Dantrolene mitigates caerulein-induced pancreatitis in vivo in mice

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Acute pancreatitis is a painful, inflammatory disorder for which adequate treatments are lacking. An early, critical step in its development is the aberrant signaling of Ca^{2+} within the pancreatic acinar cell. This Ca^{2+} release is modulated by the intracellular Ca^{2+} channel the ryanodine receptor (RYR). We have previously shown that RYR inhibition reduces pathological intra-acinar protease activation, an early marker of pancreatitis. In this study, we examined whether pretreatment with the RYR inhibitor dantrolene attenuates the severity of caerulein-induced pancreatitis in mice. Immunofluorescent labeling for RYR from mouse pancreatic sections showed localization to the basolateral region of the acinar cell. After 1 h of caerulein hyperstimulation in vivo, dantrolene J reduced pancreatic trypsin activity by 59% (P < 0.05) and 2) mitigated early ultrastructural derangements within the acinar cell. Eight hours after pancreatitis induction, dantrolene reduced pancreatic trypsin activity and serum amylase by 61 and 32%, respectively (P < 0.05). At this later time point, overall histological severity of pancreatitis was reduced by 63% with dantrolene pretreatment (P < 0.05). TUNEL-positive cells were reduced by 58% (P < 0.05). These data suggest that the RYR plays an important role in mediating early acinar cell events during in vivo pancreatitis and contributes to disease severity. Blockade of Ca^{2+} signals and particularly RYR-Ca^{2+} may be useful as prophylactic treatment for this disease in high-risk settings for pancreatitis.

ACUTE PANCREATITIS is a life-threatening disorder that leads to over 200,000 hospital admissions each year in the U.S., and the incidence appears to be rising in both children and adults (15, 40). Despite the growing burden of disease, treatment is still largely supportive, and there are currently no targeted therapies. Therefore, an understanding of the molecular mechanisms that initiate and propagate pancreatitis are required if directed therapy is to be developed. The earliest features of pancreatitis are seen within the pancreatic acinar cell (17). In particular, a high-amplitude, nonoscillatory pattern of cytosolic Ca^{2+} signaling occurs within seconds of exposing isolated acinar cells to conditions that cause pancreatitis in vivo, such as supraphysiological concentrations of cholecystokinin or acetylcholine (25, 32, 44, 46). The aberrant Ca^{2+} signal is necessary for subsequent pathological events within the cell, including intra-acinar protease activation, an early marker of pancreatitis, and acinar cell tissue. This is evidenced by the finding that Ca^{2+} chelation with BAPTA both in isolated acinar cells (24, 44) and in vivo (36) reduces those outcomes. In addition, the aberrant Ca^{2+} signal is observed in experimental models for the two most common causes of pancreatitis: 1) biliary (28, 36, 42, 55) and 2) alcohol-induced (9).

Ca^{2+} signals are shaped by the complex orchestration of a host of Ca^{2+} toolbox proteins, notably Ca^{2+} channels (2). A few have recently been implicated in pancreatitis, including the transient receptor potential cation channel TRPC3 (29) and the inositol 1,4,5-trisphosphate receptor (IP3R) (18). However, the role of the basally localized endoplasmic reticulum (ER)-bound ryanodine receptor (RYR) has not been fully studied. The RYR is a 565-kDa homotetramer that is responsible for the propagation of acinar cell Ca^{2+} waves from the apical into the basolateral region of the acinar cell (37, 49, 50).

We have previously shown that the RYR modulates pathological protease activation in isolated acinar cells (25). In the present study, we extend our findings from the isolated acinar cell model to the intact animal by examining whether the RYR effects in vivo protease activation and pancreatitis severity. We inhibited the RYR with dantrolene, a drug that is clinically used to treat malignant hyperthermia (31). Most patients with this disease harbor a mutation in the RYR that predisposes them to aberrant Ca^{2+} release in skeletal myocytes (45). In mice, we found that dantrolene blunted the rise in pancreatic protease activity observed after caerulein hyperstimulation and prevented several key ultrastructural derangements in the acinar cell. It also reduced the level of acinar cell apoptosis and histological severity of pancreatitis, thus implicating RYR-Ca^{2+} release in both the early and late stages of this disease.

METHODS

Reagents and animals. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Male C57BL/6 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. All animal treatments and euthanasia protocols were approved by the Animal Care and Use Committee.

Immunofluorescence for RYR. Pancreatic sections were fixed in cold acetone for 5 min and incubated with monoclonal anti-RYR antibody 34C diluted 1:25 (Hybridoma, Iowa City, IA). Primary antibodies were detected with Alexa 488-conjugated goat anti-mouse secondary antibodies diluted 1:100 (Molecular Probes, Eugene, OR). Specimens were imaged on a Zeiss LSM 510 laser scanning confocal microscope.

Expression of RYR isoforms through PCR. Total RNA was extracted and reverse transcribed from pancreatic acinar cells and brain tissue by use of an RNAskey kit (Qiagen, Valencia, CA). RT-PCR was performed with SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen, San Diego, CA). The mix was supplemented with the following forward and reverse oligonucleotide primers specific for mice: 5’-GAAAGTTCG-
GACAAACACGGG-3' and 5'-TCGCTCTTGTATGAACTTGGG-3' for RYR1, 5'-GAATCAGCGAGTTACTGGG-3' and 5'-TTGCTCGATCACTCCGCT-3' for RYR2, and 5'-TCCTTACTCTCGTGCTGGCTAAAGTCCAGG-3' for RYR3. The amplified products were electrophoresed on an agarose gel, stained with ethidium bromide, and visualized by UV illumination.

Induction of pancreatitis in vivo. After an overnight 12-h food fast with free access to water, pancreatitis was induced in mice by administering hourly intraperitoneal injections of caerulein (50 μg/kg body wt) for up to 12 h (38). In additional experiments a more severe model of pancreatitis was induced by administering six hourly caerulein injections followed by an intraperitoneal injection of LPS (10 mg/kg) as modified from Ding et al. (12). Saline-injected animals served as controls.

Preparation of serum and tissue samples. Mice were euthanized at varying intervals after the first intraperitoneal injection of caerulein. Whole blood was centrifuged at 5,000 g for 10 min at 4°C. Serum amylase was measured by use of a Phadebas kit (Amersham Pharmacia, Rochester, NY). Tissue from pancreas was fixed at room temperature for 2 h in 10% formalin solution with 125 mM phosphate buffer (pH 7.4), then transferred to 70% ethanol. Paraffin-embedded sections were stained with hematoxylin and eosin and graded at 1100x magnification over three separate fields in a blinded manner by a gastrointestinal pathologist (D. Jain) for edema, acinar cell vacuole formation, inflammation, and apoptosis (modified from Ref. 57). Another portion of paraffin-embedded pancreas was stained for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), and those positive cells were counted in three separate fields at 40x magnification. For measurement of trypsin activity, pancreas stored at −80°C was thawed and homogenized in iced medium containing 5 mM MOPS, 250 mM sucrose, and 1 mM MgSO4 (pH 7.0). Samples were then centrifuged at 5,000 g for 5 min at 4°C. Trypsin was measured from supernatant by use of a fluorogenic substrate as previously described (25).

Tissue fixation for electron microscopy. Animals were anesthetized and perfused for 5 min with 20 ml of fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Pancreas tissue was dissected, cut into 2-mm3 tissue blocks, incubated at room temperature for 2 h in the remaining fixative, and then postfixed with 1% osmium tetroxide. Tissues were blocked, stained with 2% uranyl acetate, dehydrated in acetone series, and Epon...
embedded. Thin sections were stained with lead citrate and uranyl acetate. Electron micrographs were acquired on a Philips 410 electron microscope and evaluated by an expert electron microscopist (C. Rahner).

Expression of cytokines through PCR. RNA samples were DNase treated and subjected to semiquantitative RT-PCR, as previously described (4, 6). Forward and reverse primer sequences used for detecting the specific cytokines are as follows: IL-1β 5′-GCCCATCCTCTGTGACTCAT-3′ and 5′-AGGCCACAGGTATTTTGTCG-3′; IL-2 5′-CCACTTCAAGCTCCACTTC-3′ and 5′-ATCCTGGGAGTTTCAGGTT-3′; TNF-α 5′-TTGAGGTCAACAACCCACA-3′ and 5′-CGCAATCACAGTCTTGGCTA-3′.

Statistical analysis. Statistical significance was determined by a Student’s t-test. Data are expressed as means ± SD unless otherwise stated. A P value of <0.05 was considered significant.

RESULTS

The RYR is localized to the basal region, and RYR1 is expressed in mouse acinar cells. RYR-dependent Ca2+ release in the basolateral region of the pancreatic acinar cell modulates pathological intra-acinar protease activation that overlaps with the RYR in a nonapical, supranuclear region. Although we and others have shown this in rat acinar cells (14, 25), our present in vivo studies used mice. Nevertheless, mouse acinar cells appear to exhibit similar properties in RYR-Ca2+ signaling as rat acinar cells. Specifically, mouse acinar cells pretreated for 30 min have selectively reduced basolateral Ca2+ signal when stimulated with carbachol (1 μM). We also first confirmed by immunofluorescence that the RYR is similarly distributed in the basolateral region of mouse acinar cells (Fig. 1A). Of the three known RYR isoforms, only RYR1 was expressed, as determined by PCR (Fig. 1B). This finding lends support to the use of the RYR inhibitor dantrolene, which targets RYR1 over the other isoforms (59).

Dantrolene reduces intrapancreatic protease activation in vivo. To examine the in vivo effects of RYR inhibition during pancreatitis, C57BL/6 mice were pretreated with the RYR inhibitor dantrolene (5 mg/kg), and pancreatitis was induced by use of the cholecystokinin analog caerulein (50 μg/kg; Fig. 2). Two doses of dantrolene (5 mg/kg each) were administered by intraperitoneal injection 4 h apart, modified from a protocol in mice (23). The first injection was given 1 h before caerulein 1 h before caerulein hyperstimulation as an initial prophylactic bolus, followed by an injection 4 h later to assure RYR inhibition over the 8- to 12-h course of repeated caerulein injections. The schema was extrapolated from pharmacokinetic studies that recommend using dantrolene for the treatment of malignant hyperthermia with repeated bolus injections (43). The dantrolene dose of 5 mg/kg was chosen on the following basis: 1) the recommended parenteral administration range in humans for therapy of malignant hyperthermia (31); 2) a successfully used dosing regimen in mice (11, 23, 58); and 3) solubility limitations of the compound (39). Furthermore, no additional effect on pancreatic protease activation with higher concentrations was observed (data not shown). In this experimental mouse model, activation of trypsin occurs within minutes after the first intraperitoneal caerulein dose and peaks by 0.5 to 1 h (21, 47). A second rise in trypsin activation occurs after 8 h, and extrapancreatic factors such as immune cell infiltration contribute in part to the increase (19, 20). Since we were particularly interested in determining the effects of RYR1

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Fig. 4. Dantrolene mitigates ultrastructural changes observed early on during caerulein hyperstimulation. Representative electron micrographs of pancreatic tissue 1 h after caerulein hyperstimulation in vivo. Changes include deformation of the nucleus structure, mitochondrial swelling, disruption of lateral membrane integrity, Golgi complex effacement, and microvascular damage. Arrowheads point to mitochondria or Golgi in their respective rows.
inhibition on protease activation, we examined both early and late time points during caerulein hyperstimulation. Dantrolene treatment reduced trypsin activity by 59% and 61% at 1 and 8 h postcaerulein relative to control, respectively (P < 0.05; Fig. 3, A and B). The reduction was maintained even at 12 h (P < 0.05; Fig. 3C).

**Dantrolene mitigates early ultrastructural changes associated with pancreatitis.** In the first hour, caerulein hyperstimulation caused several typical (48, 56) ultrastructural changes within the acinar cell (Fig. 4). The nuclei were pyknotic and contained dense heterochromatin. The mitochondria appeared swollen, along with loss of cristae. The lateral plasma membrane was disrupted, permitting cytoplasmic contents and large blebs to spill into the interstitial space. The Golgi was degraded in most cells; only occasional tubulovesicular remnants of the Golgi could be identified next to the nucleus in some cells. The apical microvilli were effaced (data not shown), and the ER was thickened and dilated. In addition, the intrapancreatic endothelium was enlarged and irregular. Dantrolene appeared to prevent all of the changes noted above except at the apical membrane and ER. These findings suggest that RYR inhibition attenuates many of the early morphological abnormalities observed during caerulein-induced pancreatitis.

**Dantrolene reduces pancreatitis severity.** Eight hours after the first injection, caerulein hyperstimulation caused a mild to moderate pancreatitis, characterized by pancreatic edema, appearance of inflammatory infiltrate, apoptosis, and acinar cell vacuolization (Fig. 5, A–C); this was consistent with previous observations (33). Dantrolene pretreatment caused a 61% reduction in histological damage relative to control levels (P < 0.05). These improvements were similarly noted at the 12-h time point (Fig. 5D). Recent studies have linked aberrant acinar cell Ca²⁺ signaling and trypsin activation to acinar cell death (8, 26). Although by morphometric analysis there was a modest reduction in apoptosis (Fig. 5C), with the more sensitive TUNEL assay there were 58% fewer apoptotic acinar cells in sections from dantrolene-pretreated animals (P < 0.05; Fig. 6). Serum amylase levels were reduced by 32% relative to control (P < 0.05; Fig. 7). The pancreatic content of proinflammatory cytokines IL-1β, IL-2, and IFN-γ was examined by PCR; they are known to be elevated in pancreatitis (5) (Fig. 8). Dantrolene pretreatment reduced levels by 83, 82, and 77%, respectively, compared with caerulein alone (P < 0.05). These results suggest that dantrolene reduces pancreatitis severity, inhibits apoptosis, and attenuates the inflammatory response.

To examine whether dantrolene might have a therapeutic role, mice were injected with dantrolene 2 and 6 h after the start of caerulein hyperstimulation and examined 8 h postinduction (Fig. 9A). Histological severity was reduced by 34% (P < 0.05; Fig. 9, B and C). To examine the effect of dantrolene on a more severe model of pancreatitis, mice were given six hourly caerulein injections followed by an intraperitoneal injection of LPS (10 mg/kg) as modified from Ding et al. (Fig. 4A) (12). Dantrolene given either as pre- or post-treatment reduced serum amylase levels and intrapancreatic trypsin activity by 70 and 60%, respectively (P < 0.05; Fig. 10, B and C).

![Fig. 5. Dantrolene reduces histological severity of pancreatitis. A: representative hematoxylin and eosin sections of pancreas from mice 8 h after the first caerulein dose, showing apoptosis (Apo) and inflammatory infiltrate (Inf). B: overall histological scoring of pancreatitis severity after 8 h of caerulein administration with or without dantrolene (n = 5 animals per group). C: analysis of histological parameters. A score of 0–3 was based on the degree of edema, inflammation, apoptosis, and vacuole formation. D: overall histological scoring of pancreatitis severity after 12 h of caerulein administration with or without dantrolene. *,#P < 0.05 compared with saline-treated and caerulein-alone groups, respectively.](http://ajpgi.physiology.org/)

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DISCUSSION

Our study provides a comprehensive characterization of the in vivo effects of dantrolene in experimental pancreatitis. The main findings were that dantrolene reduced in vivo protease activation, early acinar cell injury, and severity of acute pancreatitis in mice induced by caerulein hyperstimulation, presumably by inhibiting RYR1-dependent Ca\(^{2+}\) elevation. Of the three RYR isoforms, RYR1 was expressed in mouse acinar cells. By contrast, in rat acinar cells one study showed expression of RYR2 (34) and another demonstrated all three isoforms (14). Thus species-specific expression patterns of the RYR isoforms will need to be examined when translating our findings to humans. Studies from our laboratory suggest that RYR1 and RYR2 are the predominant isoforms present in human pancreatic tissue (unpublished data). The predominance of RYR1 in mouse acinar cells substantiated the use of dantrolene, which selectively inhibits RYR1 over the other isoforms (59). In contrast, our previous study using rat acinar cells demonstrated that although dantrolene inhibited basolateral signaling it mitigated only the early findings of pancreatitis (25). The long-term events were unchanged. The difference in drug effect could be related to RYR isoform expression.

Other inhibitors of the RYR include ryanodine, ruthenium red, and tetracaine. Ryanodine has highly specific inhibition at micromolar concentrations. In isolated acinar cells, we have shown that ryanodine reduced intra-acinar protease activity induced by caerulein (25), but the agent could not be used in vivo because of cardiotoxicity. Ruthenium red and tetracaine are nonspecific. The former blocks the inner mitochondrial membrane Ca\(^{2+}\) uniporter (54), whereas the latter may block several other channels including sodium- and voltage-sensitive channels (13). Although caffeine is an activator of the RYR, and caffeine-induced depletion of acinar ER-Ca\(^{2+}\) stores leads to reduced intra-acinar protease activation (25), it is difficult to assign this effect to the RYR alone, since this drug is also an antagonist of the adenosine receptor and the IP3R (1).

The inhibitory effects of dantrolene on the RYR are controversial and may be direct or indirect. Studies with heterologously expressed RYR1 suggested that dantrolene has a direct inhibitory binding site (41), and functional studies by \(^{45}\)Ca\(^{2+}\) release and \(^{3}H\)ryanodine binding activity showed high affinity, monophasic inhibition of RYR1 (16). Others, however, were unable to find any effect of dantrolene on \(^{3}H\)ryanodine binding, or of ryanodine on \(^{3}H\)dantrolene binding (39). The results implied that the two binding sites were either nonoverlapping, not allosterically linked, or on different molecules. Dantrolene might inhibit RYR1-mediated Ca\(^{2+}\) release by

Fig. 6. Dantrolene reduces acinar cell apoptosis observed with caerulein hyperstimulation. Top and bottom left: representative terminal deoxynucleotidytranferase dUTP nick-end labeling (TUNEL)-stained sections of pancreas from mice 8 h after the first caerulein dose. Bottom right: quantification of apoptosis derived by counting TUNEL-positive cells from 3 separate fields (n = 5 animals per group). *#P < 0.05 compared with saline-treated and caerulein-alone groups, respectively.

Fig. 7. Dantrolene reduces serum amylase elevations. Serum was assayed for amylase 8 h after the first caerulein dose (n = 3–5 animals per group). *P < 0.005; #P < 0.05 compared with saline-treated and caerulein-alone groups, respectively. Serum values from dantrolene-alone treated animals did not rise above control values.
stabilizing domain interactions within the receptor, thus preventing the domain unzipping required for channel opening (30). However, dantrolene failed to reduce channel activity of purified RYR1 in lipid bilayers (51). The conflicting results might be due to degradation or loss of the dantrolene binding site during the purification, the need for accessory elements in the RYR1 complex for dantrolene binding to the channel, or, possibly, an indirect inhibition of the RYR1 altogether (31). Indeed, recent evidence from experiments in mouse skeletal muscle demonstrated that the dantrolene congener azumolene had no effect on sarcoplasmic reticulum Ca\(^{2+}\) flux, whereas it inhibited a component of store-operated Ca\(^{2+}\) entry that is tightly coupled to RYR1-dependent Ca\(^{2+}\) release (60). Thus dantrolene can be used as an effective inhibitor of an RYR-dependent Ca\(^{2+}\) rise in vivo, although its mode of channel inhibition is not fully defined.

Fig. 8. Dantrolene reduces the proinflammatory cytokine profile observed with caerulein hyperstimulation. A: PCR for expression of IL-1\(\beta\), IL-2, and IFN-\(\gamma\) from pancreatic tissue \((n = 3–5\) animals per group\). \(\beta\)-Actin serves as a loading control. B–D: densitometric measurements normalized to \(\beta\)-actin. IL-1\(\beta\) \#\(P < 0.01\) compared with caerulein-alone group; IL-2 \#\(P < 0.01\) compared with caerulein-alone group; IFN-\(\gamma\) \#\(P < 0.01\) compared with caerulein-alone group.

Fig. 9. Dantrolene posttreatment reduces histological severity of pancreatitis. A: schema for posttreatment with dantrolene \((5\) mg/kg\) and pancreatitis induction with caerulein \((50\) \(\mu\)g/kg\). Mice were euthanized at the designated time points. B: representative hematoxylin and eosin sections of pancreas from mice 8 h after the first caerulein dose, showing apoptosis and inflammatory infiltrate. C: overall histological scoring of pancreatitis severity after 8 h of caerulein administration with or without dantrolene \((n = 5\) animals per group\). *, \#\(P < 0.05\) compared with saline-treated and caerulein-alone animals, respectively.
An alternate approach to studying the role of RYR would be to utilize mice with genetic deletions of the various isoforms. However, we are limited by the nonavailability of relevant RYR-deficient animals. RYR1- and RYR2-deficient mice do not live beyond the neonatal or embryonic period, respectively (52, 53). RYR3-deficient mice live to adulthood (3), but these mice may not be helpful because acinar cell expression of RYR3 was undetectable from our study using (3), but these mice may not be helpful because acinar cell expression of RYR3 was undetectable from our study using (3). Nevertheless, our study shows a protective effect of dantrolene on pancreatic protease activation and cell injury within the first hour of pancreatitis induction and, although incomplete, a moderate amelioration of the severity of the induced pancreatitis. The results lend support to the notion that RYR-dependent increases in acinar cell cytoplasmic Ca\(^{2+}\) contribute to both the initiation and transduction of pancreatitis. Further studies are required to determine whether the RYR contribute to other Ca\(^{2+}\)-dependent pathways independent of pathological protease activation.

In summary, we have shown that using dantrolene in vivo significantly retards the development of pathological protease activation, acinar cell injury, and apoptosis early in pancreatitis and moderately mitigates pancreatitis severity and inflammation hours after disease induction. We believe that RYR-Ca\(^{2+}\) channel blockers might be particularly useful as prophylaxis for patients at high risk for pancreatitis, such as those undergoing endoscopic retrograde cholangiopancreatography. Overall, the results point to the importance of examining therapies that target aberrant acinar cell Ca\(^{2+}\) signals and particularly RYR-Ca\(^{2+}\) in the pathogenesis of this disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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