Alcohol/Cholecystokinin-evoked Pancreatic Acinar Basolateral Exocytosis Is Mediated by Protein Kinase Cα Phosphorylation of Munc18c

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The pancreatic acinus is the functional unit of the exocrine pancreas whose role is to secrete zymogen granules into the gut lumen for food digestion via apical exocytosis. We previously reported that supramaximal CCK induced apical blockade and redirected exocytosis to ectopic sites on the basolateral plasma membrane (BPM) of this polarized cell, leading to pancreatitis. Basolateral exocytosis was mediated by protein kinase C phosphorylation of BPM Munc18c, causing its displacement into the cytosol and activation of BPM-bound Syntaxin-4 to form a SNARE complex. To mimic the conditions of alcoholic pancreatitis, we now examined whether 20 mM alcohol followed by submaximal CCK might mimic supramaximal CCK in inducing these pathologic exocytotic events. We show that a non-secretory but clinically relevant alcohol concentration (20 mM) inhibited submaximal CCK (50 pM)-stimulated amylase secretion by blocking apical exocytosis and redirecting exocytosis to less efficient BPM, indeed mimicking supramaximal CCK (10 nM) stimulation. We further demonstrate that basolateral exocytosis caused by both stimulation protocols is mediated by PKCα/β-induced phosphorylation of Munc18c: 1) PKCα is activated, which binds and induces phosphorylation of PM-Munc18c at a Thr site, and these events can be inhibited by PKCα blockade; 2) PKCα inhibition blocks Munc18c displacement from the BPM; 3) PKCα inhibition prevents basolateral exocytosis but does not rescue apical exocytosis. We conclude that 20 mM alcohol/submaximal CCK as well supramaximal CCK stimulation can trigger pathologic basolateral exocytosis in pancreatic acinar cells via PKCα-mediated activation of Munc18c, which enables Syntaxin-4 to become receptive in forming a SNARE complex in the BPM; and we further postulate this to be an underlying mechanism contributing to alcoholic pancreatitis.

The pancreatic acinar cell is a highly polarized epithelial cell designed for zymogen granules (ZG)3 to undergo regulated exocytosis at the apical pole, thereby emptying the digestive zymogen granules into the gut lumen for food digestion. This well orchestrated exocytic pathway can be altered by supramaximal stimulation with cholecystokinin (CCK), which causes apical blockade and redirection of exocytosis to the basolateral plasma membrane (BPM) surface. Specifically, using epifluorescence imaging of the FM1–43 dye in pancreatic acinar cells, we showed real-time visualization of apical exocytosis induced by maximal CCK, and aberrant exocytosis at the BPM caused by supramaximal CCK stimulation (1). We further found that this basolateral exocytosis was consistent with the paradigm of the SNARE Hypothesis (2–5), involving a distinct set of cognate SNARE partners, Syntaxin-4 (Syn-4) and SNAP 23 on the BPM, and VAMP proteins on the ZG (1, 6, 7), and whose interactions were regulated by the SM protein Munc18c (1). Here, Munc18c on the acinar BPM binds Syn-4, and upon supramaximal CCK stimulation, becomes displaced into the cytosol via a PKC-mediated mechanism; which activates Syn-4 to become capable of forming a SNARE complex with its cognate partners, then proceeding to basolateral exocytosis (1). The precise PKC isoform acting on Munc18c to mediate basolateral exocytosis is unknown.

Supramaximal CCK treatment induces pancreatitis in rodents (8), which however is not a clinically relevant form of the disease in humans. Alcoholic pancreatitis is the most common clinical disease of the human exocrine pancreas, but neither acute nor chronic exposure to alcohol in rodents causes pancreatitis (8–12). Instead, chronic alcohol diet followed by low-dose CCK in rodents induced pancreatitis (8), but the precise cellular mechanism remains vague. Interestingly, in intact acinar cells of human alcoholic chronic pancreatitis, we found that Munc18c was displaced into the cytosol, while t-SNARE proteins Syn-4 and SNAP 23 remained intact in the BPM (13). Taken together, this led us to hypothesize that alcohol pretreatment followed by low-dose CCK might mimic supramaximal CCK in inducing basolateral exocytosis by inducing Munc18c displacement into the cytosol, and that this process may be...
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required to activate the BPM SNARE complex. Here, we performed parallel studies for these two stimulatory models (supramaximal CCK and alcohol pretreatment/low-dose CCK stimulation) in dispersed rat pancreatic acini. Our results demonstrate that: 1) similarly to supramaximal CCK, pretreatment of acini with a non-stimulatory concentration of EtOH (EtOH) reduced subsequent submaximal CCK-stimulated enzyme secretion by blocking apical exocytosis and redirecting exocytosis to less efficient ectopic BPM sites. 2) In both models, these events were caused by Munc18c displacement from the BPM into the cytosol through a PKCa-induced threonine phosphorylation of Munc18c, and which remarkably were blocked by PKCa inhibition.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

Antibodies used include those generated against Munc18c (a gift from Y. Tamori, Kobe University, Japan), Syntaxin-4 (a gift from J. Pessin, Stony Brook University, NY), SNAP 23 (generated by us), Na⁺/K⁺-ATPase (Upstate Biotechnology-Chemicon Int., Temecula, CA), phosphothreonine (Zymed Laboratories Inc., San Francisco, CA), PKCa, and tubulin (Sigma). Sulfated CCK octapeptide and 12-O-tetradecanoylphorbol-13-acetate (TPA) were from Sigma. Calphostin C, G60976, and the Non-Radioactive Protein Kinase Alpha Assay kit were from Calbiochem. FM1-43 was from Molecular Probes Inc (Eugene, OR). PKCa myristoylated pseudosubstrate inhibitor (myr-PKCα 14 amino acid peptide unique to PKCa) was a gift from Driss Zoukhi (Tufts University, Boston, MA) (14). PKCδ translocation inhibitor δV1-1 and PKCe translocation inhibitor εV1–2 were reported by J. Reeve Jr. (University of California, Los Angeles) (15).

Dispersed Acini Preparation and Amylase Secretion

The preparation of isolated pancreatic acini from male Sprague-Dawley rats (125–150 g) was performed by a mechanical and enzymatic dissociation technique we previously described (1, 16). The acini were suspended in oxygenated Krebs-Ringer-HEPES (KRH) buffer, consisting of (in mM): 104 NaCl, 5 KCl, 1 KH₂PO₄, 1.2 MgCl₂, 2 CaCl₂, 0.2% (w/v) bovine serum albumin, 0.01% (w/v) soybean trypsin inhibitor, 10 glucose, 25 HEPES/NaOH, pH 7.4, supplemented with minimal essential and non-essential amino acid solution and glutamine. The acini (~10⁶ cells) were stimulated with the indicated agonists for 1 h at 37 °C. Amylase released into the supernatant and amylase content of the acini pellet were determined by a colorimetric method. Total amylase is defined as the summation of the amylase content in the respective cell pellet plus supernatant, and the amylase secreted into the supernatant is expressed as a percent of total amylase. The data obtained were collected from three separate portions of acini per experiment (from one rat) of at least five different acini preparations, which were then pooled (n > 15) and analyzed by Student’s t test, with significance determined to be p < 0.05. Animal care and all the procedures were approved by the Institutional Animal Care and Use Committees of the University of Toronto.

Confocal Immunofluorescence Microscopy

This was performed as we previously described (1, 13). Dispersed acini were plated on glass coverslips, fixed with 4% paraformaldehyde, rinsed in phosphate-buffered saline for 5 min followed by 10 min in 100 mM glycine. The acini were then blocked with 5% normal goat serum with 0.1% saponin for 0.5 h at 25 °C. The cells were then washed and incubated with the indicated primary antibodies (1:100 dilution, 1 h, 25 °C) followed by appropriate fluorochrome (Cy3)-conjugated secondary antisera (1:1000 dilution, 1 h, 25 °C), along with FITC-conjugated phalloidin (0.2 μM) (Molecular Probes, Eugene, OR) added at the secondary antibody step. The coverslips were mounted on slides with DAKO Fluorescence Mounting Medium (Carpinteria, CA) and examined by a laser scanning confocal imaging system (Zeiss LSM 510) equipped with the LSM software version 5.00 (Carl Zeiss, Oberkochen, Germany). All immunostaining experiments were repeated at least five times with similar results.

Subcellular Fractionation, Immunoprecipitation, and Immunoblotting

Subcellular Fractionation—Isolated pancreatic acini, after equilibration (20 min, 37 °C) were subjected to the indicated agonist stimulation, and then terminated by adding excess volume of ice-cold KRH buffer. The acini were then pelleted by centrifugation (300 g, 4 °C). Whole cell lysates and purified PMs and ZGs were prepared from the treated dispersed acini by sucrose density gradient centrifugation as previously described (1). Briefly, the sucrose buffer consisted of 0.3 M sucrose, 0.01% soybean trypsin inhibitor, 0.5 M phenylmethylsulfonyl fluoride, and 5 mM β-mercaptoethanol. The acini or pancreatic tissues were homogenized in a Potter-Elvehjem homogenizer, followed by a 5-min centrifugation (14,000 × g, 4 °C) to separate the nuclei pellets from supernatants. The supernatant fractions were centrifuged (15 min, 14,000 × g, 4 °C) to separate the ZG pellets, and the resulting supernatants subjected to ultracentrifugation (3 h, 39,000 × g, 4 °C) to obtain the PM pellet and cytosol supernatant fractions.

Immunoprecipitation—For immunoprecipitation, lysates containing equal amounts of protein were clarified by centrifugation (12,000 × g, 10 min). The resulting supernatants, pre-cleared for 40 min using 40 μl of 50% suspension of protein G-Sepharose beads (Amersham Biosciences) were incubated with the indicated antibodies (Munc18c or Syn-4) for 1 h. Immunoprecipitates were captured by rotating the samples for 1 h at 4 °C with 40 μl of protein G-Sepharose beads, which were then washed four times with lysis buffer containing 1 mM Na₃VO₄.

Immunoblotting—Protein content of all these samples was determined by the BCA method. Samples of subcellular fractions or immunoprecipitated proteins were dissolved in Laemmli buffer and boiled for 5 min. Equal amounts of protein were separated on 8% SDS-PAGE and transferred to nitrocellulose membranes (Bio–Rad). Blots were blocked for 1 h in Tris-buffered saline containing 5% bovine serum albumin and then incubated with the corresponding primary antibody. The bound antibody was visualized by relevant peroxidase-coupled secondary antibodies using the enhanced chemiluminescence...
method (Amersham Biosciences). Quantification was performed by densitometry using the NIH-Image software.

**FM1–43 Epifluorescence Imaging of Exocytosis**

Single ZG exocytosis was visualized in real-time using this imaging assay technique as previously reported (1, 17). Dispersed acini were plated on glass coverslips and mounted in a heated chamber kept at 37 °C. The cells were then incubated in 2\textsubscript{H}9262M FM1–43 (15 min, 37 °C) until a stable basal fluorescence staining of the acinar PM was attained. Imaging of the cells was carried out on a Nikon TE2000E inverted microscope imaging system, with a high numerical aperture lens (N.A., 1.45) executing a greater light cone that enabled a thinner and higher resolution optical section. Fluorescence images of the FM1–43 stained cells were obtained with 490/20 nm excitation provided by a Tills Photonics monochromator and 617/73 nm emission filters, with image acquisition at 1s exposure (1Hz) using an ORCA-ER CCD-camera. We determined the size and intensity of the FM1–43 fluorescence within indicated regions of interest at the X-Y axis to monitor ZG exocytosis occurring at the different sites of the acinar cell. Quantitative analysis of fluorescent signal of individual hotspots on the PM was measured using the Compix Simple PCI and NIH-Image J softwares. The intensity for each HS was normalized to the intensity of the PM adjacent to it.

**Determination of PKC\textalpha Activity**

PKC\textalpha activity assay was performed on lysates from isolated acinar cells obtained and processed following the protocol indicated by the manufacturer using an ELISA-based detection method that is as sensitive as the radioactive one and specific to PKC\textalpha. Protein concentration of each sample was determined by the BCA method, and 10\textsubscript{H}11007\text{g} of protein were run in the assay. PKC\textalpha activity is directly proportional to the color intensity developed that was read at 490 nm with the Thermo max microplate reader using the Soft max program. Measurements of each sample were performed in triplicates Activity values were normalized to the basal activity in unstimulated control acini. The data obtained were collected from three separate portions of acini per experiment (from one rat) of three different acini preparations, which were then pooled (n = 9) and analyzed by Student’s t test, with significance determined to be p < 0.05.

**Statistical Analysis**

All data are presented as means ± S.D. Statistical analysis was done by Student’s t test. Significance was assumed at a p value of less than 0.05.

**RESULTS**

**Twenty Millimolar Alcohol Inhibits CCK-stimulated Amylase Release, Which Can Be Further Influenced by PKC\textalpha Blockade**—We determined that 1 h was the minimal incubation time for
submaximal CCK (50 pm) to maximally release amylase from dispersed pancreatic acini (19.6 ± 0.7% in Fig. 1A). We then examined the effects of alcohol on 50 pm CCK stimulated amylase secretion. Here, we have used 20 mM ethanol (EtOH), a concentration well within the blood alcohol levels observed with clinical alcohol intoxication (18), but which does not stimulate amylase secretion. In Fig. 1B, we found that it takes 1 h of preincubation with 20 mM EtOH to achieve maximal reduction of 50 pm CCK stimulated secretion from 20.2 ± 0.8% to 13.4 ± 0.5%, which is a 33.6% reduction (p < 0.05). In Fig. 1D, 20 mM EtOH also inhibited maximal CCK (0.8 nm)-stimulated secretion from 30.1 ± 1.2% to 16.8 ± 0.9%, a 44.2% reduction (p < 0.05), but did not affect supramaximal CCK (10 nm)-stimulated secretion (data not shown).

Because we had previously shown that nonspecific PKC activation by low level phorbol ester concentrations could induce a similar effect of reducing maximal CCK stimulated secretion, mimicking supramaximal CCK inhibition of secretion (19), we here explored which PKC isoform might be mediating this effect by using membrane-permeable isoform-specific PKC inhibitors to dominant PKC isofoms reported in pancreatic acini, including PKCα, δ, and ε (15). For this purpose we used the following PKC inhibitors: PKCδ translocation inhibitor peptide δVI–1 (15), PKCe translocation inhibitor peptide εVI–2 (10 μM, 3 h) (15), PKCα myristilated pseudosubstrate inhibitor (myr-PKC-α 14 amino acid peptide unique to PKCα, 5 μM, 30 min) (14), pharmacologic PKCα inhibitor Gö6976 (200 nm, 30 min), and a general potent PKC inhibitor Calphostin C (500 nm, 40 min). Acini were pretreated with each of these PKC inhibitors under optimal conditions previously described (14, 15) prior to stimulation with either 10 nm CCK or 20 mM EtOH preincubation (1 h) followed by 50 pm CCK stimulation.

In Fig. 1, C, D, and E, we examined the effects of these PKC inhibitors on submaximal (50 pm), maximal (0.8 nm), and supramaximal CCK (10 nm) stimulated release, in the presence or absence of 20 mM EtOH pretreatments. PKCδ and ε inhibition had no effects on CCK-stimulated secretion in the presence of 20 mM EtOH. However, PKCα inhibition had profound effects. Specifically, preincubation of acini with PKCα pseudosubstrate inhibitor or Gö6976 prior to the addition of 10 nm CCK inhibited secretion from 13.1 ± 0.7% to 8.6 ± 0.6% and 8.1 ± 0.8%, respectively, which is a reduction of 34.4% and 38.2%, respectively (p < 0.05) (Fig. 1E). Of note, these PKCα inhibitors did not affect submaximal (50 pm CCK) or maximal CCK (0.8 nm)-stimulated release.

We next examined whether EtOH pretreatment might inhibit submaximal or maximal CCK-stimulated amylase release. In Fig. 1, C and D, we confirmed that 20 mM EtOH did not stimulate significant secretion over basal release (either KRH or Me2SO vehicle control). In Fig. 1C, 20 mM EtOH inhibited submaximal 50 pm CCK-stimulated secretion from 18.3 ± 1.3% to 12.6 ± 0.5%, a 31.1% reduction (p < 0.05). Interestingly, pre-incubation of acini with PKCα pseudosubstrate inhibitor or Gö6976 resulted in a further decrease in amylase secretion to 8.2 ± 0.5% and 8.0 ± 0.8%, respectively, which is a further reduction of 55.2% and 56.3%, respectively. In Fig. 1D, 20 mM EtOH inhibition of maximal 0.8 nm CCK-stimulated secretion (from 30.1 ± 1.2% to 16.8 ± 0.9%) was similarly further inhibited by PKCα pseudosubstrate inhibitor and Gö6976 to 10.1 ± 1.1% and 9.9 ± 0.8%, respectively, a further reduction of 66.4% and 67.1%, respectively.

**PKCα Inhibition Blocks Basolateral Exocytosis Caused by Supramaximal CCK or 20 Millimolar Alcohol/Submaximal CCK Stimulation, but Does Not Rescue Apical Exocytosis**—We next examined the exocytotic events corresponding to the amylase secretion data in Fig. 1 using the epifluorescence microscopy of the FM1–43 dye, which fluoresces upon contact with the lipid PM, and appears as hotspots on the PM when the dye further permeates into the ZGs undergoing exocytosis (17). While large acini of 20–30 cells often interfere with the dye penetration and visualization of the dye-tracked exocytic events, 4–7 cell acini allows a good permeation rendering good real-time visualization of exocytosis as we had previously reported (1). To clearly indicate the appearance of PM hotspots we present the selected images with fluorescence intensity shown in pseudocolor.

Similar to our previous report (1), normal regulated exocytosis was stimulated by 50 pm CCK at the apical poles of acinar cells, observed as increased fluorescence intensity starting at the apical lumen, which then permeates into the ZGs within the apical poles (ZGs encompassing the entire apical pole of each acinar cell can be observed on phase contrast optics) (1) (Fig. 2A, panels i and ii). This apical exocytosis was not blocked by either PKCα pseudosubstrate inhibitor (Fig. 2A, panels iii and iv) or Gö6976 (Fig. 2A, panels v and vi). As previously reported (1), 10 nm CCK stimulation blocked apical exocytosis and redirected exocytosis to the basal PM of this 4-cell acinus ((Fig. 2B, panels i and ii), indicated by arrows pointing to 4 hotspots: HS1–HS4). Fig 2B, panel i shows a timed sequence of selected static images with fluorescence intensity shown in pseudocolor and Fig. 2B, panel ii shows the analysis of these hotspots (HS1–HS4) together with the corresponding apical lumens and apical poles of each of the four acinar cells. The fluorescence excursions of the 4 HS were remarkably synchronous, with...
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HS2 and HS3 exhibiting higher peak amplitudes. We have previously shown that these basal PM FM1–43 hotspots colocalized with exocytosed amylase (1). Most remarkably, this basal PM exocytosis was blocked by PKCα pseudosubstrate inhibitor (Fig. 2B, panels iii and iv) and Gö6976 (Fig. 2B, panels v and vi). Of note, apical exocytosis was however not rescued by the PKCα inhibition, which would explain the further inhibition of 10 nM CCK-stimulated amylase secretion in Fig. 1E.

We then examined whether EtOH-induced inhibition submaximal CCK-stimulated amylase secretion would evoke exocytic events similar to those observed with supramaximal CCK. For this purpose, we first examined exocytosis stimulated by 20 mM EtOH, which, like KRH control, caused no detectable exocytosis in the apical pole or basal PM (data not shown). Pretreatment of acini with 20 mM EtOH followed by 50 pM CCK-stimulation showed a blockade of apical exocytosis, and instead, exocytosis occurred on the basal PM (Fig. 2C, panel i). Fig. 2C, panel i, which is very similar to the exocytic events evoked by supramaximal CCK stimulation (Fig. 2B, panels i and ii), shows a timed sequence of selected static images of a 4-cell acinus with two basal PM hotspots indicated - HS1 and HS2, and analyzed in Fig. 2C, panel ii, which exhibit synchronous rhythmic excursions as similarly observed with 10 nM CCK stimulation (Fig. 2B, panel ii). The latter suggests cycles of single ZG exocytosis and content emptied by waiting for recruitment of oncoming single ZGs to the same exocytotic sites. It therefore appears that alcohol pretreatment of the acinus causes submaximal CCK-evoked exocytosis to be redirected from the apical pole to BPM sites, mimicking supramaximal CCK-evoked exocytosis (1). We then pretreated the acini with PKCα pseudosubstrate inhibitor (Fig. 2C, panels iii and iv) or Gö6976 (Fig. 2C, panels v and vi), and both completely blocked the basal PM exocytosis caused by subsequent 20 mM EtOH/50 pM CCK stimulation, but did not rescue apical exocytosis. These events were remarkably similar to those observed with the supramaximal CCK exocytotic studies (Fig. 2B, panels iii and iv).

We observed additional differences between apical and basal PM exocytoses. Given that there are much fewer ZGs in the vicinity of the BPM surface and longer distance of ZGs deeper in the cell interior to get to the BPM, a much longer time (the observations in Fig. 2 are over >30 min) was required to observe these exocytic events on the BPM. This is in contrast to the highly efficient apical exocytosis, which undergoes rapid sequential granule-granule fusions (1, 20). Furthermore, it appears that not the entire BPM surface is equipped for exocytosis, because the ZGs were recruited preferentially to previous exocytic sites rather than de novo exocytic sites on the basal PM. For these reasons, the cumulative exocytosis at the BPM is much less efficient than apical exocytosis, which would explain why alcohol inhibited CCK-stimulated amylase release in Fig. 1. With epifluorescence FM1–43 imaging, mostly basal PM exocytosis was observed, while lateral exocytosis was not well appreciated here because the extracellular FM dye has limited access to this compartment, even with the use of smaller acini.

PKCα Inhibition Prevents Displacement of Munc18c from the Basal Plasma Membrane into the Cytosol—We next examined the molecules that may be mediating the exocytic events observed in Fig. 2. We had postulated that the molecular basis of basolateral exocytosis is mediated by the SNARE complex proteins: Syn-4 and SNAP 23 on BPM, and undefined VAMPs on ZGs approaching the BPM surface (1, 6). Presumably Munc18c binds Syn-4 to prevent its assembly with cognate SNARE proteins, and the process of PKC-mediated Munc18c dissociation from Syn-4 remodels Syn-4 in a manner that enables its assembly with cognate SNARE proteins to form a SNARE complex capable of effecting exocytotic membrane fusion (1). We examined the behavior of these molecules in dispersed acini subjected to the stimulatory protocols in Fig. 2, specifically 10 nM CCK and 20 mM EtOH + 50 pm CCK, by subcellular fractionation (Fig. 3) and confocal microscopy (Fig. 4), and also examined how they are affected by the PKC isoform-specific inhibitors.

Fig. 3A shows that 10 nM CCK stimulation caused Munc18c normally residing in the BPM of pancreatic acinar cells (Me2SO control) to dissociate from the BPM into the cytosol (10 mM...
CCK in KRH). Remarkably, this Munc18c displacement from the BPM was blocked by a general PKC inhibitor, Calphostin C, as we had reported (1), and more specifically, also by PKC\(\alpha\) pseudosubstrate inhibitor and Go6976 (Fig. 3A). Importantly, in both stimulatory protocols, total lysate Munc18c levels (67 kDa) were reduced because of degradation as evidenced by the appearance of a smaller 35-kDa degradation product in the cytosol fraction. This latter result would indicate that 20 mM EtOH/50 pM CCK also induced the Munc18c degradation.

Neither PKC\(\delta\) or \(\epsilon\) inhibitors affected 10 nM CCK or 20 mM EtOH/50 pM CCK-induced displacement of PM-bound Munc18c nor total lysate Munc18c levels. Of note, PM levels of Syn-4 and SNAP 23 were not affected by the PKC\(\alpha\) (or PKC\(\delta\) and \(\epsilon\)) inhibition, and therefore, they would be relieved to bind each other in the BPM to form a scaffold that can then bind VAMPs of oncoming ZGs to consummate basolateral exocytosis (shown later in Fig. 7). As controls, irrelevant proteins in the PM and cytosol, including \(\text{Na}^+/\text{K}^+\)-ATPase and tubulin respectively, were unchanged by any of the treatments in both stimulation conditions. These figures are representative of three independent experiments in which samples were performed in duplicates (\(n = 6\)), and the densitometry analysis of all these experiments is shown in supplemental Fig. S1.

FIGURE 4. Confocal microscopy: 20 mM alcohol + submaximal CCK stimulation, like supramaximal CCK, displaces Munc18c from the BPM. Dispersed acini were treated with either control buffers (KRH) or PKC isoform-specific inhibitors as described in Fig. 1, C–E and then stimulated with either 10 nM CCK for 30 min (A) or 20 mM EtOH (1 h) + 50 pM CCK (1 h) (B). The acini are then subjected to confocal microscopy with double labeling for Munc18c and actin. Note that Munc18c displacement from the PM is blocked by PKC\(\alpha\) inhibition but not by PKC\(\delta\) or \(\epsilon\) inhibition. This is representative of four independent experiments performed in triplicate per experiment. Scale bars correspond to 10 \(\mu\)m.

CCK from the PM was prevented by PKC\(\alpha\) pseudosubstrate inhibitor and Go6976, and also by Calphostin C. Disruption of actin cytoskeleton is an early cellular event observed with supramaximal CCK stimulation of pancreatic acinar cells and pancreatitis (21), which was also observed here with 10 nM CCK stimulation as well as with 20 mM EtOH + 50 pM CCK stimulation. Pretreatment of the acini with PKC\(\alpha\) pseudosubstrate inhibitor and Go6976, blocked both 10 nM CCK (Fig. 4A) and 20 mM EtOH + 50 pM CCK (Fig. 4B) induced displacement of Munc18c from the BPM. Surprisingly, the PKC\(\alpha\) inhibition seemed to also prevent actin disassembly caused by these stimulatory protocols. This raises the possibil-
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Supramaximal CCK or alcohol followed by submaximal CCK stimulation induces threonine-phosphorylation of Munc18c by a PKCα-mediated mechanism. Threonine phosphorylation of Munc18c is blocked by PKCa inhibition. Similar to Fig. 1, C–E, dispersed acini were incubated with control buffers (KRH or DMSO) or PKC isoform-specific inhibitors; and then stimulated with 10 nM CCK (10 min) (A) or 20 mM EtOH (1 h) + 50 pm CCK (10 min) (B); or 1 μM TPA for 10 min in both A and B. Whole cell lysates were immunoprecipitated with anti-Munc18c antibody; and resulting precipitated proteins separated on SDS-PAGE and blotted with anti-phosphothreonine, Munc18c and PKCa antibodies. Each blot is representative of three independent experiments, n = 3 analyses are shown in supplemental Fig. S2.

FIGURE 5. Supramaximal CCK or alcohol followed by submaximal CCK stimulation induces threonine-phosphorylation of Munc18c by a PKCα-mediated mechanism. Threonine phosphorylation of Munc18c is blocked by PKCa inhibition. Similar to Fig. 1, C–E, dispersed acini were incubated with control buffers (KRH or DMSO) or PKC isoform-specific inhibitors; and then stimulated with 10 nM CCK (10 min) (A) or 20 mM EtOH (1 h) + 50 pm CCK (10 min) (B); or 1 μM TPA for 10 min in both A and B. Whole cell lysates were immunoprecipitated with anti-Munc18c antibody; and resulting precipitated proteins separated on SDS-PAGE and blotted with anti-phosphothreonine, Munc18c and PKCa antibodies. Each blot is representative of three independent experiments, n = 3 analyses are shown in supplemental Fig. S2.

PKCa
Phospho-Threonine
Munc18c-67 kDa

A

PKCa
Phospho-Threonine
Munc18c-67 kDa

B

Supramaximal CCK and EtOH/CCK-induced Munc18c Displacement from BPM Causing Basolateral Exocytosis Is Mediated by PKCa Phosphorylation of Munc18c—Because this is a PKC-mediated event, we proceeded to directly examine Munc18c phosphorylation and its interaction with PKCa (Fig. 5). Parental Munc18a serine phosphorylation is required to modulate Syntaxin 1A conformation into an activated form capable of binding cognate SNARE proteins (22, 23). This Munc18a Ser313 phosphorylation site is conserved in Munc18c as a threonine residue (Thr314). We therefore evaluated the threonine phosphorylation status of immunoprecipitated Munc18c. From acini treated with phorbol ester TPA (1 μM, 10 min) to induce PKC phosphorylation, immunoprecipitated Munc18c was indeed recognized by a phosphothreonine antibody, 10 nM CCK and 20 μM EtOH + 50 pm CCK induced a similarly strong threonine phosphorylation of Munc18c, but none with 50 pm CCK alone. Remarkably, the threonine phosphorylation of Munc18c induced by 10 nM CCK or EtOH + 50 pm CCK was completely suppressed by both PKCa pseudosubstrate inhibitor and Gö6976 indicating that this is PKCa-dependent. We then investigated the interaction between threonine-phosphorylated Munc18c and PKCa, and found that the immunoprecipitated phosphorylated Munc18c co-precipitated PKCa, and that these protein interactions could be blocked by PKCa inhibition (PKCa pseudosubstrate inhibitor or Gö6976) but not PKCd or ε inhibition.

The sequence of events would be that PKCa activation (Fig. 6A) leads to its translocation to the PM (Fig. 6B) to bind and phosphorylate PM-bound Munc18c (Fig. 5); and these events would precede Munc18c displacement from the PM. Because PKCa occurs very rapidly and becomes maximal at 10 min CCK stimulation as had been determined by Li et al. (24), we have used this optimal condition of 10 min CCK stimulation for PKCa activation in our studies (Figs. 5 and 6). We had previously reported that 10 nM CCK induced Munc18c PM displacement over time starting or noticeable at 5 min and progressively increased toward 15 min (1); and thus, our studies in Figs. 3 and 4 examining Munc18c translocation were performed after 30–60 min of CCK stimulation. Taken together, Munc18c displacement from the PM would follow and lag behind these events of PKCa activation, translocation, and phosphorylation of PM-bound Munc18c. To further track these postulated events, we next examined PKCa activation per se by determining its activity (Fig. 6A) and translocation from the cytosol to the PM (Fig. 6B) where it would then bind and activate BPM-bound Munc18c (Fig. 5). Compared with KRH control, PKCa activity was increased by 10 nM CCK stimulation (1.67 ± 0.2 fold, p < 0.05) (Fig. 6A, panel i), 20 μM EtOH + 50 pm CCK (2.15 ± 0.13 fold, p < 0.05) and 20 μM EtOH + 0.8 nM CCK (1.30 ± 0.17 fold, p < 0.05) (Fig. 6A, panel ii). This correlated well with PKCa translocation from the cytosol to the PM (Fig. 6B, panels i and ii) observed as reduced cytosol levels and increased PM levels. 20 μM EtOH alone had very little effect on PKCa activation or translocation to the PM. Both PKCa inhibitors abrogated 10 nM CCK, 20 μM EtOH + 50 pm, and 20 μM EtOH + 0.8 nM CCK induced PKCa activation (Fig. 6A). Interestingly, a higher EtOH (120 mM) concentration followed by 50 pm CCK or 0.8 nM CCK stimulation did not cause an increase in PKCa activity (Fig. 6A, panel ii). The densitometry analysis of PKCa expression is shown in the supplemental data Fig. S2. (Fig. 6B experiment is representative of three independent experiments in which samples were performed in duplicates, n = 6.)

Supramaximal CCK and EtOH/CCK Stimulation Induce SNARE Complex Assembly—For basolateral exocytosis to occur, the cognate SNARE proteins (Syntaxin 4, SNAP-23, VAMPs) would have to come together to form a SNARE com-
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20 mM EtOH + 50 pm CCK stimulation (Fig. 7B) caused great reduction of Munc18c levels in the PM (by displacement into the cytosol) and cell lysate (by cytosolic degradation), which confirm our above results; but more remarkably, these treatments enabled the PM SNARE proteins: Syntaxin-4, SNAP-23, and VAMPs (VAMP2 and VAMP8) to form the exocytotic SNARE complex. The relative low levels of VAMP proteins in the SNARE complex is explained by the limited number of ZGs containing the VAMP proteins that are actually fusing with the PM during stimulation. Nonetheless, the abundant SNAP-23 complexed with Syntaxin-4 would render the BPM extremely receptive to fusion with ZG VAMPs. Consistent with all our above results with PKCα inhibition, pretreatment of the acini with PKCα pseudosubstrate inhibitor or Gö6976 completely blocked this process, wherein PM Munc18c levels were retained to bind PM Syntaxin-4, and thereby blocked the exocytotic BPM SNARE complex assembly from forming.

DISCUSSION

We previously reported that supramaximal CCK-stimulated amylase secretion to be less than maximal because of apical blockade and redirection of exocytosis to less efficient basal PM sites (1). Here, we show that non-stimulatory EtOH (20 mM) pretreatment followed by submaximal or maximal CCK stimulation also reduced amylase secretion in a manner mimicking supramaximal CCK. We had shown that this inhibition was caused by PKC activation (19). We have now determined a process, wherein PM Munc18c levels were retained to bind PM Syntaxin-4, and thereby blocked the exocytotic BPM SNARE complex assembly from forming.

FIGURE 6. PKCα activation and distribution caused by supramaximal CCK or 20 mM alcohol followed by submaximal CCK stimulation. A, PKCα activity in dispersed acini resulting from stimulation with 10 nM CCK (10 min) (panel i) or 20 mM EtOH (1 h) + 50 pm CCK (10 min) (panel ii), in the absence or presence of the indicated PKC isoform-specific inhibitor. 10 μg of protein of lysates from dispersed acini subjected to the indicated treatments was loaded into each well and PKCα activity determined by EIA, with each sample performed in triplicate. Data shown in panels i and ii are the means ± S.D. from three separate portions of acini per experiment (from one rat) of three different acini preparations (n = 9), and values shown were normalized to basal activity in unstimulated (KRH only) control acini. Results were analyzed by Student’s t test, with significance determined to be p < 0.05. B, subcellular fractionation of dispersed acini stimulated with panel i, 10 nM CCK (10 min) or panel ii, 20 mM EtOH (1 h) + 50 pm CCK (10 min) showing PKCα translocation from the cytosol to the PM. 10 μg of protein of each fraction was separated on SDS-PAGE and immunoblotted with antibodies to PKCα and other indicated proteins. These blots are representative of three independent experiments n = 3, whose analyses are shown in supplemental Fig. S3.

plex capable of inducing fusion between the ZGs and PM. Our above results indicate that 10 nM CCK or 20 mM EtOH/50 pm CCK induction of Munc18c phosphorylation and displacement from the PM-bound Syntaxin 4 into the cytosol (where it becomes degraded) should enable such SNARE complex assembly to take place on the BPM. Fig. 7 directly examines this. Acini stimulated with KRH, 20 mM EtOH or 50 pm CCK did not induce SNARE complex assembly; and under these conditions, the abundant PM Munc18c remained tightly bound to Syntaxin-4, disabling Syntaxin 4 from binding the cognate SNARE proteins in the PM. In contrast, both 10 nM CCK (Fig. 7A) and PKCα inhibition could block basal PM exocytosis evoked by either supramaximal CCK or 20 mM EtOH/submaximal to maximal CCK stimulation. Surprisingly, apical exocytosis was not rescued in either stimulation protocols resulting in even further inhibition of secretion by the PKCα inhibitors. In fact, PKCα inhibition had no effect on amylase release or apical exocytosis evoked by submaximal or maximal CCK (in absence of EtOH pretreatment) (Figs. 1, C and D and 2A), indicating that apical exocytosis neither involves PKCα or the Munc18c/Sym-4 complex (see below). Apical exocytosis is likely mediated by a different complement of Munc18/SNARE complex (i.e.
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Munc18b, Syn-2, and Syn-3) (13, 25, 26) as postulated by a corollary hypothesis of compartmental specificity of the SNARE Hypothesis (5).

What is the mechanism that mediated basal PM exocytosis in pancreatic acinar cells? We previously postulated and demonstrated that basal PM exocytosis caused by supramaximal CCK stimulation involves the displacement of Munc18c from the BPM, a process that seemed to be required at least in pancreatic acinar cells to activate BPM-bound Syn-4 (1); and there, Munc18c displacement from the BPM could be prevented by Calphostin C, a nonspecific PKC inhibitor (1). Here, we have not only determined the precise PKC isoform mediating this action, but also demonstrated this to be the same putative molecular mechanism by which 20 mM EtOH altered submaximal (and maximal) CCK-evoked exocytosis to be redirected to the basal PM. The principal PKC isoforms present in pancreatic acini are conventional PKCα (DAG- and Ca\(^{2+}\)-sensitive); atypical PKCζ (DAG- and Ca\(^{2+}\)-insensitive) and novel PKCδ and PKCe (DAG-sensitive, Ca\(^{2+}\)-insensitive) (15, 27). PKCδ and PKCe have been reported to regulate cytokine-mediated inflammation and pancreatic acinar cell injury causing pancreatitis (15). Here, we showed that PKCδ and ε to have no role in exocytosis in pancreatic acinar cells.

We show that PKCα is the putative PKC that mediates Munc18c phosphorylation. Subcellular fractionation and confocal microscopy showed that both PKCα pseudosubstrate inhibitor and Go6976 were as effective as Calphostin C (1) in completely preventing Munc18c displacement from the BPM into the cytosol (and degradation). Both 10 nM CCK and 20 mM EtOH + 50 pM CCK stimulation uniformly activated PKCα, causing PKCα to translocate from the cytosol to the PM to directly bind and induce threonine phosphorylation of BPM-bound Munc18c, the latter shown by immunoprecipitation studies. Remarkably, all of these PKCα events activated by 10 nM CCK and 20 mM EtOH + 50 pM CCK stimulation were blocked by specific PKCα inhibition either by PKCα pseudosubstrate inhibitor or pharmacologic inhibitor and Go6976.

Other molecules are also involved in apical and basolateral exocytosis such as Ca\(^{2+}\) release and cytoskeleton disassembly, however these are not within the scope of this study. ZG v-SNAREs include VAMP2 and endobrevin/VAMP8 (7, 28); however, VAMP is specifically targeted to the different exocytotic sites has still to be determined. Considering that VAMP8 deletion ameliorated pancreatitis induced by supramaximal CCK (7), raises a possible role of VAMP8 in basolateral exocytosis. Consistently with this thinking, the immunoprecipitated SNARE complex in our study contained both VAMP2 and VAMP8 (Fig. 7). The FM1–43 imaging study showed a sequence of hotspots occurring at the sites on the BPM exhibiting similar amplitudes. This pattern suggests single ZG exocytosis at the basal PM occurs at only restricted sites and is capable of recruiting additional single ZGs but only after long pauses (several min) suggesting that longer intervals were required for ZGs to travel toward these BPM exocytic sites. However, the exocytic events at the BPM of adjacent cells of an acinus were remarkably synchronized suggesting that the signaling events regulating the vectorial transport of these ZGs to basal PM sites within an acinus must be tightly controlled. In contrast to this pattern of inefficient exocytosis at the BPM, apical exocytosis is much more efficient, where ~70% of ZGs within the apical poles can undergo sequential and compound fusions within just 1–2 min (1, 20), which would explain the differences in amylase secretion in these different stimulation protocols.

**FIGURE 7.** Supramaximal CCK or 20 mM alcohol + submaximal CCK stimulation can induce SNARE complex assembly. Dispersed acini were treated with either control buffers or PKC isoform-specific inhibitors as described in Fig. 1, C–E and then stimulated with either 10 nM CCK for 30 min (A) or 20 mM EtOH (1 h) + 50 pm CCK (1 h) (B). The acini were then fractionated into a PM fraction and whole cell lysates. Equal amounts of protein (200 µg) from the PM fractions were immunoprecipitated with anti-Syn-4 antibody, and the precipitated PM proteins and total cell lysates (8 µg) were separated on SDS-PAGE and blotted with the indicated antibodies. Total cell lysate lanes show similar levels of SNARE proteins in the various treatments and serve as input controls; however Munc18c levels are reduced only in the KRH-pretreated acini stimulated with 10 nM CCK or 20 mM EtOH + 50 pm CCK (see text for explanation). These blots are representative of three independent experiments n = 3, whose analyses are shown in supplemental Fig. 54.
This work leads us to postulate a model that partly explains a mechanism by which 20 mM alcohol, mimicking clinical alcohol intoxication (acute and chronic), acts as a susceptibility factor that induces pancreatic acinar cells to become sensitive to triggering factors (i.e. postprandial CCK stimulation), and which would evoke similar exocytotic events as supramaximal CCK stimulation employing the same set of exocytotic molecules. Of note, both these stimulatory protocols in vivo induce mild acute pancreatitis in rodents (8). Because CCK receptors are found on human pancreatic acinar cells (29), this would be a susceptibility mechanism by which alcohol intoxication would predispose to human pancreatitis (1, 13). In this model, EtOH treatment alone has little effect on Munc18c (minimal PKCα induced phosphorylation), but in the presence of submaximal CCK stimulation, induces maximal PKCα-induced threonine phosphorylation of Munc18c, likely at Thr314, as this site is conserved in the parental Munc18a at Ser313 (23). Phosphorylation of Munc18c would remodel Syn-4, activating it to promote its assembly with SNAP23, which would render the BPM receptive to exocytosis, by their further assembly with oncoming ZG VAMPs (VAMP2 and/or VAMP8) to form a SNARE complex. In fact, this work precisely demonstrated that the above postulated sequence of events do occur after both 20 mM EtOH/50 pm CCK and supramaximal CCK stimulation, and which indeed led to the formation of the BPM SNARE complex (Fig. 7) and ensuing basolateral exocytosis.

Our results are consistent with the very recent report showing specific domain-specific interactions between Munc18c and Syn-4, which greatly facilitated Syn-4 assembly with SNAP 23 and VAMP (30). That study is distinct from earlier reports of synaptic SNARE complex regulation by parental Munc18a-Syn-1A interactions (3, 4, 22). Here we show that PKCα-mediated Munc18c phosphorylation is required for basolateral exocytosis per se, but whether Munc18c displacement from the BPM is also required or simply a downstream event occurring after SNARE complex assembly is effected, would require further study. It is possible that phosphorylated Munc18c in pancreatic acinar cells may be more susceptible to cytosolic enzymes native to acinar cells or that such cytosolic enzymes could be independently activated by PKCα in acinar cells, either of which would cleave Munc18c from the PM (appearing as displacement into the cytosol) to then undergo further cytosolic proteolytic degradation. In pancreatic acinar cells, upon assembly of the Syn-4/SNAP23/VAMP SNARE complex the presence of Ca2+ would trigger exocytotic fusion at the BPM into the interstitial space. Whereas basal PM exocytosis normally occurs, this would likely be rather minimal. However, excessive exocytosis into the interstitial space that is not cleared by the microcirculation and ensuing unchecked zymogen activation would then lead to interstitial pancreatitis. This mechanism would contribute to the other cellular mechanisms (i.e. trypsinogen activation, cytokines, etc.) of alcohol-induced injury to the exocrine pancreas (9–12, 31).

Munc18c and its cognate SNARE complex (Syn-4, SNAP 23) are key molecules regulating GLUT transporters in adipose tissues and muscles (30, 32, 33), and recently also in pancreatic islet beta cell insulin granule exocytosis (34). This role of Munc18c in inducing aberrant basal PM exocytosis in pancre-atic acinar cells raises a possibility that Munc18c could be pathologically regulated in diabetes (or perhaps by alcohol) to distort GLUT4 transport or insulin exocytosis in these tissues. Furthermore, other primary alcohol-targeted secretory cells, such as neurons and hepatocytes could also be susceptible to this novel mechanism (i.e. PKCα activation) of alcohol or its metabolites acting on Munc18 proteins present in these tissues (4, 35) to modulate exocytosis. Further studies would be required to examine whether these insights into Munc18c biology in pancreatic acinar cells could be applied to these tissues as well.

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