ETHANOL ENHANCES CARBACHOL-INDUCED PROTEASE ACTIVATION AND ACCELERATES Ca\textsuperscript{2+} WAVES IN ISOLATED RAT PANCREATIC ACINI

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Alcohol abuse is a leading cause of pancreatic injury, accounting for 30% of acute cases and 70-90% of chronic cases, yet the mechanisms leading to alcohol-associated injury are unclear. An early and critical feature of pancreatitis is the aberrant signaling of Ca\textsuperscript{2+} within the pancreatic acinar cell. An important conductor of this Ca\textsuperscript{2+} is the basolaterally localized, intracellular Ca\textsuperscript{2+} channel ryanodine receptor (RYR). In this study, we examined the effect of ethanol on mediating both pathologic intra-acinar protease activation, a precursor to pancreatitis, as well as RYR Ca\textsuperscript{2+} signals. We hypothesized that ethanol sensitizes the acinar cell to protease activation by modulating RYR Ca\textsuperscript{2+}. Acinar cells were freshly isolated from rat, pre-treated with ethanol, and stimulated with the muscarinic agonist carbachol (1 uM). Ethanol caused a doubling in the carbachol-induced activation of the proteases trypsin and chymotrypsin (P<0.02). The RYR inhibitor dantrolene abrogated the enhancement of trypsin and chymotrypsin activity by ethanol (P<0.005 for both proteases). Further, ethanol accelerated the speed of the apical to basolateral Ca\textsuperscript{2+} wave from 9 um/sec to 18 um/sec (P<0.0005; n=18-22 cells/group); an increase in Ca\textsuperscript{2+} wave speed was also observed with a change from physiologic concentrations of carbachol (1 uM) to a supraphysiologic concentration (1 mM) that leads to protease activation. Dantrolene abrogated the ethanol-induced acceleration of wave speed (P<0.05; n=10-16 cells/group). Our results suggest that the enhancement of pathologic protease activation by ethanol is dependent on the RYR and that a novel mechanism for this enhancement may involve RYR-mediated acceleration of Ca\textsuperscript{2+} waves.

Pancreatitis is a life threatening inflammatory disorder of the pancreas which leads to more than 30,000 deaths per year (1). Alcohol-associated pancreatitis accounts for 30% of acute cases and 70-90% of chronic cases. Further, alcoholic pancreatitis carries the highest mortality rate among all etiologies (2). However, the mechanisms by which ethanol mediates pathology are largely unknown.

Alcohol can exert diverse effects on the pancreas. Ethanol exposure has been linked to abnormal blood flow, leading to ischemic changes, and increased sphincter of Oddi dysfunction, resulting in pancreatic duct hypertension (2).

In addition, ethanol appears to directly predispose the acinar cell to pathologic changes including oxidant stress (3), membrane fragility (4), mitochondrial uncoupling (5,6), and basolateral exocytosis (7). Several lines of evidence also link ethanol to aberrant Ca\textsuperscript{2+} signaling (6). High amplitude, aberrant, intra-cellular Ca\textsuperscript{2+} waves that propagate from the apical to basolateral region of the acinar cell predispose to early features of pancreatitis, particularly intra-acinar protease activation (8,9). We know that intra-cellular Ca\textsuperscript{2+} release is responsible for the onset of this aberrant Ca\textsuperscript{2+} signal (10). In addition, we have previously shown that the ryanodine receptor (RYR), a major intracellular Ca\textsuperscript{2+} channel is localized to the basolateral region of the acinar cell and, more importantly, is linked to the onset of pathologic protease activation (8).

Though ethanol has been reported to cause small increases in resting acinar cell Ca\textsuperscript{2+} and low amplitude Ca\textsuperscript{2+} transients, more prominent changes were associated with its metabolites, notably fatty acid ethyl esters (FAEEs) (6,11). Recent studies demonstrate that administration of FAEEs evoke large Ca\textsuperscript{2+} transients that were IP3R-sensitive (5,6). However, the effect of ethanol on RYR Ca\textsuperscript{2+} has not been examined.
Cellular Ca\textsuperscript{2+} homeostasis is governed by the interaction of a number of factors, including Ca\textsuperscript{2+} pools, release channels, pumps, exchangers, and buffers (12). Following secretagogue-induced stimulation of acinar cells, the initial rise in Ca\textsuperscript{2+} is predominantly controlled by two types of ER Ca\textsuperscript{2+} channels: (1) the inositol 1,4,5-trisphosphate receptor (IP3R) channel, which is activated by inositol 1,4,5 trisphosphate (IP3), and (2) the RYR, which is structurally similar to the IP3R but responds to different ligands (13). We and others have shown that the RYR is excluded from the apical region but localized to the basal region, importantly, where pancreaticzymogens, particularly proteases, are first activated during acute pancreatitis (8,14). Cytosolic Ca\textsuperscript{2+} is the most potent activator, thus making the RYR the prototypic Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release (CICR) channel.

We have linked RYR Ca\textsuperscript{2+} release in the basal region of the acinar cell to pathologic, premature intra-acinar protease activation, a critical and primary event in the pathogenesis of pancreatitis (15). Protease activation is dependent on cytosolic Ca\textsuperscript{2+} signals. This is supported by evidence demonstrating that the cytosolic Ca\textsuperscript{2+} chelator BAPTA completely blocks activation (9,16,17). In the current study, we examined the role of ethanol in modulating the RYR Ca\textsuperscript{2+} signal and its effect on mediating protease activation. Our data demonstrate that ethanol (1) enhances intracellular protease activation and (2) accelerates carbachol-induced Ca\textsuperscript{2+} waves. Both effects of ethanol appear to be working through the RYR.

**EXPERIMENTAL PROCEDURES**

Preparation of pancreatic acini for enzyme kinetics/secretion assays- Groups of pancreatic acinar cells were isolated as previously described (8) with modifications. Briefly, Sprague-Dawley rats were euthanized using a protocol approved by the Animal Care and Use Committee. The pancreas was removed and minced for 5 min in Dulbecco’s Modified Eagle Medium (DMEM)/F12 1X buffer without phenol (Gibco Invitrogen, Carlsbad CA) containing 15 mM HEPES (pH 7.4), 120.61 mM NaCl, 4.16 mM KCl, 0.301 mM MgCl\textsubscript{2}, 1.05 mM CaCl\textsubscript{2}, 17.51 mM dextrose, 0.05 M HCl, 2 mM sodium pyruvate, and 2.5 mM glutamine, plus 0.1% BSA and 2mg/ml type-4 collagenase (Worthington, Freehold, NJ). The tissue was briefly oxygenated, and incubated for 5 min at 37°C with shaking (90 rpm), buffer was removed and replaced with fresh collagenase buffer, then briefly oxygenated, and incubated for 35 min. The tissue digest was triturated and filtered through a 300 µm mesh (Sefar American, Depew, NY). Acinar cells were washed three times with collagenase-free buffer. Acinar cells were allowed to equilibrate for 5 min at 37°C before treatment.

Preparation of pancreatic acini for Ca\textsuperscript{2+} imaging- Groups of pancreatic acinar cells were isolated as previously described (8) with minor modifications. Briefly, Sprague-Dawley rats weighing 50-100 g (Charles River Labs, Wilmington, MA) were euthanized using a protocol approved by the Animal Care and Use Committee. The pancreas was removed and minced for 5 min in buffer containing 20 mM HEPES (pH 7.4), 95 mM NaCl, 4.7 mM KCl, 0.6 mM MgCl\textsubscript{2}, 1.3 mM CaCl\textsubscript{2}, 10 mM glucose, and 2 mM glutamine, plus 1% BSA, 1X MEM nonessential amino acids (GIBCO/BRL), 200 units/ml type-4 collagenase (Worthington, Freehold, NJ), and 1 mg/ml Soybean Trypsin Inhibitor. The tissue was incubated for 60 min at 37°C with shaking (90 rpm). The digest was transferred to a 15 mL conical tube and washed three times with collagenase-free and BSA-free buffer. The cells were vigorously shaken and then filtered through a 300 µm mesh (Sefar American, Depew, NY) to separate the cells into smaller clusters.

Stimulation of Acini and Enzyme Activation Assays- Acini were pre-treated with ethanol (1, 10, 50, 100 mM) or dantrolene (10, 50, 100 uM) at 37°C for 15 and 30 min, respectively. Acini were then stimulated with the acetylcholine analog carbachol (Carb) for 60 min at 37°C. Protease activity assays were performed at room temperature using fluorogenic substrates as previously described (8). Briefly, 50 ul of 400 uM enzyme substrate were added to each homogenized sample and accumulation of fluorescence was measured over 10 minutes using a fluorescent plate reader (Infinite M200, Tecan) at 380-nm excitation and 440-nm emission wavelengths. The trypsin substrate was supplied by Peptides International (Louisville, KY) and had the amino acid sequence Boc-Gln-Ala-Arg-MCA.
Chymotrypsin substrate was supplied by CalBiochem (Gibbstown, NJ) and had the amino acid sequence Suc-Ala-Ala-Pro-Phe-AMC. Protease activity was collected as relative fluorescent units per second (RFU/sec), normalized to total Amylase (RFU/sec/ug Amylase), and expressed as a fold increase relative to either the control or a maximum condition. Amylase secretion was measured at room temperature from the media and cell homogenates using a Phadebas kit (Magle life sciences, Lund, Sweden). Total amylase values were used for normalization of chymotrypsin and trypsin results, and percent amylase secretion into the media was calculated as a measure of enzyme secretion.

Detection of cellular Ca\textsuperscript{2+} signals- Acinar cells were loaded at room temperature with the high-affinity Ca\textsuperscript{2+}-sensing dye fluo-4/AM (K_{Ca}=345 nM; Molecular Probes). Cells were then pre-treated with or without one or more of the following for 30 or 60 min at room temperature: ethanol (100 mM); the RYR inhibitor dantrolene (100 uM). Acinar cells were plated on acid-washed glass coverslips and then mounted on a perfusion chamber. Thereupon, they were stimulated at room temperature with the muscarinic agonist carbachol at the concentrations indicated. A Zeiss LSM510 laser scanning confocal microscope was used with a 63X, 1.4 numerical aperture objectives. The dye was excited at 488 nm wavelength, and emission signals of >515 nm were collected at frame speeds of 200-300 m/sec/frame. Fluorescence from individual acinar cells as well as apical and basal sub-cellular regions was recorded. For experiments performed at 37°C, cells were loaded at room temperature and mounted on a Zeiss 710 heating stage. Images were collected as described above.

Determination of Ca\textsuperscript{2+} wave speed- Apical and basal regions of interest in the acinar cell recordings were chosen using the Image J software (NIH), and mean fluorescence over time in each region was graphed. Ca\textsuperscript{2+} wave speed was calculated by dividing the distance along the long axis of the acinar cell by the time it took for the Ca\textsuperscript{2+} wave to travel from the apical to the basal region.

Cell injury assays. Acinar cell injury was quantified as LDH release using the non-radioactive cytotoxicity assay (Promega, Madison, WI). Absorbance was measured at 490 nm within 15 min of stopping the enzyme reaction. Results are expressed as percent LDH released into the media.

Statistics- Data represent mean ± SEM of at least three individual experiments with multiple cells from each experiment. Statistical significance was determined by Student’s t test analysis.

RESULTS

Ethanol enhances carbachol-induced protease activation. Supraphysiologic concentrations of the secretagogues cholecystokinin (CCK) and acetylcholine (Ach), or their analogs, induce pathologic intra-acinar protease activation. It is known that ethanol enhances CCK-induced protease activation within acinar cells (18). However, there are conflicting reports as to whether the cholinergic pathways are similarly affected by ethanol (19,20). It would be of particular interest to know this because Ach stimulation of acinar cells may be a more physiologically relevant situation than CCK stimulation because: (1) Ach is the primary stimulant for pancreatic secretion in human acinar cells and (2) even in rodents, neurogenic pathways that locally secrete Ach likely contribute to a major component of CCK-induced secretion as well as pancreatitis (20,21). In addition, ethanol was recently shown to disrupt Ach-induced acinar cell events such as secretion by redirecting exocytosis from the apical to the basolateral membrane (22). For these reasons, we examined whether ethanol can modulate intracellular events initiated by stimulation with the Ach analog carbachol. Assays for trypsin and chymotrypsin activity were measured because the generation of these proteases closely follows first order kinetics over a 30-60 min period of incubation (18).

Treatment of acinar cells with ethanol alone (100 mM) for 15 min had no effect on protease activity, a result consistent with previous work (18). However, administration of ethanol (100 mM) for 15 min before physiologic carbachol (1 uM) stimulation caused a two-fold increase in both trypsin and chymotrypsin activities above unstimulated controls (P<0.02; Fig. 1). This effect was also observed during supraphysiologic stimulation with carbachol (1 mM) (P<0.05; Supp
Fig. 1A) as well as with 1 hr ethanol pretreatment (P<0.05; Supp Fig. 2).

To know whether the enhancement of protease activation by ethanol is seen over a broad range of concentrations, acinar cells were incubated with biologically relevant ethanol concentrations (1-100 mM) prior to stimulation with carbachol (1 uM). These ethanol concentrations are achievable in serum during acute ingestion, corresponding to blood alcohol concentrations of 0.005-0.5%. We observed a concentration-dependent increase in chymotrypsin activity with ethanol (P<0.01;Fig 2). For all subsequent experiments we used 100 mM concentration to provoke the maximal ethanol effect. These results indicate that biologically relevant ethanol concentrations sensitize the pancreatic acinar cell to carbachol-induced protease activation.

Dantrolene reduces ethanol-enhanced protease activation but does not affect amylase secretion. In previous work we’ve shown that aberrant Ca^{2+} signaling is mediated by the intracellular Ca^{2+} channel, the ryanodine receptor (RYR). In the current study, we tested the hypothesis that ethanol triggers protease activation through RYR-mediated aberrant Ca^{2+} signals. Pancreatic acinar cells were pre-treated with the RYR inhibitor dantrolene for 30 min prior to ethanol incubation. We observe that the enhancement of protease activity by ethanol was abrogated by dantrolene during physiologic carbachol stimulation (P<0.01;Fig. 3). The reduction with dantrolene was also seen during supraphysiologic carbachol stimulation (P<0.05; Supp Fig. 1) and 1 hr ethanol pretreatment (P<0.05;Supp Fig. 2). The predominance of RYR1 in rat acinar cells substantiated the use of dantrolene (8), which selectively inhibits RYR1 over other isoforms (23). Further, unlike other RYR inhibitors dantrolene does not disrupt mitochondrial pathways at concentrations above 100 uM. Nevertheless, in limited studies with lower concentrations, dantrolene (10-100 uM) caused a concentration-dependent reduction in the enhancement of protease activation by ethanol (P<0.01;Fig. 4). The results indicate that ethanol sensitizes the pancreatic acinar cell to enhanced protease activation via the RYR.

Early pancreatic acinar cell injury is thought to result from a combination of intracellular protease activation and concurrent secretory inhibition of those enzymes from the acinar cell. Cosen-Binker et.al. demonstrated that pretreatment of isolated acinar cells with ethanol for 1 hr caused an inhibition of amylase secretion (24). However, we found that brief pretreatment did not affect either constitutive or regulated (i.e. carbachol-stimulated) enzyme secretion (Fig. 5). These data are consistent with our hypothesis that RYR-mediated Ca^{2+} signaling from the basolateral region enhances protease activation, but does not affect apical secretory events.

We also demonstrate that cells pre-treated with ethanol prior to supraphysiologic carbachol stimulation undergo cell injury, evidenced by an increase in LDH release. However, dantrolene treatment completely abrogated the enhancement by ethanol (P<0.05; Supp Fig. 1B)

Supraphysiologic carbachol stimulation leads to increased speed of the apical to basolateral Ca^{2+} wave compared to physiologic stimulation. Acinar cells perfused with carbachol evoke a Ca^{2+} wave that originates in the apical region and propagates in a coordinated fashion to the basolateral region. Although there also exists a coordinated release of Ca^{2+} between cells (25-27), each individual cell propagates an apical to basolateral Ca^{2+} wave. We examined the speed of the acinar cell Ca^{2+} wave induced by carbachol with or without ethanol because, among other factors in the Ca^{2+} toolbox (28), the speed is dependent on the serial activation of the basolaterally localized intracellular Ca^{2+} channel the RYR (29). Tracings from apical and basolateral regions of interest from multiple cells in each field were used to calculate the speed of this Ca^{2+} transient, as described in the Methods. Physiologic carbachol (1 uM) evoked a Ca^{2+} wave speed of 9 um/sec, while supraphysiologic (1 mM) stimulation, a concentration that causes pathologic protease activation, markedly increased the speed to 43 um/sec (P<0.05;Fig. 6). Although no causal relationship has been established, the results correlate an increase in Ca^{2+} wave speed with a change from increasing carbachol concentrations that cause protease activation.

Ethanol accelerates the speed of the carbachol-induced Ca^{2+} wave via the RYR. Next, we examined whether ethanol affected Ca^{2+} waves generated by physiologic carbachol (1 uM). Cells were pre-treated with ethanol (100 mM) for 30
min prior to carbachol (1 uM) stimulation. We observed that cells pre-treated with ethanol still evoked a Ca²⁺ transient that began in the apical region and propagated to the basolateral region, however, Ca²⁺ wave speed was significantly enhanced above control levels (P<0.05; Fig. 7). This effect was also observed after 1 hr of ethanol pretreatment or at 37°C (P<0.05; Supp Fig. 4-5). In addition, ethanol caused a two fold increase in Ca²⁺ wavespeed after supraphysiologic carbachol (1 mM) stimulation (P<0.05; Supp Fig. 8). To know whether the RYR mediates the ethanol-induced acceleration of Ca²⁺ waves, acinar cells were pre-treated with dantrolene (100 uM). In this condition, the acceleration of Ca²⁺ wave speed by ethanol was abrogated (P<0.05;Fig. 8), indicating a dependency on the RYR.

**DISCUSSION**

Our study advances four important findings that relate ethanol to early events in pancreatitis: (1) Ethanol accelerates the speed of the apical to basolateral Ca²⁺ wave generated by physiologic carbachol (1 uM), a previously undescribed effect of ethanol; (2) An increase in Ca²⁺ wave speed correlates with conditions that cause protease activation (e.g. supraphysiologic carbachol); (3) Ethanol enhances carbachol-induced protease activation; (4) Both the acceleration of Ca²⁺ wave speed and the enhancement of protease activation are dependent on RYR activation.

**Ethanol’s effect on the acinar cell.** Ethanol has multiple effects on the acinar cell. Cosen-Binker et. al. have shown that ethanol inhibits enzyme secretion stimulated by sub-maximal carbachol concentrations (30). The reduction is associated with a partial redirection of zymogen granule exocytosis to the basolateral membrane. Ethanol phosphorylates PKC α, leading to phosphorylation of a SNARE protein, Munc18C. This results in displacement of Munc18C and allows a SNARE complex to form between zymogen granules and the basolateral membrane, thereby permitting basolateral exocytosis (30). Satoh et. al. showed that another PKC isoform PKC ε could be phosphorylated by ethanol and that this caused NF-κB activation (31). In addition, ethanol causes lysosomal membrane fragility, resulting in the leakage of lysosomal enzymes capable of activating trypsinogen within the cytosol (32).

**Ethanol’s effect on protease activity.** Our results demonstrate that ethanol pretreatment enhances intra-acinar protease activation by carbachol. The findings confirm recent work by Lugea et. al. (20). Previous studies from Gorelick and colleagues demonstrated that ethanol sensitizes the acinar cell to CCK-induced protease activation (18,33), but they were not able to detect sensitization of ethanol to carbachol using a carboxypeptidase A1 zymogen conversion assay (33). However, both our study and that of Lugea et. al. used selective fluorescent protease substrates, which are more sensitive in measuring changes in zymogen activity. We used a physiologic secretagogue concentration (1 uM), which evokes maximal pancreatic enzyme secretion, only a small amount of protease activation, and no acinar cell damage. The pathologic effects of ethanol on this condition were observed over a clinically relevant range of ethanol concentrations (10-100 mM) that are achievable in serum during intoxication (34).

**Ethanol’s effect on Ca²⁺ signaling.** Ethanol also affects acinar cell Ca²⁺. By itself, a concentration of 850 mM was required to evoke a small Ca²⁺ transient (6). There is also evidence that ethanol either directly or through generation of reactive oxygen species (ROS) causes Ca²⁺ influx (35,36). However, several reports implicate a role for ethanol metabolites as the actual mediators of aberrant Ca²⁺ signaling in acinar cells (34,37). Ethanol is readily metabolized by tissues into oxidative metabolites, notably acetaldehyde, and the non-oxidative metabolites, phosphatidylethanol and fatty acid ethyl esters (FAEES) (38).

**The role of FAEES in pathology.** Notably, FAEES are found at highest levels in the pancreas, as produced by pancreatic acinar cells (37), and can induce pancreatic damage (39,40) and intra-acinar protease activation (6,41). In more recent studies, administration of FAEES evoked large Ca²⁺ transients that were both IP3R and RYR sensitive (42). Further, sustained cytosolic Ca²⁺ levels by FAEES were associated with necrosis (5,6).

We confirmed that ethanol alone (100 mM) did not evoke a Ca²⁺ transient. However, brief pretreatment nearly doubled the speed of the
apical to basolateral Ca\textsuperscript{2+} wave induced by physiologic carbachol. To our knowledge, this is the first demonstration that ethanol accelerates the speed of the apical to basolateral acinar cell Ca\textsuperscript{2+} wave. Further, an increased Ca\textsuperscript{2+} wave speed was associated with conditions leading to protease activation. It is intriguing to postulate how this acceleration of Ca\textsuperscript{2+} waves might decode signals leading to pancreatitis. Specifically, what pathologic targets are modulated by the change in the shape of the Ca\textsuperscript{2+} signal? A prototypic demonstration is that the frequency of Ca\textsuperscript{2+} oscillations can cause differential changes in gene expression (43). Although ethanol did not affect the frequency of acinar cell Ca\textsuperscript{2+} oscillations (Supp Fig. 6D), the concept is proof of principal that apparently subtle biophysical changes in the Ca\textsuperscript{2+} signal can translate to biologically important outcomes. Putative targets of acinar cell Ca\textsuperscript{2+} pathology could include the Ca\textsuperscript{2+}-activated phosphatase calcineurin, which has recently been shown to mediate protease activation and pancreatitis (17,44), the calpains (45-47), or the classical protein kinase C (48). Future work using targeted deletion of these proteins as well as simultaneous imaging of both Ca\textsuperscript{2+} and protease activation will help examine pathways linking an acceleration of Ca\textsuperscript{2+} waves to pancreatitis.

The origins and determinants of the Ca\textsuperscript{2+} wave. Two theories explain the origins of the Ca\textsuperscript{2+} wave. Kasai et. al. postulated that high sensitivity IP3Rs at the apical “trigger zone” generate the Ca\textsuperscript{2+} transient, which then propagates throughout the cell as a result of Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release from low-sensitivity IP3R in the granular and basal regions (49,50). A second theory postulates that RYRs distributed in the basolateral pole are responsible for the Ca\textsuperscript{2+} wave propagation once a Ca\textsuperscript{2+} transient is triggered by apical IP3Rs (25,26,29). Our results, demonstrating that RYR inhibition abrogates the acceleration of Ca\textsuperscript{2+} waves by ethanol supports the latter possibility. These data also suggest that ethanol, either directly or indirectly, causes the RYR to shift to a higher sub-conductance state for Ca\textsuperscript{2+} opening.

The RYR as a mediator of pathology. The current work is consistent with our previous findings demonstrating that (1) high amplitude Ca\textsuperscript{2+} transients in the basolateral region induced by supraphysiologic carbachol are dependent on the RYR (8), (2) RYR inhibition reduces protease activation in those conditions (8), and (3) in vivo administration of the RYR inhibitor dantrolene alleviates disease outcomes of pancreatitis (51). Factors that might modulate RYR opening in the acinar cell include phosphorylation, nitrosylation, and oxidation. The former two post-translational modifications are associated with disorders of Ca\textsuperscript{2+} leak in myocytes leading to cardiac arrhythmias and muscular dystrophy, respectively (52).

Although the RYR can be phosphorylated at different sites, it will be interesting to examine whether a specific PKA site extensively described in cardiomyocytes is modulated by ethanol. This is because cAMP has been shown to accelerate the acinar cell Ca\textsuperscript{2+} wave in an RYR-dependent manner (53).

Although the RYR may play a central role in accelerating acinar cell Ca\textsuperscript{2+} waves, there are several other potential modifiers. Activation of PKC, without regard to isoform specificity, or lowering cytosolic pH can reduce Ca\textsuperscript{2+} wave speed (54,55). Conversely, inhibition of mitochondria (25) or SERCA pumps (27) increase wave speed. It will be of interest to know whether ethanol affects one or more of these pathways to modulate Ca\textsuperscript{2+} waves or whether its metabolites are involved.

In summary, we report that ethanol accelerates the speed of acinar cell Ca\textsuperscript{2+} waves induced by carbachol and that it also enhances carbachol-induced protease activation. Both effects of ethanol are dependent on the RYR. We speculate that RYR inhibition with dantrolene might, therefore, function as treatment for alcohol-induced pancreatitis, a disease for which only supportive care is currently available.

REFERENCES


FOOTNOTES

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The abbreviations used are: BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; Ca$^{2+}$, cytosolic Ca$^{2+}$ unless otherwise stated; CCh, carbachol; Ach, acetylcholine; CCK, cholecystokinin; CICR, Ca$^{2+}$ induced Ca$^{2+}$ release; ER, endoplasmic reticulum; FAEEs, fatty acid ethyl esters; IP3, inositol 1,4,5 trisphosphate; IP3R, inositol 1,4,5 trisphosphate receptor; ROS, reactive oxygen species; RYR, ryanodine receptor; SERCA, sarco-endoplasmic reticulum Ca$^{2+}$ ATPase; SOCE, store-operated Ca$^{2+}$ entry.

**FIGURE LEGENDS**

**Fig. 1.** Ethanol enhances carbachol-induced pancreatic protease activation. Acinar cells were pre-treated with ethanol (100 mM) for 15 min prior to carbachol (1 uM) stimulation. Activities of the proteases relative to carbachol alone. *A.* trypsin and *B.* chymotrypsin were normalized to total amylase content (n=4). *, P<0.05 with respect to carbachol alone.

**Fig. 2.** Ethanol causes a concentration-dependent enhancement of protease activation induced by carbachol. Acinar cells were treated with ethanol for 15 min prior to carbachol (1 uM) stimulation. Activity of chymotrypsin was normalized to total amylase content and represented relative to carbachol alone (n=3). *, P<0.01 with respect to carbachol alone.

**Fig. 3.** Ethanol-enhanced protease activation is dependent on the ryanodine receptor (RYR). Acinar cells were pre-treated with the RYR inhibitor dantrolene (100 uM) for 30 min prior to ethanol (100 mM) and carbachol (1 uM) administration. Activities of the proteases *A.* trypsin and *B.* chymotrypsin were normalized to total amylase content and represented relative to carbachol alone (n=3). *, P<0.005; **, P<0.01, relative to carbachol alone and carbachol with ethanol, respectively.

**Fig. 4.** Dantrolene reduces ethanol-enhanced protease activation over a range of inhibitory concentrations. Acinar cells were pre-treated with dantrolene (10-100 uM) for 30 min prior to ethanol (100 mM) and carbachol (1 uM) administration (n=3). Activity of chymotrypsin was normalized to total amylase content and represented relative to the maximum condition. *, P<0.005; #, P<0.01, relative to carbachol alone and carbachol with ethanol respectively.

**Fig. 5.** Amylase secretion is unaffected by short term ethanol incubation or RYR inhibition. Acinar cells were pre-treated with dantrolene (100 uM) for 30 min prior to ethanol (100 mM) and carbachol (1 uM) administration (n=3). Percent amylase release was assayed from cell lysate and media samples.

**Fig. 6.** Supraphysiologic carbachol stimulation leads to increased speed of the apical to basolateral Ca$^{2+}$ wave compared to physiologic stimulation. Acinar cells were stimulated with *A.* Carbachol (1 uM), from left to right: Bright field view of an acinus labeled at the (A)palic and (B)asolateral regions of interest from an acinar cell. Cells were loaded with the Ca$^{2+}$ indicator fluo-4 (5 uM). Upon stimulation with physiologic carbachol (1 uM), subsequent images show the initiation of the Ca$^{2+}$ signal in the apical region followed by propagation to the basal region. *B.* Each paneled image (1-4), corresponds to a frame along a representative tracing of change in fluorescence over time for each region of interest. Left and right arrows show time of first Ca$^{2+}$ rise in the apical and basal regions, respectively. *(C-D)* Cells were stimulated with supraphysiologic carbachol (1 mM). *E.* Quantitation of difference in Ca$^{2+}$ wave speed between the two carbachol conditions (n=10 cells in each). *, P<0.05.

**Fig. 7.** Ethanol accelerates the physiologic carbachol-stimulated Ca$^{2+}$ wave. Acinar cells were treated with or without ethanol (100 mM) for 30 min prior to carbachol. *A.* Carbachol (1 uM) stimulation. From left to right: Bright field view of an acinus labeled at the (A)palic and (B)asolateral regions of interest from an acinar cell. Cells were loaded with the Ca$^{2+}$ indicator fluo-4 (5 uM). Upon stimulation with physiologic carbachol (1 uM), subsequent images show the initiation of the Ca$^{2+}$ signal in the apical
region followed by propagation to the basal region. B. Each paneled image (1-4), corresponds to a frame along a representative tracing of change in fluorescence over time for each region of interest. Left and right arrows show time of first Ca\textsuperscript{2+} rise in the apical and basal regions, respectively. (C-D) Cells were pre-treated with ethanol (100 mM) for 30 minutes. E. Quantitation of difference in Ca\textsuperscript{2+} wave speed between the two carbachol conditions (n=13 cells in each). *, P<0.005.

Fig. 8. The acceleration of Ca\textsuperscript{2+} wave speed by ethanol is dependent on the RYR. In addition to pretreatment with ethanol (100 mM), a third group of acinar cells was incubated dantrolene (100 uM) for 30 min prior to carbachol (1 uM) stimulation (n = 13-14 cells in each group). *, P<0.005; **, P<0.0005, with respect to carbachol alone and, carbachol with ethanol, respectively.

Fig. 9. Proposed mechanism by which ethanol evokes pathological effects on the pancreatic acinar cell. In the presence of carbachol, ethanol targets the RYR to induce aberrant Ca\textsuperscript{2+} waves that propagate from the apical to basolateral region of the acinar cell. This change in the shape of the Ca\textsuperscript{2+} signal predisposes to premature intra-acinar protease activation, and results in pancreatic injury, leading to pancreatitis.
Fig. 1

A  Trypsin

B  Chymotrypsin

Relative activity

Unstimulated  Carb

+ EtOH

*
Fig. 2

![Bar graph showing relative activity levels with different concentrations of Carb (1 uM) and EtOH (mM).]
**Fig. 3**

**A**

**Trypsin**

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<th>Condition</th>
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<tr>
<td>Carb (1 uM)</td>
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<td>EtOH (100 mM)</td>
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<tr>
<td>Dant (100 uM)</td>
<td></td>
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</tbody>
</table>

Relative activity

* P < 0.05
** P < 0.01
B

**Chymotrypsin**

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<tr>
<td>Dant (100 uM)</td>
<td></td>
<td></td>
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<td>**</td>
</tr>
</tbody>
</table>
Fig. 5

Pancreatic Enzyme Secretion

% Amylase secretion

Carb (1 uM) - + + +
EtOH (100 mM) - - + +
Dant (100 uM) - - - +

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Fig. 6

A

Physiologic carbachol (1 μM)

B

Apical Basolateral

C

Supra-physiologic carbachol (1 mM)

D

Apical Basolateral
**Graph:**

- **Y-axis:** Carbachol
  - 1 μM
  - 1 mM

- **X-axis:** Ca\(^{2+}\) wave speed (μm/sec)
  - 0
  - 10
  - 20
  - 30
  - 40
  - 50
  - 60

**Note:**
- Significant difference indicated by asterisk (*)
Fig. 7

A

100% ΔF/F₀
2 sec

1 2 3 4

Carbachol (1 uM)

B

Est. [Ca²⁺]ᵢ
min max

1 2 3 4

Apical Basolateral

C

D

100% ΔF/F₀
2 sec

1 2 3 4

Carbachol + EtOH

Apical Basolateral
Fig. 8

Ca\textsuperscript{2+} wave speed (um/sec)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Carb (1 uM)</th>
<th>EtOH (100 mM)</th>
<th>Dant (100 uM)</th>
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<tr>
<td>-</td>
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</table>
Fig. 9

Apical

Ca^{2+} wave

Basolateral

Pancreatitis

Protease Activation

EtOH

RYR

Ca^{2+}

ER

Carbachol
Ethanol enhances carbachol-induced protease activation and accelerates Ca\textsuperscript{2+} waves in isolated rat pancreatic Acini

Abrahim I. Orabi, Ahsan U. Shah, Kamaldeen Muili, Yuhuan Luo, Syeda Maham Mahmood, Asim Ahmad, Anamika Reed and Sohail Z. Husain

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