Pharmacological and genetic inhibition of calcineurin protects against carbachol-induced pathological zymogen activation and acinar cell injury

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Submitted 23 December 2011; accepted in final form 31 January 2012

Pharmacological and genetic inhibition of calcineurin protects against carbachol-induced pathological zymogen activation and acinar cell injury. Am J Physiol Gastrointest Liver Physiol 302: G898–G905, 2012. First published February 9, 2012; doi:10.1152/ajpgi.00545.2011.—Acute pancreatitis is a major health burden for which there are currently no targeted therapies. Premature activation of digestive proenzymes, orzymogens, within the pancreatic acinar cell is an early and critical event in this disease. A high-amplitude, sustained rise in acinar cell Ca2+ is required for zymogen activation. We previously showed in a cholecystokinin-induced pancreatitis model that a potential target of this aberrant Ca2+ signaling is the Ca2+-activated phosphatase calcineurin (Cn). However, in this study, we examined the role of Cn on both zymogen activation and injury, in the clinically relevant condition of neurogenic stimulation (by giving the acetylcholine analog carbachol) using three different Cn inhibitors or Cn-deficient acinar cells. In freshly isolated mouse acinar cells, pretreatment with FK506, calcineurin inhibitory peptide (CiP), or cyclosporine (CsA) blocked intra-acinar zymogen activation (n = 3; P < 0.05). The Cn inhibitors also reduced leakage of lactate dehydrogenase (LDH) by 79%, 62%, and 63%, respectively (n = 3; P < 0.05). The CnA0.05)-deficient acinar cells were also protected against zymogen activation and cell injury induced by the cholecystokinin analog carbeuline. Importantly, amylase secretion was generally not affected by either the Cn inhibitors or Cn deficiency. These data provide both pharmacological and genetic evidence that implicates Cn in intra-acinar zymogen activation and cell injury during pancreatitis.

MATERIALS AND METHODS

Reagents and animals. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. CnAβ1–7 mice were of the B6129J/F1 strain (Harlan Laboratories, Boston, MA). Age-, sex-, and strain-matched control mice (generated by JDM; Ref. 1) were used as wild-type (WT) controls. All mice weighed 20–25 g at the time of use and were supplied with food and water ad libitum and maintained on a 12-h:12-h light/dark cycle. The complete protocol was approved by the Institutional Animal Care and Use Committee.

Preparation of pancreatic acini. Groups of pancreatic acinar cells were isolated as previously described (27) with modifications. Briefly, the pancreas was removed from euthanized mice and minced for 5 min in DMEM/F12 1× buffer without phenol (GIBCO Invitrogen, Carlsbad, CA) containing 15 mM HEPES (pH 7.4), 120 mM NaCl, 4 mM KCl, 0.3 mM MgCl2, 1 mM CaCl2, 18 mM dextrose, and 3 mM glucose, plus 0.1% BSA, and 2 mg/ml type-4 collagenase ( Wor-
thington, Freehold, NJ). The tissue was briefly oxygenated and incubated for 5 min at 37°C with shaking (90 revolution/min). Buffer was removed and replaced with fresh collagenase buffer, briefly oxygenated, and incubated for 35 min. The tissue digest was resuspended to obtain dispersed acini and then filtered through a 300-μm mesh (Sefar American, Depew, NY). Acinar cells were washed three times with collagenase-free buffer and then allowed to equilibrate for 5 min at 37°C before treatment.

**Stimulation of acini and enzyme activity assays.** Acini were pre-treated with Cn inhibitors (FK506, 1–20 μM) or Cn inhibitory peptide (CiP, 1–20 μM), or cyclosporine A (CsA, 0.1–10 μM) at 37°C for 30 min. They were then stimulated with carbachol (0.1 μM-1 mM) for 30 min to 2 h. Zymogen activity assays were performed at room temperature using fluorogenic substrates as previously described (4) with modifications. Briefly, 50 μl of 400 μM enzyme substrate was added to each homogenized sample, and accumulation of fluorescence was measured over 10 min using a fluorescent plate reader (Infinite M200; Tecan, Gratz, Austria) 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. Zymogen activity was normalized to total amylase content using a Phadebas Kit (Magle Life Sciences, Lund, Sweden) and expressed as relative fluorescent units per second. Acinar cell injury was quantified by the amount of lactate dehydrogenase (LDH) released into the media using a nonradioactive cytotoxicity assay (Promega, Madison, WI). Absorbance was measured at 490 nm within 15 min of stopping the enzyme reaction. Results were expressed as a percentage of the total LDH that is released into the media.

**Genomic DNA preparation and genotyping.** For genotyping, 1–2-mm sections of mouse tail were dissolved in 0.1 ml of 50 mM Tris (pH 8.0), 15 mM EDTA, 0.5% SDS, and 0.5 mg/ml proteinase K (Roche, Mannheim, Germany) solution at 55°C for at least 4 h. The DNA was purified with DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Briefly, tail digest was purified once with phenol/chloroform-isooamyl alcohol, and the DNA was precipitated by adding 1.5 ml of 100% ethanol and then dissolved in 0.2 ml of Tris-EDTA solution. CnAβ−/− mice were confirmed by tail cDNA genotyping using the following primers obtained from Ref. 1: 636 (3′) 5′-CTCTGCTAGGGACCAGATCTG-3′ and 5′-TGAAGCCTCTTTTCGGGGTG-3′; CnB2, 5′-GAGGAGAGTA-CCCACCATCATCT-3′ and 5′-TCA GTA AGG TCA GTA AGG AGG TGT CTG CAT TC-3′; CnB1, 5′-TGAAGCCTCTTTTCGGGGTG-3′ and 5′-AAGGTTGCTCTCATATTG-3′; CnAβ, 5′-CTCTGCTAGGGACCAGATCTG-3′ and 5′-TGAAGCCTCTTTTCGGGGTG-3′; CnB2, 5′-GAGGAGAGTA-CCCACCATCATCT-3′ and 5′-TCA GTA AGG TCA GTA AGG AGG TGT CTG CAT TC-3′; CnAβ, 5′-TGAAGCCTCTTTTCGGGGTG-3′ and 5′-AAGGTTGCTCTCATATTG-3′; CnB2, 5′-GAGGAGAGTA-CCCACCATCATCT-3′ and 5′-TCA GTA AGG TCA GTA AGG AGG TGT CTG CAT TC-3′; CnAβ, 5′-TGAAGCCTCTTTTCGGGGTG-3′ and 5′-AAGGTTGCTCTCATATTG-3′; CnB2, 5′-GAGGAGAGTA-CCCACCATCATCT-3′ and 5′-TCA GTA AGG TCA GTA AGG AGG TGT CTG CAT TC-3′; CnAβ, 5′-TGAAGCCTCTTTTCGGGGTG-3′ and 5′-AAGGTTGCTCTCATATTG-3′; CnB2, 5′-GAGGAGAGTA-CCCACCATCATCT-3′ and 5′-TCA GTA AGG TCA GTA AGG AGG TGT CTG CAT TC-3′.

**RESULTS**

One of the earliest features of pancreatitis is the activation of zymogens, particularly, proteases, within the pancreatic acinar cell (20). These events occur in vivo and can be recapitulated in isolated acinar cells. The long-acting ACh analog carbachol can induce intra-acinar zymogen activation at supraphysiological concentrations in the high-micromolar or low-millimolar range (Figs. 1 and 2). In this situation, muscarinic receptors on the acinar cell signal to cause the opening of intracellular Ca2+ channels, including the inositol 1,4,5-trisphosphate receptor, the ryanodine receptor, and the subsequent opening of store-operated Ca2+ channels (16, 30). To examine whether these Ca2+ events trigger zymogen activation via Cn, freshly isolated mouse acinar cells were pretreated with each of three Cn inhibitors. FK506 and CsA inhibit Cn by forming a complex with FK506 binding protein 12 and cyclophilin, respectively, which then binds Cn and blocks access of substrates to the catalytic site on Cn (24). CiP is a short peptide that mimics the autoinhibitory domain of Cn. We have used a cell-permeable form of CiP that is made by covalently attaching an arginine tail to the peptide (43). Figure 1 provides concentration-dependence data of the Cn inhibitors on zymogen activation, whereas Fig. 2 shows concentration dependence of carbachol. There was a 2.5- and 30-fold increase in the intra-acinar activation of the zymogens trypsin and chymotrypsin, respectively, with carbachol (1 mM). FK506, CiP, and CsA reduced the pathological activation of trypsin by 81%, 50%, and 88%, respectively (P < 0.05; Fig. 1). The reduction was observed over a range of carbachol concentrations that are known to induce zymogen activation (Fig. 2). Carbachol stimulates the secretion of pancreatic enzymes from the acinar cell in a biphasic fashion, with maximal secretion at 1 μM (Fig. 2C) (51). However, none of the Cn inhibitors significantly affected the secretory pattern of carbachol-stimulated amylase release. The results with the Cn inhibitors indicate that Cn mediates carbachol-induced zymogen activation and cell injury.
but does not affect the primary physiological function of the cell in secreting pancreatic enzymes.

The intra-acinar activation of zymogens is followed by acinar cell injury, which can be followed over several hours in cultured acini by measuring leakage of LDH into the media (35). Carbachol (1 mM) induced 10\(^{1/2}\)1.2% LDH release over a 2-h period. Compared with 3\(^{1/2}\)0.17% in unstimulated cells and 5\(^{1/2}\)0.27% in cells receiving a generally noninjurious concentration of carbachol (1 \(\mu\)M; Fig. 3). Pretreatment with the Cn inhibitors each reduced cell injury on average by 68% (\(P<0.05\)).

To confirm the results with the three different inhibitors, we also pursued a genetic approach to provide complimentary evidence for a role of Cn in acinar cell pathology. Of the CnA subunit isoforms, we found by semi-quantitative PCR that CnA\(^{\gamma}\) is strongly expressed in the acinar cell (Fig. 4A). For this reason, we obtained acinar cells from CnA\(^{\alpha\beta\gamma}/-/\) mice. These mice have no overt phenotypic defects at baseline, but they display defective T cell development (1) and have a reduced ability to mount a cardiac hypertrophy response (2). Tail genotyping confirmed the presence or absence of CnA\(^{\alpha\beta}\) in WT or CnA\(^{\alpha\beta}\)/-/ mice, respectively (data not shown). Although CnA\(^{\gamma}\) is also expressed in the acinar cell, the CnA\(^{\alpha\beta}/-/\) acinar cells had an 89% reduction in Cn phosphatase activity compared with WT controls (\(P<0.05\); Fig. 4B). This represented a greater reduction in activity than the 43% and 51% seen in the whole pancreas or splenic lymphocytes, respectively, from the CnA\(^{\alpha\beta}/-/\) mice. The findings indicate that CnA\(^{\alpha\beta}/-/\) acinar cells can be used as a model of Cn deficiency to examine the role of Cn in acinar cell events leading to pancreatitis.

Compared to WT acinar cells, CnA\(^{\alpha\beta}\)-deficient acinar cells had an 84% and 50% reduction in trypsin and chymotrypsin activation, respectively (Fig. 5, A and B). Cell injury after a 2-h incubation with 1 mM carbachol was reduced by 63% compared with the WT cells (\(P<0.05\); Fig. 5C). Differences in amylase secretion were modest although secretion in the

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**Fig. 1.** Calcineurin (Cn) inhibitors at varying concentrations reduce intra-acinar zymogen activation. Acinar cells were treated with FK506 (A), Cn inhibitory peptide (CiP) (B), or cyclosporine A (CsA) (C) at the indicated concentrations for 30 min before carbachol (1 mM) stimulation. Activities of trypsin (left) and chymotrypsin (right) were measured 30 min after carbachol stimulation and normalized to total amylase content (n = 3). *\(P<0.05\) with respect to control or carbachol alone, respectively. Data are means ± SE. RFU, relative fluorescence units.
CnA−/− cells was significantly higher at maximal stimulatory concentrations of carbachol (1 mM). However, there was no particular shift in the biphasic secretion curve in the CnA−/− deficient cells compared with WT.

We have previously demonstrated that pharmacological Cn inhibition reduces CCK-induced intra-acinar zymogen activation (15, 38). In the present study, we now examined the effects of CnAB deficiency on CCK responses. As with carbachol, the CCK analog caerulein caused intra-acinar zymogen activation in a concentration-dependent manner in WT cells (Fig. 6, A and B). Interestingly, the CnAB−/− cell responses to caerulein were comparatively flat-lined, and cell injury was virtually abrogated (Fig. 6C). In contrast to the secretory response with carbachol, maximal secretion was reduced in the CnA−/− cells. Overall, the results with the CnAB−/− cells provide compelling evidence that Cn influences carbachol- and caerulein-induced zymogen activation and cell injury.

DISCUSSION

The key findings of the present study are novel because previous studies implicated a role for Cn in mediating CCK-induced zymogen activation (15, 38). Here, we demonstrate...
that either Cn inhibition or genetic deficiency of a predominant Cn isoform (CnA/H9252) protects the pancreatic acinar cell from neurogenic-induced zymogen activation as well as cell injury. Cn (also known as PP2B) is a CAM-dependent serine/threonine phosphatase. It was first identified in brain, but its importance is now recognized in a diverse number of physiological and pathological states ranging from neuronal and muscle development, lymphocyte activation, cardiac hypertrophy, and growth of the pancreas (33). Regarding the latter, Williams and colleagues (42) demonstrated that the adaptive growth of the pancreas in mice fed camostat, a soybean trypsin inhibitor, could be blocked by CsA and FK506 (42), as well as in mice that conditionally overexpressed in acinar cells an endogenous Cn inhibitor, Rcan1 (regulator of calcineurin 1) (13, 14). The role of Cn in lymphocyte activation has been clinically exploited with the advent of the Cn inhibitors CsA and FK506 (18). They are widely used as immunosuppressants after solid organ transplantation and have other indications in the treatment of several autoimmune disorders. Thus our findings in a neurogenic model of acinar cell injury suggest the Cn inhibitors could have a multimodal effect on acute pancreatitis at both the level of early acinar cell events and the ensuing inflammatory cascade. It is important to note, however, that the Cn inhibitors were given for brief periods before or during the induction of acinar cell injury. However, chronic administration of Cn inhibitors exacerbates pancreatitis or pancreatic fibrosis in several clinical (37, 46) and experimental reports (6, 12, 47).

Cn activity is dependent on the formation of a heterodimer, a catalytic A subunit (CnA) and a regulatory B subunit (CnB). Of the three CnA isoforms, we saw strong expression in the pancreatic acinar cell of CnAβ. Even though CnAy was also

Fig. 5. CnAβ-deficient mice have reduced intra-acinar zymogen activation and cell injury induced by carbachol. Acinar cells from WT or CnAβ-/- mice were treated with carbachol (0.1 μM-1.0 mM) for 30 min, and trypsin (A) and chymotrypsin (B) activities were normalized to total amylase content (n = 3). #P < 0.05 with respect to the unstimulated condition or WT acinar cells, respectively. C: acinar cells were treated with 1 mM carbachol for 2 h, and cell injury was measured as percent LDH released (n = 2). #P < 0.001, with respect to WT acinar cells receiving 1 mM carbachol. D: biphasic amylase secretion curve (n = 3). Data are means ± SE.
expressed, Cn phosphatase activity in the CnA−/− acinar cell was reduced by 89%, demonstrating that CnA is a functionally important acinar cell Cn isoform. In addition, CnA may have selective effects as a stress-response isoform, as described in heart (2, 41). The findings in these cells compared with WT mostly paralleled those seen with administration of the Cn inhibitors. With regard to amylase secretion, however, both the present study and previous reports expose a heterogeneity of findings. Doi et al (5) and Waschulewski et al (50) reported that both FK506 and CsA reduced CCK-stimulated amylase secretion. Later, Groblewski et al (11) demonstrated a reduction with CsA but not FK506, and this discrepancy roughly correlated with the degree of pancreatic Cn phosphatase inhibition. In a previous study, we also saw no reduction in caerulein-stimulated amylase secretion with FK506 (15), consistent with the present findings of the Cn inhibitors with carbachol stimulation. However, it is not clear why there was a marked reduction in amylase secretion observed in the CnA−/− acini stimulated with caerulein but no decrease with carbachol. It is possible that, compared with muscarinic receptor stimulation, CCK receptor-mediated Ca2+ release selectively signals to the Cn complex containing CnA to induce pancreatic enzyme secretion. Another explanation for the difference could be that CCK receptors, but not muscarinic receptors, are coupled to adenylate cyclase (23). Thus the cAMP rise from the CCK receptor stimulation could potentiate the effects of Cn on downstream targets such as the phosphatase PP1 by posttranslationally modifying a Cn substrate DARPP-32 (10). It is also notable that CnA−/− deficiency caused a more profound reduction in trypsin and chymotrypsin activation with caerulein than with carbachol, indicating the Cn plays a more prominent role in the CCK receptor pathway.
In previous work, we showed that Cn inhibition does not modulate acinar cell Ca\(^{2+}\) signals, suggesting that Cn is downstream to Ca\(^{2+}\) (15). Together with the present work, however, the findings raise several questions for further study, including what pattern of Ca\(^{2+}\) signaling would favor Cn activation in the acinar cell and what modulates this response to Ca\(^{2+}\). Further downstream, what are the targets of Cn activation in the acinar cell that lead to pathological zymogen activation and cell injury? Cn has a number of known phosphoprotein targets. The most well-described is the transcription factor nuclear factor of activated T cells (NFAT) (22). However, our zymogen activation and injury responses were assayed within 2 h of administering carbachol, and thus NFAT is unlikely a target of these early events. Nonetheless, its role in vivo, hours after the onset of pancreatitis, either within the acinar cell or in other cell types, has not been determined. In summary, we have used a model of acinar cell injury with carbachol that mimics neurogenic hyperstimulation and demonstrates that both pharmacological inhibition and genetic deficiency of Cn protect against intra-acinar zymogen activation and cell injury. The findings implicate a role of Cn in clinically relevant models of acinar cell injury leading to acute pancreatitis and the potential role of Cn inhibitors in treating pancreatitis.

ACKNOWLEDGMENTS

The authors thank Drs. Fred Gorelick, Michael Nathanson, Mark Lowe, Vijay Singh, and Vineet Bhandari for helpful discussion.

GRANTS

This work was supported by National Institutes of Health Grants DK093491, DK083327, HD001401 (Yale Child Health Research Center), DK34989 (Yale Liver Center), and a Children’s Digestive Health and Nutrition Young Investigator Award (to S. Husain).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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