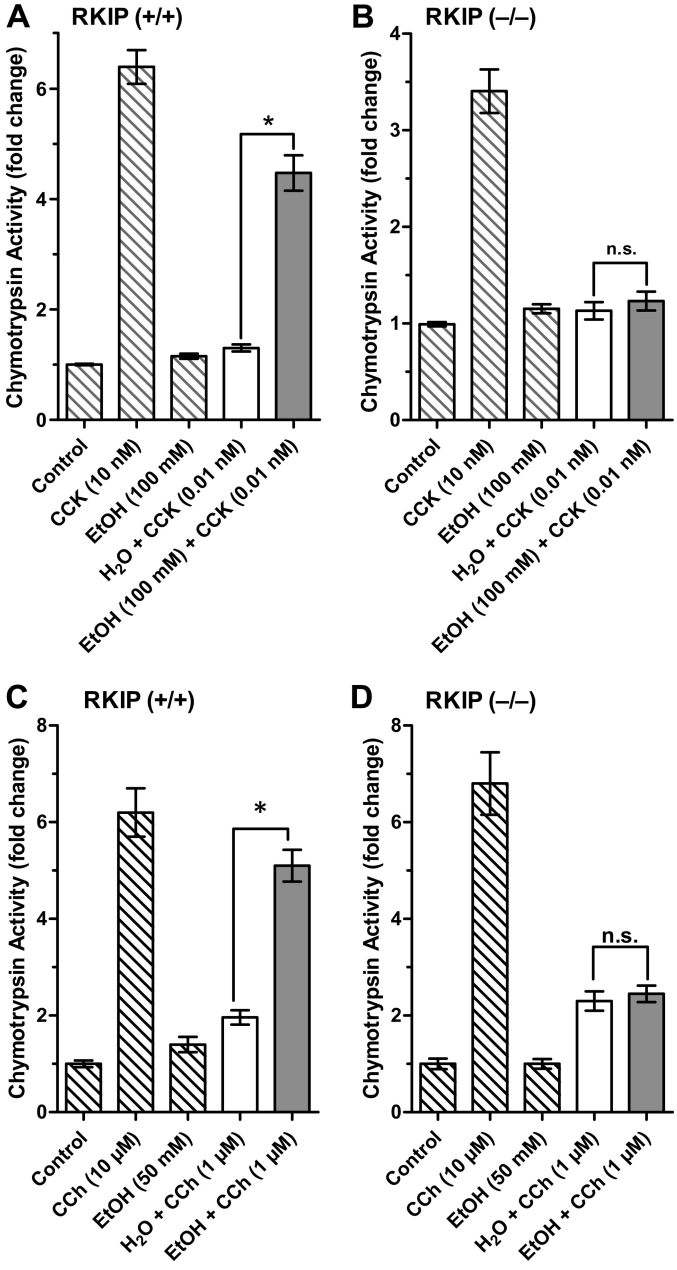

FIGURE 6. **Effect of RKIP expression on EtOH-induced sensitization of the CCK (*A* and *B*)- and carbachol (*CCh*)-stimulated (*C* and *D*) chymotrypsin activation in freshly isolated mouse pancreatic acinar cells.** *, $p < 0.0001$ *H_2O + CCK* or *CCh* (*open bar*) *versus EtOH + CCK* or *CCh* (*gray bar*). *n.s.*, not significant.

either from $RKIP^{+/+}$ or $RKIP^{-/-}$ mice with H_2O followed by stimulation with a physiologic concentration of CCK (0.1 nM) had no effect on basal chymotrypsin activity (Fig. 6, *A* and *B*, *Control versus H_2O + CCK*, $t = 60$ min). In contrast, acinar cells from $RKIP^{+/+}$ mice exposed to EtOH (100 mM) showed an ~3.5-fold increase in chymotrypsin activity in response to 0. 1 nM CCK compared with controls (Fig. 6*A*, *EtOH + CCK versus H_2O + CCK* or *Control*, $t = 60$). EtOH pretreatment, however, failed to potentiate CCK-induced chymotrypsin activity in acinar cells from $RKIP^{-/-}$ mice (Fig. 6*B*, *EtOH + CCK versus H_2O + CCK* or *Control*, $t = 60$). A similar RKIP-dependent aberrant intracellular chymotrypsin activation was observed in

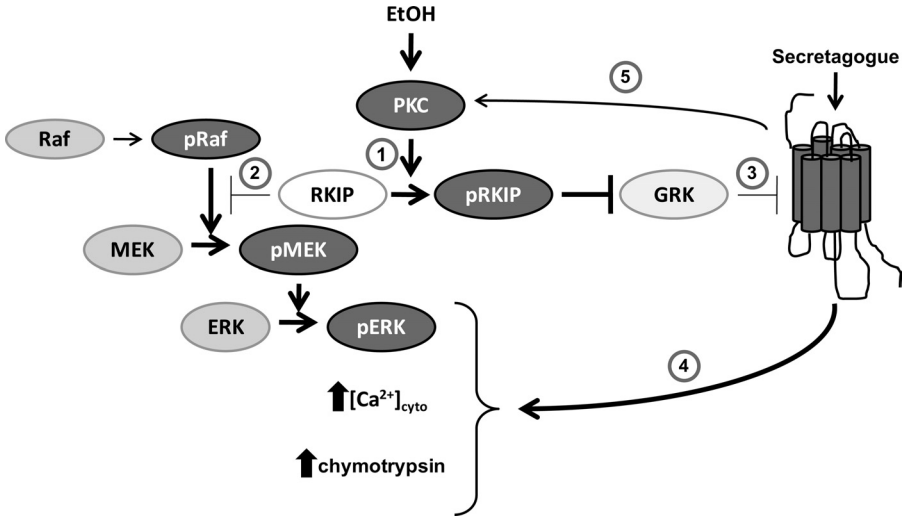

FIGURE 7. **Diagram illustrating proposed role of RKIP in EtOH-induced sensitization of secretagogue signaling in pancreatic acinar cells.** *Raf*, Ras-dependent mitogen-activated protein kinase kinase kinase; *pRaf*, phosphorylated Raf.

acinar cells pretreated for 10 min with 50 mM EtOH and stimulated with a low concentration (1 μM) of carbachol (Fig. 6, *C* and *D*). Together, these data demonstrate that RKIP expression is critical for EtOH-induced sensitization of secretagogue-stimulated chymotrypsin activation in isolated pancreatic acinar cells.

DISCUSSION

Although alcohol abuse has been long recognized as a risk factor for acute and chronic pancreatitis, the molecular mechanism mediating the sensitizing affect of EtOH on secretagogue signaling in acinar cells has remained largely unresolved (26, 27). In this study we show that RKIP is a substrate for EtOH-induced PKC-dependent phosphorylation and a critical mediator of EtOH-induced sensitization of secretagogue signaling. Collectively, the data presented are consistent with the model diagramed in Fig. 7. Acute exposure of pancreatic acinar cells to EtOH induces the PKC-dependent phosphorylation of RKIP (*pRKIP*) (Fig. 7, *1*), decreasing both RKIP-mediated inhibition of the Raf/MEK/ERK pathway (Fig. 7, *2*), and GRK-mediated secretagogue receptor desensitization (Fig. 7, *3*), resulting in an increase in basal ERK activation as well as secretagogue-stimulated increases in $[Ca^{2+}]_{cyto}$, ERK phosphorylation, and aberrant intracellular chymotrypsin activation (Fig. 7, *4*), a hallmark of pancreatitis. Additionally, CCK stimulation alone increased cellular levels of phosphorylated RKIP, suggesting that RKIP is a physiological modulator of agonist-induced receptor desensitization (Fig. 7, *5*).

To elucidate the molecular mechanism by which EtOH sensitizes secretagogue signaling, we have focused on the peptide hormone CCK, which binds with high affinity to the CCK1 receptor, a Class A or rhodopsin-like GPCR. Similar to other members of this class of GPCRs, agonist activation of CCK1 receptor is followed by its rapid phosphorylation and desensitization (60, 61). Agonist-dependent GPCR desensitization, in general, is initiated by GRKs, a family of serine/threonine kinases (62) that phosphorylates specific amino acid residues within the third intracellular loop domain and/or C terminus of the activated receptor, creating binding sites for the adapter protein β-arrestin, which then blocks further receptor interactions with heterotrimeric G proteins and signaling (for review, Kelly *et al.* 2008 (63)).

RKIP has been identified as an antagonist of GRK-dependent GPCR desensitization (47) and thus functions to enhance or prolong agonist-induced signaling. For example, PKC-phosphorylated RKIP has been shown to inhibit GRK2-mediated phosphorylation of the isoproterenol (agonist)-activated β_2-adrenergic receptors with the result of potentiating agonist-stimulated cAMP production and inhibiting receptor internalization (47). The potential for RKIP to enhance GPCR signaling led us to investigate its role in EtOH-induced sensitization of secretagogue signaling in pancreatic acinar cells. Herein, we show that RKIP mediates the toxic effects of EtOH on CCK signaling by enhancing both agonist-induced increases in ERK activation and $[Ca^{2+}]_{cyto}$. The dysregulation of acinar cell Ca^{2+} homeostasis is a critical event in acinar cell death and the early pathophysiology of pancreatitis (36, 64). We show that EtOH stimulates the PKC-dependent phosphorylation of RKIP, which is associated with both enhanced ERK activation and CCK-induced increases in $[Ca^{2+}]_{cyto}$. Furthermore, we showed either inhibition of PKC or loss of RKIP expression is sufficient to prevent the sensitizing effects of EtOH on both CCK-induced Ca^{2+} signaling and, importantly, on intracellular chymotrypsin activation, a hallmark of pancreatitis.

Previous studies have shown that EtOH activates both the classical PKC isozymes PKCα and the novel PKCϵ (26, 50, 51). Consistent with these observations, we show that EtOH-induced phosphorylation of RKIP is inhibited in cells treated with the classical PKC inhibitor, GF109203X, as well as the PKCϵ-specific inhibitory peptide (Figs. 1*C* and 2*B*). Although the phosphorylation of RKIP by classical PKC isozymes is relatively well documented in the scientific literature, the role of the novel PKC isozymes is ambiguous. This is due in part to an earlier study by Corbit *et al.* (44) in which they evaluated the ability of various PKC isozymes to phosphorylate RKIP using purified recombinant proteins in a cell-free kinase assay. In that study they show that RKIP is robustly phosphorylated by the classical

PKC isozymes -α, -βI, -βII, -γ, and atypical PKCζ primarily at Ser-153, as the substitution of Ser-153 with alanine prevented most of RKIP phosphorylation. By comparison, the phosphorylation of RKIP by the purified novel PKC isozymes -δ, and -ϵ was very weak but, based on the data shown, specific for Ser-153. However, despite the evidence for weak Ser-153 phosphorylation by PKCδ and -ϵ, the authors concluded that RKIP is not a substrate for novel PKC isozymes (44). Our own *in silico* analyses of RKIP protein sequences from human, rat, and mouse using the Scansite 2.0 software (65) identified Ser-153 in humans and rats and Thr-153 in mice with high stringency for classical PKC consensus sites but low stringency PKCδ sites. Neither Ser-153 nor Thr-153 was predicted to a PKCϵ consensus site using the Scansite software. High stringency indicates that the sequence motif indentified in the query is within the top 0.2% of all matched sequences contained in the Swiss-Prot protein database. Low stringency motifs sequences are in the top 5%.

How can the PKCϵ-dependent phosphorylation of RKIP induced by EtOH that we observed be explained? A recent structural analysis of RKIP protein may provide one possible explanation (66). Mammalian RKIP, like other members of the evolutionarily conserved phosphatidylethanolamine-binding protein (PEBP) superfamily, is structurally characterized by a conserved ligand binding pocket that is capable of binding chemically distinct ligands, including but not limited to phosphatidylethanolamine and the protein Raf-1. Importantly, occupancy of the RKIP binding pocket appears to exert an allosteric effect on RKIP, modulating both its association with and phosphorylation by specific protein kinases involved in its regulation (66). Allosteric regulation of pancreatic acinar cell RKIP by EtOH or its metabolites may explain the enhanced phosphorylation by PKCϵ observed in Fig. 2. Alternatively, it is also possible that the cell-free assay used by Corbit *et al.* (44) lacked critical cellular components, such as specific phospholipids or yet to be identified proteins present in pancreatic acinar cells that occupy the RKIP binding site, altering RKIP affinity for specific PKC isozymes.

Finally, our data suggest that in addition to mediating the toxic effects of EtOH, RKIP also regulates normal physiological secretagogue signaling in pancreatic acinar cells. Physiologic concentrations of secretagogues such as acetylcholine and CCK trigger zymogen exocytosis by repetitively releasing Ca^{2+} from multiple intracellular stores (20). The demonstration that stimulation of AR42J cells with a physiologic concentration of CCK (0.01 nM) induced an increase in Ser(P)-153-RKIP (Fig. 1*D*) levels suggests that under physiologic conditions CCK can modulate its own receptor desensitization (homologous desensitization) by stimulating the phosphorylation of RKIP that in turn reduces the activity of GRK at the agonist-activated receptor. Although the physiological role of RKIP-mediated attenuation of CCK receptor desensitization was not specifically addressed in our study, we hypothesize that RKIP-dependent modulation of GRK may be part of the mechanism by which pancreatic secretagogues generate the oscillatory changes in $[Ca^{2+}]_{cyto}$ associated with normal meal-induced secretion. Future studies will examine the validity of this hypothesis.

Alcohol abuse is a leading risk factor for both recurrent acute and chronic pancreatitis. Understanding the molecular mechanisms by which EtOH and its metabolites exert their toxic effects on pancreatic acinar cells can help to inform the development of new therapeutic strategies for the prevention or treatment of alcohol-associated pancreatitis. Modulation of RKIP expression may have future utility in this endeavor.

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