

Bile Acids Induce Pancreatic Acinar Cell Injury and Pancreatitis by Activating Calcineurin^{*,§}

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Background: Bile acid exposure causes pancreatic acinar cell injury through a sustained rise in cytosolic Ca²⁺.

Results: Pharmacologic and genetic inhibition of the Ca²⁺-activated phosphatase calcineurin dramatically reduces acinar cell injury and *in vivo* pancreatitis resulting from bile acid exposure.

Conclusion: Acinar cell calcineurin mediates acinar cell injury and pancreatitis resulting from bile acid exposure.

Significance: Calcineurin inhibitors may provide an adjunctive therapy for biliary pancreatitis.

Biliary pancreatitis is the leading cause of acute pancreatitis in both children and adults. A proposed mechanism is the reflux of bile into the pancreatic duct. Bile acid exposure causes pancreatic acinar cell injury through a sustained rise in cytosolic Ca²⁺. Thus, it would be clinically relevant to know the targets of this aberrant Ca²⁺ signal. We hypothesized that the Ca²⁺-activated phosphatase calcineurin is such a Ca²⁺ target. To examine calcineurin activation, we infected primary acinar cells from mice with an adenovirus expressing the promoter for a downstream calcineurin effector, nuclear factor of activated T-cells (NFAT). The bile acid tauro lithocholic acid-3-sulfate (TLCS) was primarily used to examine bile acid responses. TLCS caused calcineurin activation only at concentrations that cause acinar cell injury. The activation of calcineurin by TLCS was abolished by chelating intracellular Ca²⁺. Pretreatment with 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (acetoxymethyl ester) (BAPTA-AM) or the three specific calcineurin inhibitors FK506, cyclosporine A, or calcineurin inhibitory peptide prevented bile acid-induced acinar cell injury as measured by lactate dehydrogenase leakage and propidium iodide uptake. The calcineurin inhibitors reduced the intra-acinar activation of chymotrypsinogen within 30 min of TLCS administration, and they also prevented NF- κ B activation. *In vivo*, mice that received FK506 or were deficient in the calcineurin isoform A β (CnA β) subunit had reduced pancreatitis severity after infusion of TLCS or taurocholic acid into the pancreatic duct. In summary, we

demonstrate that acinar cell calcineurin is activated in response to Ca²⁺ generated by bile acid exposure, bile acid-induced pancreatic injury is dependent on calcineurin activation, and calcineurin inhibitors may provide an adjunctive therapy for biliary pancreatitis.

Acute pancreatitis is a painful, necro-inflammatory disorder that is triggered by numerous insults (1). The most common inciting factor in both children and adults accounting for 30–50% of cases (2–4) is the presence of gallstones or sludge within the distal common bile duct (5). A dominant mechanism for this etiology of pancreatitis known as acute biliary pancreatitis is thought to be the reflux of bile into the pancreatic duct (6, 7). The major component of bile is the amphipathic bile acids (8, 9). The primary bile acids are cholic acid and chenodeoxycholic acid. Secondary bile acids are the more hydrophobic forms, which include the dehydroxylated lithocholic acid and deoxycholic acid. Most bile acids are conjugated to either taurine or glycine. Administration of submillimolar concentrations of chenodeoxycholic acid (CDC)³ to the lumen of *ex vivo* ducts elicits reactive mechanisms within the pancreatic duct cell to stimulate bicarbonate secretion (10, 11). However, higher concentrations of CDC induce acinar cell pathology, leading to pancreatitis (12, 13).

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³ The abbreviations used are: CDC, chenodeoxycholic acid; TCDC, taurochenodeoxycholic acid; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; BAPTA-AM, BAPTA acetoxymethyl ester; CnA, calcineurin A; CnB1, calcineurin B1; CnA $\beta^{-/-}$, calcineurin A β deficient; Δ CnA, constitutively active calcineurin; Gpbar1, G-protein coupled bile acid receptor; LDH, lactate dehydrogenase; Mcip1, myocyte-enriched calcineurin-interacting protein; NFAT, nuclear factor of activated T-cells; PI, propidium iodide; TC, taurocholate; TLCS, tauro lithocholic acid-3 sulfate; CIP, calcineurin inhibitory peptide; CsA, cyclosporine A; Ad, adenovirus; ERCP, endoscopic retrograde cholangiopancreatography.

Bile acids cause acinar cell injury by inducing a host of cellular changes, including reduced mitochondrial membrane potential (14), depletion of ATP levels (15), and increased production of reactive oxygen species (16). However, the most immediate effect of bile acids is their ability to elicit acinar cell cytosolic Ca^{2+} signals. The signals are observed at bile acid concentrations below the critical micellar concentration (12, 17) and occur even in the absence of external Ca^{2+} (18), albeit with lower amplitude. Thus, bile acids appear to transduce the Ca^{2+} signals not by permeabilizing the plasma membrane but by causing the opening of Ca^{2+} channels. Although the mechanism by which bile acids transduce Ca^{2+} signals is not clear, recent work suggests that bile acids exert their effects through an apically localized bile acid receptor, the G-protein-coupled bile acid receptor Gpbar1 (17). High micromolar concentrations of bile acids induce high amplitude, sustained Ca^{2+} signals, which are necessary to initiate early events in pancreatitis such as intra-acinar protease activation and NF- κ B activation (19–21). The immediate targets of this Ca^{2+} signal in acinar cells are not clear. In other cell types, the Ca^{2+} -activated phosphatase calcineurin is activated in response to a sustained increase in cytosolic Ca^{2+} (22, 23). For this reason, we hypothesized that bile acids induce acinar cell injury by activating calcineurin. In this study, we demonstrate 1) that bile acids induce calcineurin activation only at concentrations that cause acinar cell injury; 2) that calcineurin activation is dependent upon intracellular Ca^{2+} ; and 3) that pharmacologically or genetically blocking calcineurin reduces acinar cell injury from bile acid exposure in isolated acini and *in vivo* with retrograde bile acid infusion.

EXPERIMENTAL PROCEDURES

Reagents and Animals—All reagents were purchased from Sigma-Aldrich unless otherwise stated. Male Swiss Webster mice weighing 20–25 g (Harlan Laboratories, Boston, MA) were fed standard laboratory chow, given free access to water, and randomly assigned to control or experimental groups. NFAT-luciferase, constitutively active calcineurin (ΔCnA), myocyte-enriched calcineurin-interacting protein (Mcip1), and NF- κ B-luciferase adenoviruses were constructed as described previously (24).

Preparation of Pancreatic Acini—Groups of pancreatic acinar cells were isolated as described previously (25) with minor modifications. Briefly, the pancreas was removed and then minced for 5 min in Dulbecco's modified Eagle's medium (DMEM)/F12 $1\times$ buffer without phenol red (Invitrogen), plus 0.1% BSA and 2 mg/ml type 4 collagenase (Worthington). The suspension was briefly incubated with for 5 min at 37 °C while shaking at 90 rpm. The buffer was removed and replaced with new collagenase buffer and then incubated for 35 min. The suspension was filtered through a 300- μm mesh (Sefar American, Depew, NY) and then washed three times with collagenase-free buffer. Acinar cells were allowed to equilibrate for 5 min at 37 °C before use.

NFAT-Luciferase Activity Assay—Acinar cells were infected with Ad-NFAT-luciferase following a previously described procedure (26). The construct includes a luciferase gene placed downstream of an IL-4 promoter that contains nine tandem

NFAT binding sites. The luciferase gene is expressed when NFAT binds to the IL-4 promoter. Acinar cells were incubated with the NFAT-driven luciferase adenovirus for 1.5 h prior to stimulation. All of the stated inhibitors were added for 30 min prior to a stimulation with TLCS (5–500 μM). NFAT-luciferase was measured using the luciferase assay system (see Fig. 1A). Briefly, cells were spun at 1,000 rpm for 5 min, washed with PBS, and lysed using reporter lysis $5\times$ buffer (catalog number E397A, Promega, Madison, WI). Samples were vortexed and spun at $12,000\times g$ for 2 min. Supernatant was plated, and luminescence was measured using a Synergy H1 plate reader (BioTek, Winooski, VT) and normalized to total protein.

Cell Injury Assays—Acinar cell injury was measured using a cytotoxicity assay for lactate dehydrogenase (LDH) leakage (Promega). Absorbance was measured at 490 nm, 15 min after stopping the enzyme reaction. Results were expressed as the percentage of LDH released into the media. For propidium iodide (PI) uptake, acinar cells were incubated in a 48-well plate with 50 $\mu\text{g}/\text{ml}$ PI (Sigma) for 30 min prior to the addition of 500 μM TLCS. Fluorescence was measured at 536-nm excitation and 617-nm emission wavelengths over time (0–6 h). To ensure that the same amount of cells was placed in each well, total fluorescence was measured after cell lysis with 0.5% Triton X-100.

Intraductal Bile Acid Infusion Model of Pancreatitis—Pancreatitis was induced by retrograde infusion of the bile acid TLCS (3 mM) or taurocholate (TC) (37 mM) dissolved in saline into the distal common bile duct and pancreatic duct, as recently described (27). Briefly, mice between 8 and 12 weeks (20–25 g) were anesthetized with a ketamine (120 mg/kg)/xylazine (12 mg/kg) mixture (Butler Schein, Chicago, IL). A ventral incision was made to reveal the abdominal cavity. The duodenum was flipped to reveal its distal side and held in place by ligatures. The bile duct was identified, and a 30-gauge needle was inserted through the antimesenteric aspect of the duodenum to cannulate the biliopancreatic duct. TLCS was infused at 10 $\mu\text{l}/\text{min}$ for 5 min using a P33 perfusion pump (Harvard Apparatus, Holliston, MA). The exterior wound was closed using 7-mm wound clips, and a single injection of buprenorphine (0.075 mg/kg) was given immediately after the surgery. Normal saline-infused animals served as shams. Animals were allowed to recover on a heating pad for 90 min after the procedure. Mice were euthanized 6 or 24 h after induction.

NF- κ B-Luciferase Activity Assay—Acinar cells were infected with Ad-NF- κ B-luciferase 24 h prior to stimulation using a previously described procedure (28). Following a wash with DMEM/F12 media, acinar cells were evenly distributed in a 48-well plate and incubated for 30 min at 37 °C. Acinar cells were stimulated for 6 h with TLCS (500 μM) in the presence or absence of inhibitors. NF- κ B-luciferase was assayed using the same method described for NFAT-luciferase.

Tissue Preparation and Histological Grading—Pancreas, duodenum, and spleen were fixed at room temperature for 24 h in 10% formalin solution and transferred to 70% ethanol. Paraffin-embedded sections were stained with hematoxylin and eosin and graded using a $20\times$ objective over 6–10 separate

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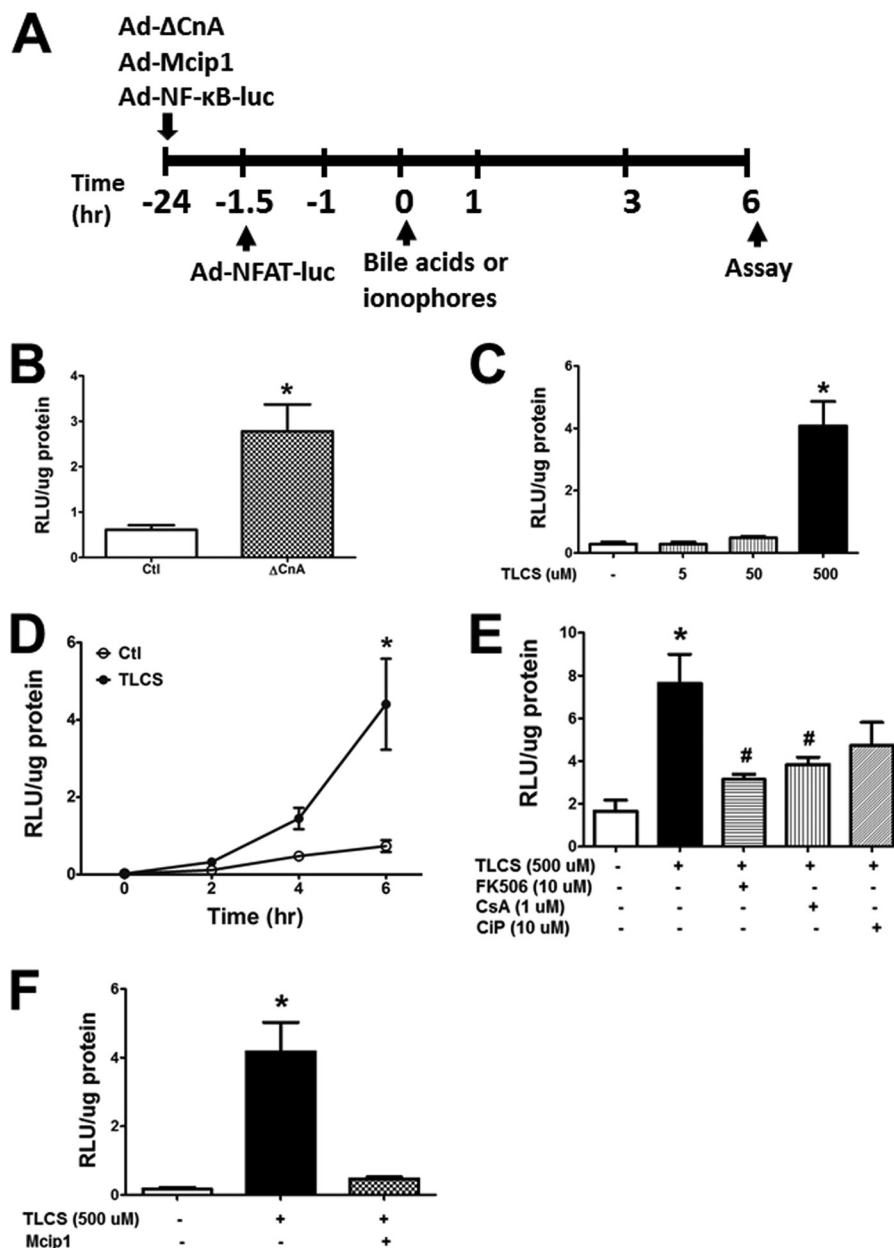


FIGURE 1. TLCS causes acinar cell calcineurin activation. *A*, schema for the transfection of primary mouse pancreatic acinar cells with adenoviral vectors. *Luc*, luciferase. *B*, expression of Δ CnA resulted in acinar cell NFAT-luciferase activity, confirming that the assay can be used to detect calcineurin activation. *RLU*, relative luciferase units; *Ctl*, control. *C*, administration of TLCS (500 μ M) induced NFAT-luciferase activity. *D*, time course demonstrating accumulation of luminescence with TLCS (500 μ M) given over a 6-h period. *E* and *F*, TLCS-induced NFAT-luciferase activity was prevented by pretreatment with the pharmacologic calcineurin inhibitors (*E*) or by overexpression of the endogenous calcineurin inhibitor Mcip1 (Ad-Mcip1) (*F*). $n = 3$. *, $p < 0.05$, relative to the control or TLCS alone, respectively.

fields in a blinded fashion. Only pancreas tissue adjacent to the duodenum (*i.e.* pancreatic head) was graded. The grading scale was adapted from Wildi *et al.* (29), in which pancreas was assessed for edema, acinar cell vacuole formation, inflammatory infiltrate, and necrosis on a scale of 0–4. The scores of each slide were totaled to calculate an overall severity score. Analysis was also conducted for each scoring parameter.

Statistical Analysis—Data were expressed as mean \pm S.E. unless otherwise stated. Statistical analysis was performed using a Student's *t* test or two-way analysis of variance for a comparison of multiple groups. Statistical significance was defined as a *p* value < 0.05 .

RESULTS

TLCS Activates Acinar Cell Calcineurin—Calcineurin activates several substrates, notably a family of transcription factors known as nuclear factor of activated T-cells (NFATc1–c4) that have been best characterized in the immune system (30, 31). We used a reporter system to evaluate calcineurin activation by infecting acinar cells with an adenovirus containing an NFAT-driven luciferase gene (24). Activation of the calcineurin phosphatase induces NFAT dephosphorylation, which then causes NFAT to translocate to the nucleus. NFAT binding to its promoter sequences drives the expression of luciferase, which serves as a sensitive measure of calcineurin activation. To test

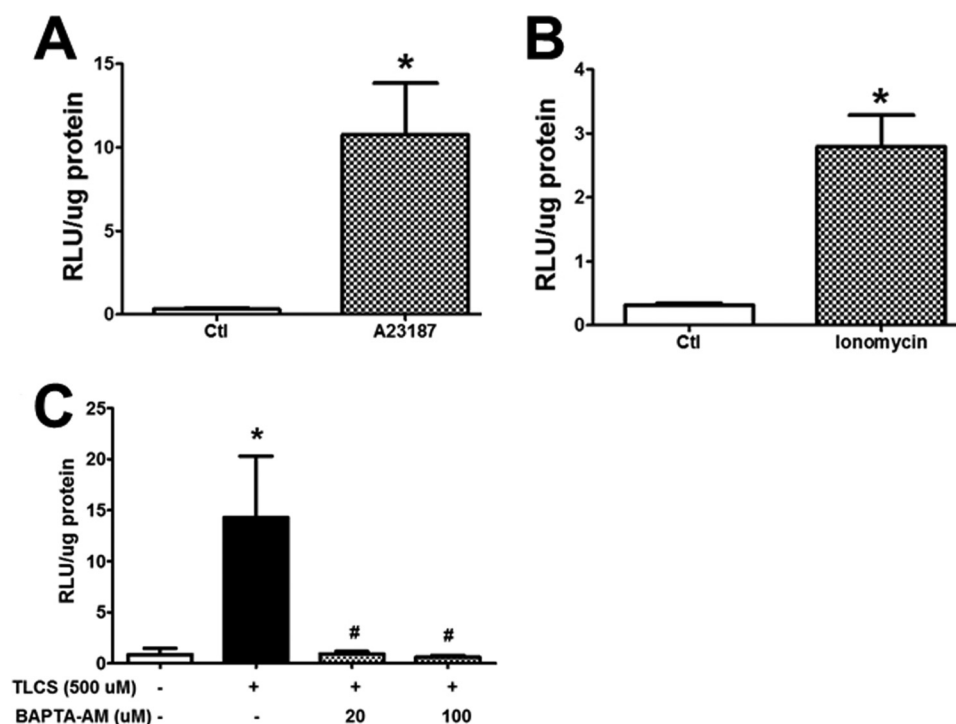


FIGURE 2. **TLCS-induced calcineurin activation is Ca²⁺-dependent.** A and B, both Ca²⁺ ionophores (A) A23187 (2 μM) and (B) ionomycin (1 μM) induced NFAT-luciferase activity. RLU, relative luciferase units; Ctl, control. C, pretreatment with BAPTA-AM prevented the increase. n = 3–4. *, #, p < 0.05, relative to the control or TLCS alone, respectively.

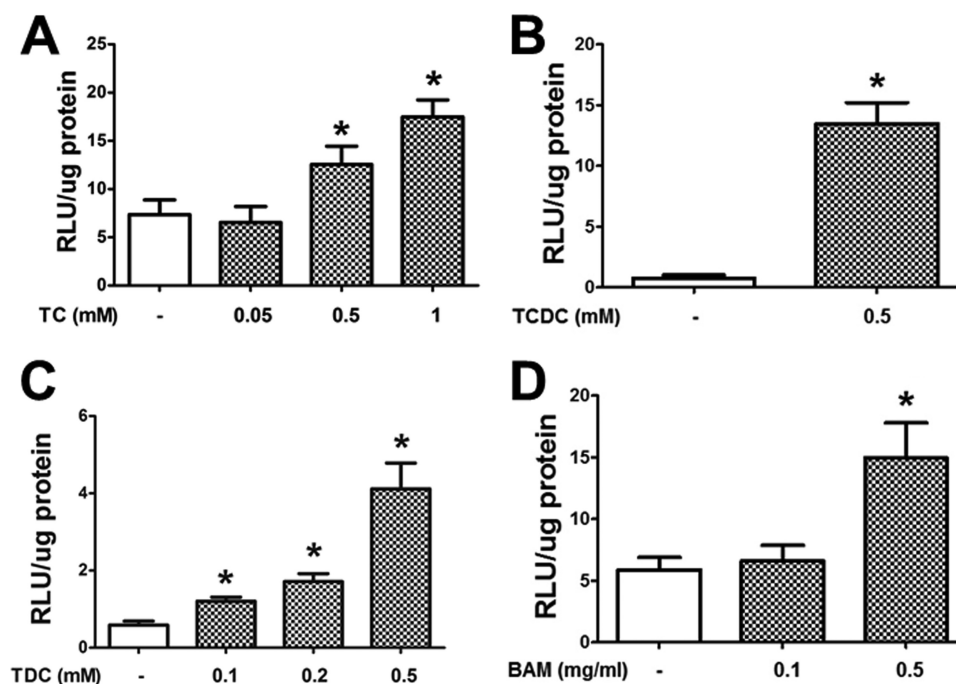


FIGURE 3. **The other naturally occurring bile acids cause acinar cell calcineurin activation.** A–D, TC (A), TCDC (B), taurodeoxycholic acid (TDC) (C), and a bile acid mixture (BAM) (D) from *Bovina Bos taurus* each induced NFAT-luciferase activity. n = 3–4. *, p < 0.05, relative to control. RLU, relative luciferase units.

this hypothesis, we co-infected cells with an adenoviral construct that expresses ΔCnA. In addition to our reporter construct, we demonstrate that NFAT-luciferase activity directly correlates with calcineurin activation (Fig. 1B). Recent studies that have examined the effects of bile acids on acinar cell Ca²⁺ signaling have used TLCS (12, 16–18). TLCS represents one of the most hydrophobic of the naturally occurring bile acids and

induces Ca²⁺ signals at submillimolar concentrations that are below the critical micellar concentration (32). We found that TLCS induced substantial increases in NFAT-luciferase activity over the course of a 6-h period. This increase is only observed at a concentration (500 μM; Fig. 1, C and D) that is also associated with acinar cell injury. To confirm that activation is mediated by calcineurin, we utilized specific calcineurin inhib-

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itors, FK506, cyclosporine A (CsA), and calcineurin inhibitory peptide (CIP) (Fig. 1E). Each of the inhibitors reduced the activation of our reporter system driven by the NFAT promoter, suggesting that TLCS injury is mediated by calcineurin. Further, infecting cells with an adenovirus that overexpresses an endogenous inhibitor of calcineurin, Mcip1, also known as regulator of calcineurin 1 (Rcan1) (33), showed similar results (Fig. 1F). We demonstrate that TLCS promotes transcriptional activation via a calcineurin-specific pathway. Thus, the NFAT-luciferase construct can be used to assay calcineurin activation, and a pathological concentration of TLCS induces calcineurin activation.

To determine whether higher levels of calcineurin activation due to bile acids resulted from increased calcineurin expression, we performed Western blot analysis from pancreatic acinar cell lysates of calcineurin expression. Stimulation with TLCS (500 μ M) over the course of 6 h did not affect calcineurin expression (data not shown).

TLCS-induced Calcineurin Activation Is Dependent on Intracellular Ca^{2+} —Calcineurin is typically activated through binding of Ca^{2+} to the regulatory subunit of calcineurin (34). To examine whether calcineurin activation is dependent on Ca^{2+} levels in pancreatic acinar cells, we used an ionophore (A23187 or ionomycin) and showed NFAT activity increased above controls by 10- and 6-fold, respectively (Fig. 2, A and B). This suggests that increases in intracellular Ca^{2+} levels stimulate calcineurin-induced activation of NFAT. Next, to examine whether TLCS-induced calcineurin activation is Ca^{2+} -dependent, acinar cells were treated with the intracellular Ca^{2+} chelator 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) 30 min prior to TLCS exposure. BAPTA prevented calcineurin activation in our model system (Fig. 2C). These data suggest that Ca^{2+} activates acinar cell calcineurin and that calcineurin activation due to TLCS is Ca^{2+} -dependent.

We used TLCS primarily because it is the most potent naturally occurring bile acid (32). In further studies, however, we assessed the ability of the other naturally occurring bile acids to activate calcineurin. TC, taurochenodeoxycholic acid (TCDC), and a bile acid mixture could each activate NFAT-luciferase (Fig. 3).

TLCS-induced Acinar Cell Injury Is Dependent on Ca^{2+} and Calcineurin—To examine whether Ca^{2+} and calcineurin are necessary for mediating acinar cell injury, we assayed LDH leakage from and PI uptake into acinar cells. Pretreatment of acini with BAPTA prior to TLCS caused a significant reduction in both measures of injury (Fig. 4). These data are in agreement with that of Booth *et al.* (16), in which similar effects of Ca^{2+} chelation with BAPTA on TLCS-induced acinar cell necrosis were observed. In addition, adenoviral overexpression of the endogenous calcineurin inhibitor Mcip1 reduced acinar cell injury with TLCS (Fig. 5A). Furthermore, the calcineurin inhibitors reduced injury induced by TLCS (Fig. 5, B–D). Importantly the effect of the calcineurin inhibitors was seen with pretreatment, cotreatment, and even 30–60 min after TLCS application. The data demonstrate that acinar cell injury are dependent on Ca^{2+} as well as calcineurin activation and that calcineurin-mediated injury can be reduced after bile acid

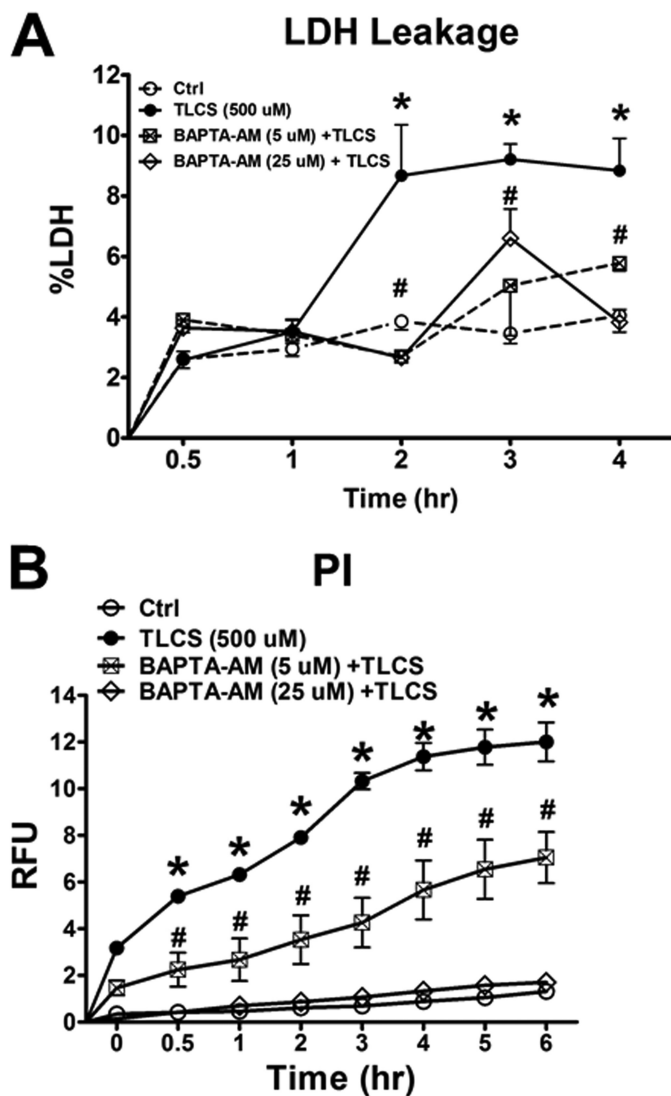


FIGURE 4. TLCS-induced acinar cell injury is Ca^{2+} -dependent. A and B, time course for LDH leakage (A) and PI uptake (B) with or without BAPTA-AM pretreatment. $n = 4$. *, #, $p < 0.05$, relative to control and TLCS alone, respectively. RFU, relative fluorescence units; Ctrl, control.

exposure. We also demonstrate that injury induced by the other bile acids was significantly reduced by BAPTA and the calcineurin inhibitors (Fig. 6).

TLCS-induced NF- κ B and Chymotrypsin Activation Are Dependent on Calcineurin—Among several injurious pathways, aberrant acinar cell Ca^{2+} signals are reported to activate two independent processes: the translocation of NF- κ B to the nucleus (35–37) and also the intra-acinar activation of pancreatic proenzymes or zymogens (20, 38, 39). We examined whether bile acids induced these two aspects through calcineurin. We demonstrate using adenoviral infection of a NF- κ B-luciferase construct that TLCS induced NF- κ B activation in a Ca^{2+} - and calcineurin-dependent manner (Fig. 7A).

To examine whether calcineurin activation is an important mediator of pathologic zymogen activation due to bile acids, acini were pretreated with calcineurin inhibitors prior to TLCS administration. We demonstrate that chymotrypsin is activated in the presence of TLCS and that this activation is abrogated in a calcineurin-dependent manner. Calcineurin inhibi-

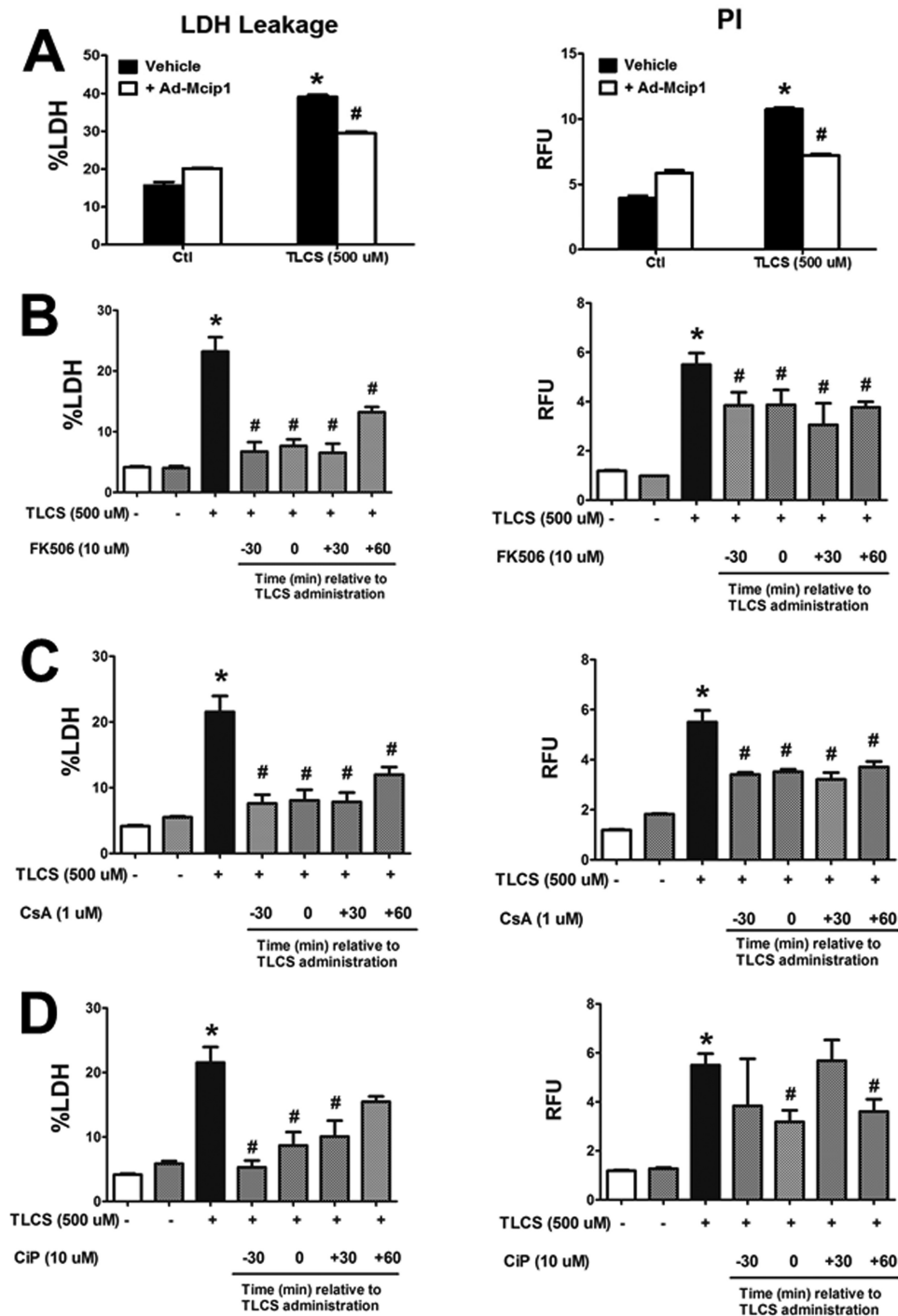


FIGURE 5. TLCS-induced acinar cell injury is dependent upon calcineurin activation. A, acinar cells were infected with Ad-Mcip1 24 h prior to TLCS (500 μ M) administration. RFU, relative fluorescence units; Ctl, control. B–D, FK506 (10 μ M) (B), CsA (1 μ M) (C), or CiP (10 μ M) (D) was administered at the stated time points relative to the start of a 2-h TLCS (500 μ M) treatment. LDH leakage (left) and PI uptake (right) were measured to assess cell injury. $n = 3–4$. *, $p < 0.05$, relative to control and TLCS alone, respectively.

tors (FK506, CsA, and CiP) reduced activation of chymotrypsin by 52, 87, and 61% down to the controls, respectively (Fig. 7B).

Previous studies have shown that, as in the case of the bile acids, calcineurin inhibitors reduce intra-acinar zymogen activation that is induced by supramaximal concentrations of caerulein and carbachol (40, 41). To confirm whether our findings, that calcineurin mediates bile acid-induced injury and NF- κ B activation, are generalizable to the secretagogue hyper-

stimulation conditions, we demonstrate that pretreatment with BAPTA-AM or FK506 reduced caerulein- and carbachol-induced LDH leakage, propidium iodide uptake, and NF- κ B activation (supplemental Fig. 1).

Pharmacologic or Genetic Inhibition of Calcineurin Attenuates Bile Acid-induced Pancreatitis in Vivo—To examine the clinical relevance of calcineurin activation in the intact animal, we employed an *in vivo* model of bile acid infusion in which

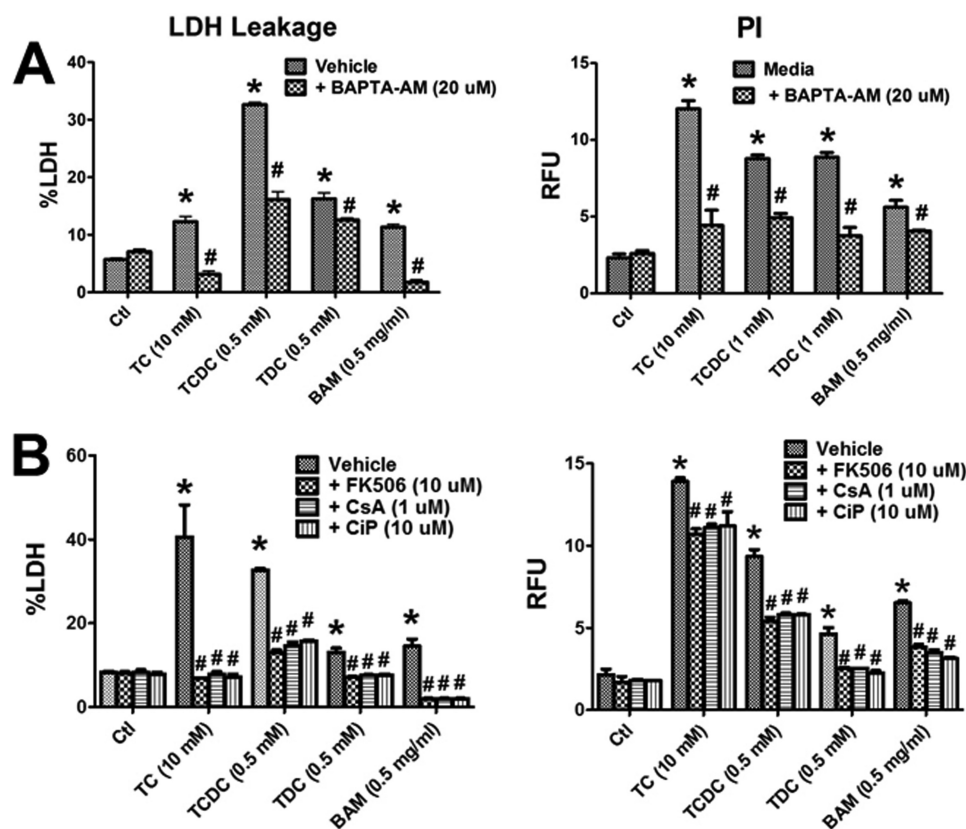


FIGURE 6. **The other naturally occurring bile acids cause acinar cell injury through a Ca²⁺ rise and calcineurin activation.** A and B, pancreatic acinar cells were stimulated with TC (10 mM), TCDC (1 mM), taurodeoxycholic acid (TDC) (1 mM), or bile acid mixture (BAM) (0.5 mg/ml) for 2 h with or without BAPTA-AM (20 μM) (A) or the calcineurin inhibitors FK506 (10 μM), CsA (1 μM), or CiP (10 μM) (B). LDH leakage (left) and PI uptake (right) were measured to assess cell injury. n = 4. *, #, p < 0.05, relative to control and bile acid alone, respectively. RFU, relative fluorescence units; Ctl, control.

anesthetized mice received brief retrograde duct infusion of either TLCS or TC. FK506 (1 mg/kg) was given 1 h prior to TLCS infusion as well as at three time points after TLCS, thus providing a cumulative dose of 4 mg/kg over a 24-h period (42) (Fig. 8). FK506 reduced TLCS- and TC-induced histological severity by 66 and 93%, respectively. To complement this pharmacologic approach, we obtained mice deficient in the predominant calcineurin isoform Aβ (CnAβ^{-/-}). We demonstrate these mice also have reduced outcomes after bile acid infusion pancreatitis using either TLCS or TC (Fig. 9). The *in vivo* studies provide evidence for a clinically important role for calcineurin in bile infusion pancreatitis.

DISCUSSION

The key findings of the present study are that bile acid exposure causes a Ca²⁺-dependent activation of calcineurin and that calcineurin inhibition using either pharmacologic calcineurin inhibitors or overexpression of an endogenous calcineurin inhibitor attenuates bile acid-induced acinar cell injury and bile acid infusion pancreatitis. Calcineurin, or PP2B, is a unique serine/threonine phosphatase among the family of type 2 protein phosphatases because it is activated by Ca²⁺ (31, 43). The enzyme complex consists of two subunits (CnA and CnB) that form a heterodimer. CnA is the large catalytic subunit that also contains the calmodulin binding domain and an autoinhibitory domain, which is mimicked by the calcineurin inhibitor CiP. CnB is the smaller regulatory subunit that con-

tains four Ca²⁺ binding EF hand motifs. Thus, Ca²⁺ appears to activate calcineurin both by direct binding to CnB and indirectly through calmodulin (34). However, the role of calmodulin in propagating calcineurin-mediated injury signals in the acinar cell is unclear because calmodulin may actually protect against ethanol-induced intra-acinar protease activation (44). There is also a study implicating an indirect mode of Ca²⁺-dependent calcineurin activation through the proteolytic activation of calcineurin by the Ca²⁺-dependent proteases the calpains (45).

Our findings that blockade of intracellular Ca²⁺ reduced calcineurin activation are in general agreement with previous reports that sustained Ca²⁺ signals are required to activate calcineurin (22, 23). The sustained Ca²⁺ signals observed with pathological concentrations of Ca²⁺-activating agonists result from Ca²⁺ release (12, 25, 46), Ca²⁺ store depletion, and the subsequent opening of store operated Ca²⁺ entry (SOCE) channels (47–49).

In this study, we examined two Ca²⁺-dependent pathways within the acinar cell that lead to acinar cell injury and pancreatitis: 1) intra-acinar protease activation (20, 39, 41, 50, 51) AND 2) NF-κB nuclear translocation (21, 52). The two appear to be independent of one another (21, 28), but we found that calcineurin is a mediator of both of these pathways. Understanding the mechanisms by which calcineurin targets protease activation and NF-κB translocation will form the basis of future

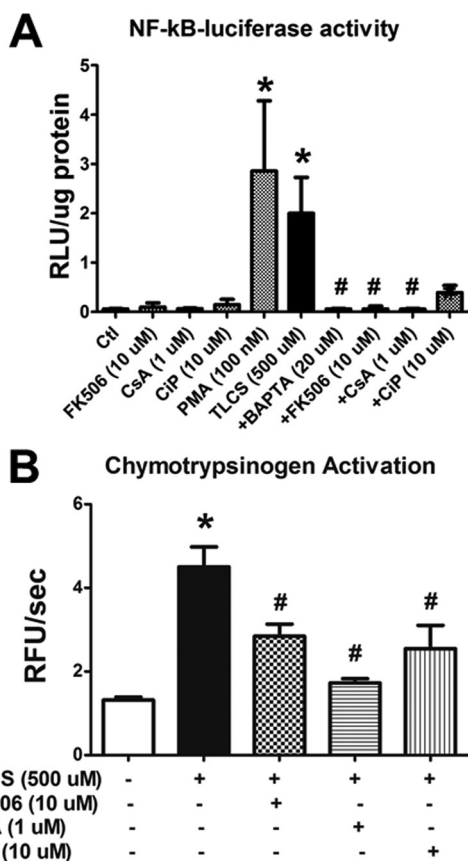


FIGURE 7. TLCS-induced NF-κB and chymotrypsinogen activation is dependent upon calcineurin. A, pretreatment with BAPTA-AM or the calcineurin inhibitors prevented NF-κB-luciferase activity. Phorbol 12-myristate 13-acetate (PMA) served as a positive control. RLU, relative luciferase units; Ctrl, control. B, acinar cells were pretreated with FK506 (10 μM), CsA (1 μM), or CiP (10 μM) for 30 min prior to TLCS (500 μM) administration. Chymotrypsin activity was normalized to total amylase content. n = 3. *, #, p < 0.05, relative to control and TLCS alone, respectively. RFU, relative fluorescence units.

work. Another important consideration is whether localized subcellular Ca²⁺ elevations and subsequent calcineurin activation mediate these downstream effects. An example in neuronal cells is that calcineurin is anchored in a complex by protein kinase A anchor protein 79 (AKAP79) to a target substrate, the Ca(V)1.2 channel; compartmentalized Ca²⁺ signals cause these channels to become inactivated by calcineurin-mediated dephosphorylation (53). Recently, a multitude of calcineurin effectors, both transcriptional and nontranscriptional, have been identified. These include not only proteins that target the nucleus as transcriptional regulators (54) but also factors that affect a host of functions including ribosomal biogenesis (55), apoptotic (56), and mitochondrial pathways (57–59). The latter is of particular interest because previous studies in both acinar (14, 15) and ductal cells (60) suggest that bile acids impair mitochondrial function.

A recent publication by Awla *et al.* (61) has examined the calcineurin target NFAT in pancreatitis. The authors demonstrate using NFAT-luciferase-expressing transgenic mice that acinar cells exhibit calcineurin activation. In our study, we provide functional data that acinar cell calcineurin mediates bile acid-induced intra-acinar protease activation, NF-κB activation, and cell injury.

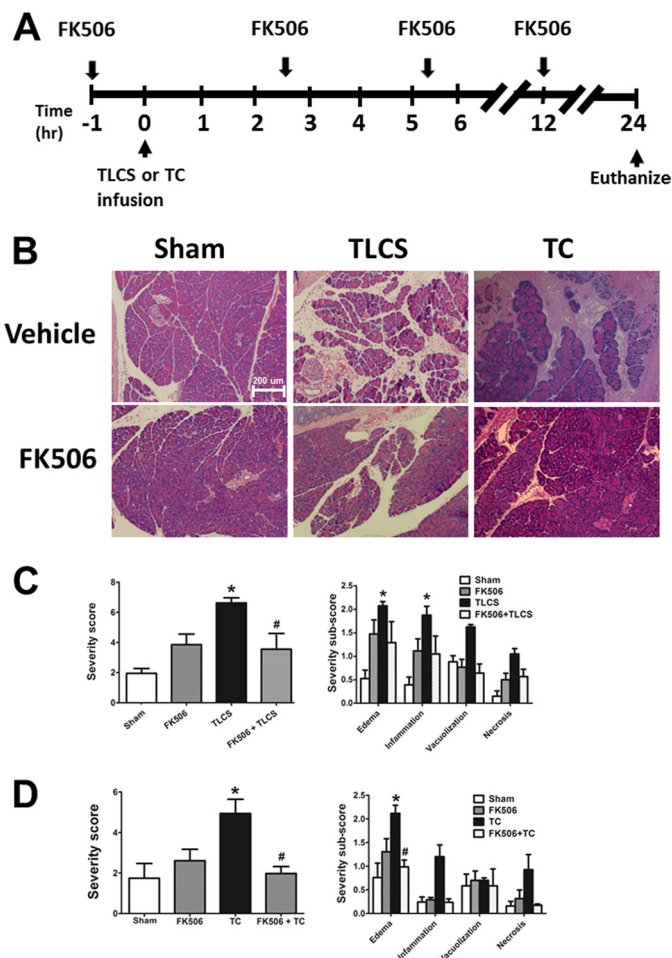


FIGURE 8. Calcineurin inhibition attenuates intraductal bile acid infusion pancreatitis. A, schema for *in vivo* calcineurin inhibition with FK506 (1 mg/kg) and pancreatitis induction using an intraductal infusion of TLCS (3 mM) or TC (37 mM). B, representative H&E sections from the pancreatic head. C and D, overall severity score (left) and sub-scores (right). n = 6 animals per group. *, #, p < 0.05 when compared with the sham and TLCS or TC alone, respectively.

Awla *et al.* (61) examine late intrapancreatic protease activation, whereas our study examines early intra-acinar protease activation. The two phenomena appear to be independent and mediated by different factors (62–64). Awla *et al.* (61) use the pharmacologic inhibitor CsA to demonstrate the importance of calcineurin pathways. Here we use FK506, which, unlike CsA, does not target cyclophilin D or the mitochondrial permeability transition pore. These off-target effects of CsA are also implicated in pancreatitis (65).

The finding that FK506 markedly attenuates *in vivo* retrograde duct infusion pancreatitis with bile acids has important clinical implications. It would be of interest to know whether Ca²⁺ modulators such as the already clinically available calcineurin inhibitors could reduce the risk of developing pancreatitis in patients admitted with common bile duct stones, or choledocholithiasis. Secondly, the intervention for a persistent common bile duct stone is extraction by endoscopic retrograde cholangiopancreatography (ERCP), which carries a 5–7% risk of causing pancreatitis (66). Thus, these preclinical animal models could be designed to examine whether calcineurin inhibition might reduce post-ERCP pancreatitis in the setting of

Calcineurin Mediates Bile Acid-induced Pancreatitis

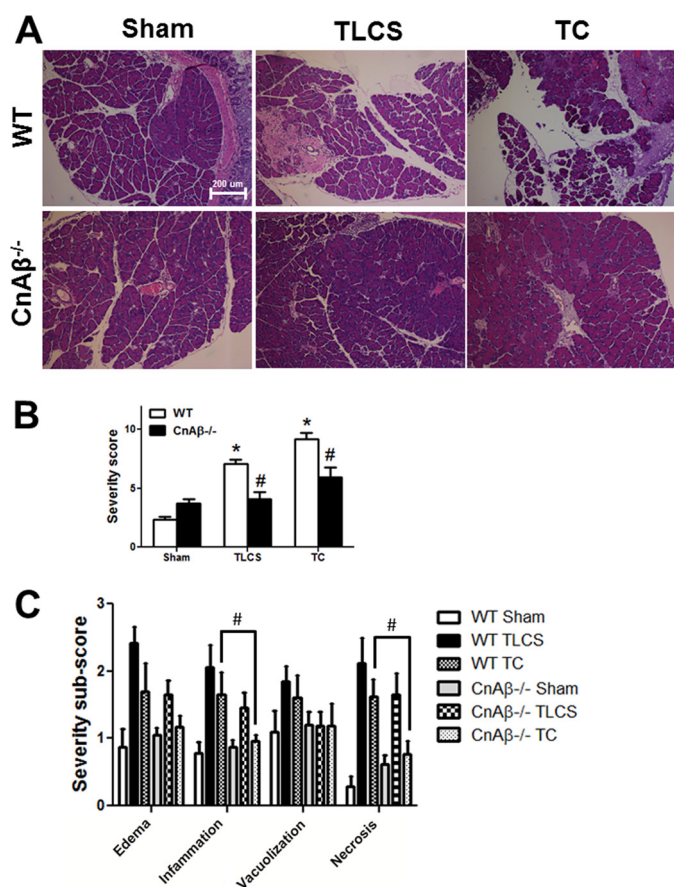


FIGURE 9. CnAβ-deficient mice are protected against bile acid infusion pancreatitis. A, representative H&E sections from the pancreatic head. B and C, overall severity score (B) and subscores (C). $n = 4-6$ animals per group. *, #, $p < 0.05$ when compared with sham and TLCS or TC alone, respectively.

choledocholithiasis, which is the most common indication for ERCP.

Notably, FK506 alone induced a mild degree of pancreatic injury. This trend, although not significant, was also observed in the CnAβ^{-/-} mice when compared with wild type mice. An explanation for this is that calcineurin inhibition or deficiency at base line may prevent the homeostatic mechanisms that preserve the normal architecture of the pancreatic parenchyma. There are a few case reports suggesting a clinical association of FK506 use with pancreatitis (67–71). However, there is considerable disparity in the experimental literature. Some demonstrate improvement of pancreatitis with FK506 (36, 72, 73), whereas others show worsening of pancreatitis (74, 75) or no effect (76). Thus, understanding the appropriate type, duration, and dosing of calcineurin inhibitors in a preclinical setting will be crucial in determining whether these drugs are helpful in treating or preventing the onset of pancreatitis.

In summary, we demonstrate that bile acids caused a Ca²⁺-dependent activation of acinar cell calcineurin and that calcineurin inhibition attenuates acinar cell injury and *in vivo* pancreatitis due to bile acids. The findings suggest that calcineurin inhibitors may be an effective adjunctive therapy for biliary pancreatitis.

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