

# The ryanodine receptor mediates early zymogen activation in pancreatitis

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**Acute pancreatitis is characterized by the pathologic activation of zymogens within pancreatic acinar cells. The process requires a rise in cytosolic Ca<sup>2+</sup> from undefined intracellular stores. We hypothesized that zymogen activation is mediated by ryanodine receptor (RYR)-regulated Ca<sup>2+</sup> release, because early zymogen activation takes place in a supranuclear compartment that overlaps in distribution with the RYR. Ca<sup>2+</sup> signals in the basolateral, but not apical, region of acinar cells observed during supraphysiologic agonist stimulation were dependent on RYR Ca<sup>2+</sup> release. Inhibition of RYR or depletion of RYR-sensitive Ca<sup>2+</sup> pools each reduced pathologic zymogen activation in isolated acinar cells, but neither treatment affected amylase secretion. Inhibition of RYR also inhibited zymogen activation *in vivo*. We propose that Ca<sup>2+</sup> release from the RYR mediates zymogen activation but not enzyme secretion. The findings imply a role for the RYR in acute pancreatitis.**

pancreatic acinar cell | dantrolene | calcium signaling

**A**cute pancreatitis is a life-threatening inflammatory disease. An early feature is the pathologic activation of zymogens, particularly of trypsinogen and other proteases, within the pancreatic acinar cell. Studies underscore the importance of zymogen activation in the pathogenesis of this disease. For example, pretreatment of rats with protease inhibitors blocks both trypsin activation and injury in the caerulein hyperstimulation model of pancreatitis (1, 2). Clinically, pretreatment with a protease inhibitor reduces procedure-related pancreatitis (3). Mice with a genetic deletion of the lysosomal hydrolase cathepsin B, an enzyme that can activate trypsinogen, have reduced trypsin activity and acinar tissue necrosis after caerulein hyperstimulation (4). Finally, hereditary pancreatitis results from mutations in the cationic trypsinogen gene that may increase trypsin activity (5). Together, such observations support a central role for pathologic intracellular zymogen activation in the pathogenesis of acute pancreatitis.

Enzyme secretion and the intracellular activation of zymogens both require an increase in acinar cell cytosolic Ca<sup>2+</sup> [(Ca<sup>2+</sup>)<sub>i</sub>], but the intracellular source of this Ca<sup>2+</sup> remains unclear (1, 6, 7). In pancreatic acinar cells, two spatially distinct Ca<sup>2+</sup> channels mediate the initial release of Ca<sup>2+</sup> from stores in the endoplasmic reticulum. The inositol 1,4,5-trisphosphate receptor (IP3R) channel is concentrated in a restricted area close to the apical membrane (8–10). The distribution of the IP3R overlaps with the sites of zymogen granule exocytosis required for enzyme secretion, although the IP3R is more apical than most of the granules (9). The ryanodine receptor (RYR) channel is diffusely distributed in the basolateral region of the acinar cell (11, 12). Early zymogen activation takes place in a supranuclear vesicular compartment that does not overlap with zymogen granules (2, 13). These results suggest that the distribution of pathologically activated zymogens would overlap with RYR, but not IP3R, channels. Based on these relationships, we hypothesized that zymogen activation, but not digestive enzyme secretion, is primarily mediated by release of Ca<sup>2+</sup> from a storage pool that is regulated by the RYR. In this study, we used the carbachol- and caerulein-stimulation models of pancreatitis to

examine the role of RYR in zymogen activation and amylase secretion.

## Materials and Methods

**Purification of Trypsinogen Activation Peptide (TAP) Antibody, Preparation of Pancreatic Sections, and Immunofluorescence.** Antibodies to the marker of zymogen activation, TAP, were prepared as described in ref. 2. All reagents were purchased from Sigma unless otherwise noted. For detection of IP3R and RYR by immunofluorescence labeling, pancreatic sections were fixed in cold acetone for 5 min. Sections were incubated with either monoclonal anti-RYR antibody 34C (diluted 1:25) or monoclonal anti-IP3R-III antibody (diluted 1:100; Transduction Laboratories, Lexington, KY). Primary antibodies were detected with Alexa 488-conjugated goat anti-mouse secondary antibodies (diluted 1:00; Molecular Probes). Tissue sections for TAP labeling were prepared by *in vivo* perfusion as described in ref. 2. After labeling with anti-TAP IgG (diluted 1:200), primary antibodies were detected with FITC-labeled goat anti-rabbit IgG F'ab fragments (diluted 1:500). Nuclei were stained with TOPRO-3 (diluted 1:200; Molecular Probes). Specimens were imaged on a Zeiss META LSM 510 laser scanning confocal microscope.

**Preparation of Pancreatic Acini.** Groups of pancreatic acinar cells, known as acini, were isolated as described in ref. 14. Briefly, fasted male Sprague–Dawley rats weighing 50–100 g (Charles River Laboratories) were killed by using a protocol approved by the Animal Care and Use Committee. The pancreas was minced in buffer containing 40 mM Tris (pH 7.4), 95 mM NaCl, 4.7 mM KCl, 0.6 mM MgCl<sub>2</sub>, 1.3 mM CaCl<sub>2</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM glucose, and 2 mM glutamine, plus 0.1% BSA, 1 × MEM nonessential amino acids (GIBCO/BRL), and 50 units/ml type-4 collagenase (Worthington) and then incubated for 60 min at 37°C.

**Stimulation of Acini.** After acini were incubated at 37°C for 60 min under constant O<sub>2</sub> with shaking (80 rpm), they were pretreated with dantrolene (10–75 μM) or ryanodine (100 μM) to inhibit the RYR. Some preparations were pretreated with either caffeine (10 mM) for 10 min or EGTA (2 mM) for 2 min. Acini then were stimulated with either the muscarinic agonist carbachol (1 μM or 1 mM) or the cholecystokinin (CCK) ortholog caerulein (100 nM) for 60 min in the presence or absence of vasointestinal polypeptide (VIP) (100 nM), secretin (100 nM), or 8-bromo-cAMP (100 μM).

**Enzyme Activity Assays.** Protease activity assays were performed by using fluorogenic substrates as described in ref. 14. Briefly, enzyme

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Abbreviations: (Ca<sup>2+</sup>)<sub>i</sub>, cytosolic Ca<sup>2+</sup>; RYR, ryanodine receptor; IP3R, inositol 1,4,5-trisphosphate receptor; CCK, cholecystokinin; TAP, trypsinogen activation peptide; VIP, vasointestinal polypeptide.

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substrate (chymotrypsin or trypsin; Calbiochem) was added to each homogenized sample and read by using 380-nm excitation and 440-nm emission wavelengths. Total amylase values were used for normalization because the enzymatic activity is stable in pancreatic homogenates and the assay is reproducible (Phadebas kit; Amersham Pharmacia Diagnostic, Rochester, NY). Amylase secretion was expressed as the percent of total released into medium.

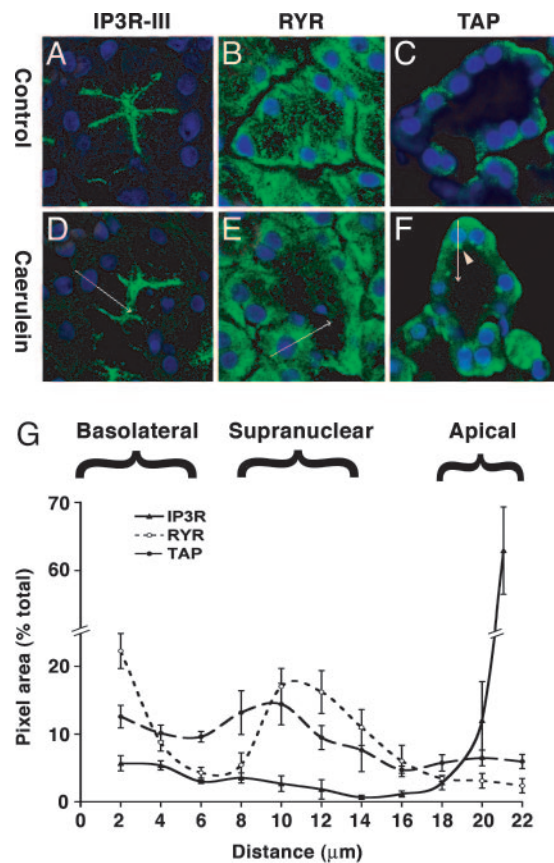
**Detection of Cellular  $\text{Ca}^{2+}$  Signals.**  $\text{Ca}^{2+}$  signals were detected in acinar cells as described in ref. 15. Acini were incubated for 30 min with the medium-affinity  $\text{Ca}^{2+}$ -sensing dye fluo-5F/AM and in limited experiments mag-fluo-4 (both at 6  $\mu\text{M}$  and from Molecular Probes). These dyes were used because ( $\text{Ca}^{2+}$ )<sub>i</sub> signals in acinar cells can exceed 1  $\mu\text{M}$  and saturate high-affinity dyes such as fluo-4 (16). A Bio-Rad MRC-1024 confocal microscope was used with a 63 $\times$ , 1.4 numerical aperture objective. An argon laser was used to excite the dye at 488-nm wavelength, and emission signals of >515 nm were collected. Signals were recorded at a rate of 2–10 frames/sec with full-screen scanning. Apical and basolateral regions were identified within individual acinar cells, and the fluorescence intensity of the  $\text{Ca}^{2+}$  signals in those regions was determined.

**In Vivo Caerulein Pancreatitis.** Based on pharmacokinetic studies, dantrolene (5 mg/kg) was given as a single tail vein injection to fasted rats (17). After 60 min, an i.p. dose of caerulein (40  $\mu\text{g}/\text{kg}$ ) was given to induce pancreatic zymogen activation. Control animals received an equivalent volume of normal saline. After another 60 min, rats were euthanized, and pancreatic homogenates were assayed for chymotrypsin and trypsin activities. In other experiments rats were given three additional hourly injections of caerulein, and the parameters of pancreatitis were evaluated 4 h after the first injection.

**Statistical Analysis.** Data represent means  $\pm$  SEM of at least three individual experiments unless otherwise noted, with each experiment performed in duplicate. Statistical significance ( $P < 0.05$ ) was determined by Student's *t* test analysis. Overlap between TAP, RYR, and IP3R was quantified by examining pixel intensity line scans in immunofluorescence sections. This value was calculated by using the Pearson overlap coefficient *r* (18).

## Results

**Zymogen Activation Overlaps with RYR but Not with IP3R.** The subcellular distribution of the RYR and IP3R was compared with TAP, a marker of zymogen activation that appears early in disease, in pancreatic tissue after saline or 30 min of caerulein treatment *in vivo*. Because the labeling patterns of all three IP3Rs are similar in the pancreatic acinar cell, labeling for only the IP3R-III was performed (8–10). We have shown that preservation of TAP immunoreactivity requires glutaraldehyde fixation (2). This fixation, however, eliminated signals for RYR or IP3R labeling (data not shown). Thus, to compare TAP with the  $\text{Ca}^{2+}$ -release channels, we performed single-label immunofluorescence for each molecule and then determined their overlaps within common cellular regions (Fig. 1). To control for the focal plane, we only analyzed images from confocal sections in which the nuclei were maximally sized. TAP labeling appeared as intense supranuclear vesicles after caerulein hyperstimulation (Fig. 1F, arrowhead). The faint, diffuse labeling of TAP in the basolateral regions of some cells was likely nonspecific. The distribution of labeled structures was quantified relative to the distance from the basolateral membrane by using the nucleus (TOPRO-3 stained) as a reference point. Overlap was measured along five parallel line scans in each section oriented basal-to-apical, which evenly dissected the nucleus (Fig. 1D–F, arrows). IP3R labeling was localized to a discrete apical compartment, whereas RYR was diffusely distributed in the basolateral two-thirds of the cell but was concentrated in the supranuclear region (Fig. 1A, B, D, and E). In contrast to IP3R, RYR was

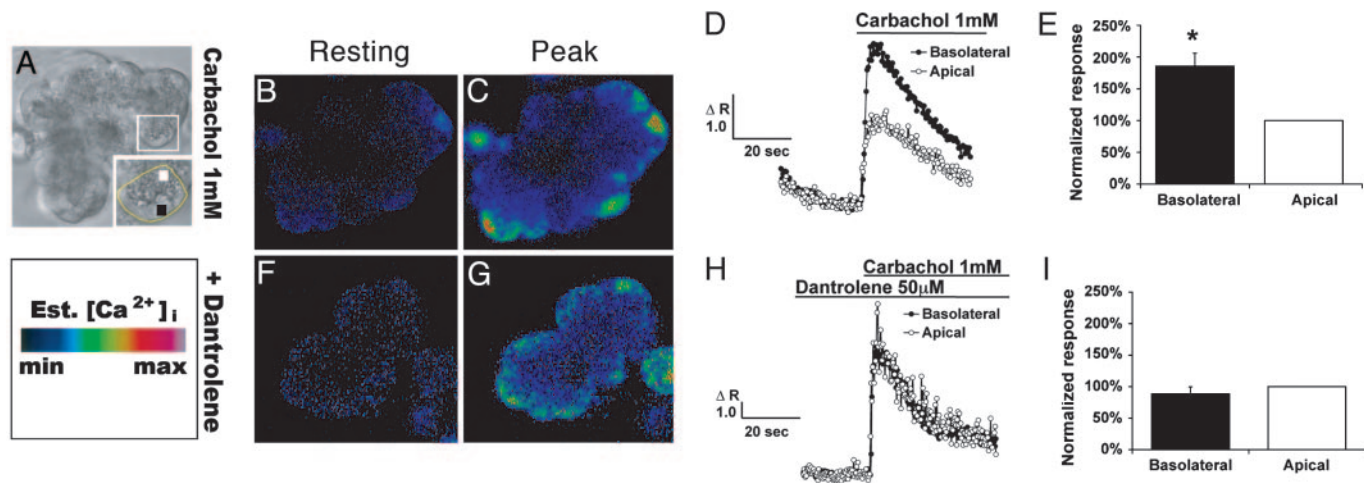


**Fig. 1.** The distribution of TAP overlaps with RYR but not with IP3R. Confocal microscopy images of pancreas sections after 30 min of normal saline (A–C) or caerulein hyperstimulation (D–F) *in vivo* were labeled for IP3R-III (A and D), RYR (B and E), or TAP (C and F); nuclei were stained blue with TOPRO-3. Basal-to-apical line scans (see arrows in D–F) show that IP3R labeling is apical; RYR is distributed in the basolateral region, concentrated in the supranuclear region but excluded from the apical region. TAP appears as discrete supranuclear structures (arrowhead). (G) Overlap between TAP and RYR, but not IP3R, in the supranuclear region is quantified in five line scans from each section, relative to the distance from the basolateral membrane with the nucleus as a reference point.

excluded from the apical region. The findings are consistent with previous localization studies in acinar cells (11, 12, 19). Significant coincidence in the distribution of TAP and RYR was demonstrated by a Pearson's overlap coefficient of  $0.78 (\pm 0.02, \text{SEM})$  (18). In contrast, the overlap between TAP and IP3R was minimal (coefficient  $0.31 \pm 0.02$ ). The difference between the two overlap coefficients was statistically significant ( $P < 0.001$ ). The data show that the cellular distribution of TAP overlaps with the distribution of RYR but not IP3R. Thus, the RYR  $\text{Ca}^{2+}$  release pool is in close proximity to the compartment in which zymogens are initially activated. The overlap between IP3R and RYR had a coefficient of  $0.21 \pm 0.03$  ( $P < 0.0001$ ). The results also demonstrate that the subcellular distribution of  $\text{Ca}^{2+}$ -release channels is not changed in the early phase (30 min) of caerulein-hyperstimulation pancreatitis.

**RYR Inhibition Selectively Reduces Basolateral  $\text{Ca}^{2+}$  Signals.** Because RYR, but not IP3R, is localized to the region of the acinar cell in which trypsinogen becomes activated, we investigated whether the RYR is responsible for the  $\text{Ca}^{2+}$  signal required for zymogen activation. Supraphysiologic concentrations of both caerulein and carbachol induce prominent zymogen activation in pancreatic acinar cells (20). Carbachol was used as a representative agonist because it may be most relevant to human disease. Specifically,





**Fig. 2.** RYR inhibition selectively reduces the  $\text{Ca}^{2+}$  peak in the basolateral region induced by carbachol (1 mM). (A) Representative phase image. *Inset* highlights regions of interest: black box, basolateral; white box, apical. (B and F) Resting  $\text{Ca}^{2+}$  fluorescence after cells were loaded with fluo-5F/AM. The images are pseudocolored using the  $\text{Ca}^{2+}$  scale. (D) Carbachol (1 mM) induced a higher basolateral than did apical  $\text{Ca}^{2+}$  signal. (E) Quantitation of studies from D ( $n = 17$ ). (H) Carbachol-induced basolateral  $\text{Ca}^{2+}$  signal is selectively reduced by dantrolene (50  $\mu\text{M}$ ). (I) Quantitation of studies from H ( $n = 6$ ). \*,  $P < 0.001$  for basolateral region peak  $\text{Ca}^{2+}$  response, compared with the apical region in carbachol-alone condition.

although rodent and human acinar cells have muscarinic receptors, only rodent acinar cells may have the  $\text{CCK}_1$  receptor that is linked to pancreatitis (21, 22). Further, muscarinic hyperstimulation is implicated in certain forms of human pancreatitis. Similar to what is described for CCK, both supraphysiologic (1 mM) and physiologic (1  $\mu\text{M}$ ) carbachol concentrations evoke a global ( $\text{Ca}^{2+}$ )<sub>i</sub> wave that travels along an apical-to-basal axis (20). In addition to this pattern, by using the intermediate sensitivity  $\text{Ca}^{2+}$  dye fluo-5F/AM ( $K_{\text{Ca}} \sim 2.3 \mu\text{M}$ ; Fig. 2 B–E) and a lower-affinity  $\text{Ca}^{2+}$  dye mag-fluo-4 ( $K_{\text{Ca}} \sim 22 \mu\text{M}$ ), we also observed an enhanced basolateral  $\text{Ca}^{2+}$  signal with supraphysiologic carbachol. However, the enhanced signal was not observed using the high-affinity  $\text{Ca}^{2+}$  dye fluo-4 ( $K_{\text{Ca}} \sim 0.35 \mu\text{M}$ ), probably because of dye saturation. To inhibit  $\text{Ca}^{2+}$  flux through the RYR, acini were preincubated for 1–2 h with the cell-permeant RYR antagonist dantrolene (23). The preincubation time resulted in the maximum inhibitory effect. The advantage of using dantrolene to inhibit the RYR over caffeine-ryanodine pretreatment is that unlike the latter, it does not deplete endoplasmic reticulum  $\text{Ca}^{2+}$  stores (24). The concentrations used (10–75  $\mu\text{M}$ ) inhibit  $\text{Ca}^{2+}$  release from all three RYR isoforms, although there is greater affinity for RYR1 (23, 25). Dantrolene (50  $\mu\text{M}$ ) selectively reduced the carbachol (1 mM)-induced  $\text{Ca}^{2+}$  signal in the basolateral, but not the apical, region of the acinar cell (Fig. 2). When peak  $\text{Ca}^{2+}$  signals were compared in the two regions, dantrolene reduced the basolateral signal relative to the apical signal by 52% ( $P < 0.001$ ). Dantrolene pretreatment did not change the baseline  $\text{Ca}^{2+}$  fluorescence in the apical or basolateral region (Fig. 2 B and F). Thus, dantrolene selectively reduced the  $\text{Ca}^{2+}$  signal in the same region of the acinar cell in which trypsinogen is activated. To examine whether extracellular  $\text{Ca}^{2+}$  contributes to the basolateral  $\text{Ca}^{2+}$  signal, cells were perfused with EGTA (2 mM) before supraphysiologic stimulation. There was no change in the comparison of apical and basolateral peak  $\text{Ca}^{2+}$  signals (data not shown). Thus, extracellular  $\text{Ca}^{2+}$  does not appear to be responsible for the basolateral  $\text{Ca}^{2+}$  signal. The finding is consistent with the observation that the initial agonist-evoked  $\text{Ca}^{2+}$  rise in acinar cells comes from intracellular stores and that the subsequent  $\text{Ca}^{2+}$  influx is needed to generate a sustained plateau (6).

**RYR Inhibition Reduces Intracellular Zymogen Activation.** To examine the effects of RYR inhibition on zymogen activation, isolated acini were preincubated with dantrolene. Zymogen activation was in-

duced by supraphysiologic carbachol (1 mM) (Fig. 3) or caerulein (100 nM) (Table 1). Assays of chymotrypsin rather than trypsin activity were measured in these cell studies because the generation of chymotrypsin more closely follows first-order kinetics over 60 min and is a more sensitive marker of zymogen activation than trypsin after 30 min of treatment (26). Dantrolene caused a concentration-dependent reduction in supraphysiologic carbachol-induced chymotrypsin activity by up to 40% (Fig. 3A). In preliminary studies, the trends for trypsin responses were similar to chymotrypsin. Because the pancreatic acinar cell has receptors that activate cAMP pathways, the effects of these ligands in combination with muscarinic stimulation were examined. We have shown that when carbachol-stimulated acini are cotreated with the cAMP agonists VIP or secretin, zymogen activation is enhanced (14, 27). We found that RYR inhibition by dantrolene (50  $\mu\text{M}$ ) reduced VIP- and secretin-enhanced chymotrypsin activity by 54% and 62%, respectively (Fig. 3B;  $P < 0.05$ ). A similar effect was seen when cAMP levels were directly increased by 8-bromo-cAMP (Fig. 3C). Although the mechanism responsible for cAMP sensitization to zymogen activation is unclear, it also appears to be inhibited by dantrolene.

Because zymogen activation and secretion can be uncoupled in the pancreatic acinar cell and during pancreatitis, the effects of RYR inhibition on both responses were assayed. Dantrolene had no effect on either basal amylase secretion or that stimulated by physiologic (1  $\mu\text{M}$ ) or supraphysiologic (1 mM) carbachol (Fig. 3D). Dantrolene also lacked an effect when carbachol-induced amylase secretion was enhanced by VIP, secretin, or 8-bromo-cAMP (Fig. 3E). To ensure that RYR inhibition was not specific to carbachol, acini were treated with the CCK ortholog caerulein (Table 1). Dantrolene decreased caerulein-induced chymotrypsin activity without affecting amylase secretion (Table 1, dantrolene treatment). To confirm dantrolene's specificity, another RYR antagonist, ryanodine (100  $\mu\text{M}$ ), was used with caerulein (28). Ryanodine reduced chymotrypsin activity by 38% ( $P < 0.01$ ) without affecting amylase secretion (Table 1, ryanodine treatment). To confirm that dantrolene and ryanodine are acting on RYR  $\text{Ca}^{2+}$  stores in the acinar cell, RYR-sensitive  $\text{Ca}^{2+}$  pools alternatively were depleted by using caffeine (10 mM) before caerulein stimulation. Previous studies have shown that this method of depletion is specific for RYR-sensitive  $\text{Ca}^{2+}$  pools (29). We found that perfusion of acinar cells with caffeine (10 mM) resulted in selective release of  $\text{Ca}^{2+}$



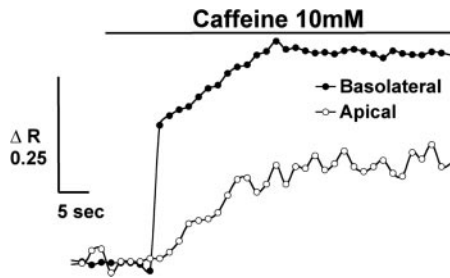


Fig. 4. Caffeine (10 mM) selectively releases  $\text{Ca}^{2+}$  from the basolateral region. Representative ( $\text{Ca}^{2+}$ )<sub>i</sub> tracing from an acinar cell shows basolateral and apical regions upon caffeine stimulation ( $n = 9$ ).

that in both a cellular and animal model of acute pancreatitis, RYR inhibition reduced pathologic zymogen activation within acinar cells but did not affect enzyme secretion. Thus,  $\text{Ca}^{2+}$  release through the RYR appears to be selectively coupled to intracellular zymogen activation, but not secretion, in the acinar cell.

In this study, a distinct pattern of ( $\text{Ca}^{2+}$ )<sub>i</sub> signaling was induced by supraphysiologic muscarinic receptor agonist stimulation. Studies in acinar cells stimulated with subphysiologic (nanomolar) carbachol concentrations have observed localized oscillations in a subcompartment of the apical region termed the trigger zone (32). These apical oscillations are sensitive to IP3R blockade (33). Confinement of these  $\text{Ca}^{2+}$  signals has been attributed to buffering of  $\text{Ca}^{2+}$  by apically distributed mitochondria (34). In contrast, physiologic (micromolar) carbachol concentrations initiate a  $\text{Ca}^{2+}$  wave that begins in the apical trigger zone and travels basolaterally (35–37). This global  $\text{Ca}^{2+}$  wave is induced by sequential activation of apical IP3R followed by basolateral RYR (15). We and others have observed that the peak level of this ( $\text{Ca}^{2+}$ )<sub>i</sub> response is equivalent throughout the cell. By using a low-affinity dye to avoid saturation by high ( $\text{Ca}^{2+}$ )<sub>i</sub> levels, it has been observed that maximum ( $\text{Ca}^{2+}$ )<sub>i</sub> peaks induced by physiologic stimulation can reach 5–10  $\mu\text{M}$  in the apical region (16). Because ( $\text{Ca}^{2+}$ )<sub>i</sub> is much higher with a supraphysiologic than physiologic concentration of carbachol, we used a similar low-affinity  $\text{Ca}^{2+}$  dye to show that high (mM) carbachol concentrations cause an apical-to-basal  $\text{Ca}^{2+}$  wave. This treatment elicits a much higher  $\text{Ca}^{2+}$  peak in the basolateral region of the acinar cell than the apical region, thus underscoring the need to use  $\text{Ca}^{2+}$  dye sensors that do not become saturated. Notably, the difference in the peak  $\text{Ca}^{2+}$  levels between the two domains was eliminated by dantrolene (Fig. 1 E and I). Although RYR2 is the principal isoform in pancreatic acinar cells (12), these cells also may express RYR1 and RYR3 (11). Because the concentrations of dantrolene used in this study inhibit all three RYR isoforms, the target of this response is unclear. Together, these findings suggest that conditions that induce pancreatitis selectively increase ( $\text{Ca}^{2+}$ )<sub>i</sub> in the basolateral, compared with the apical region

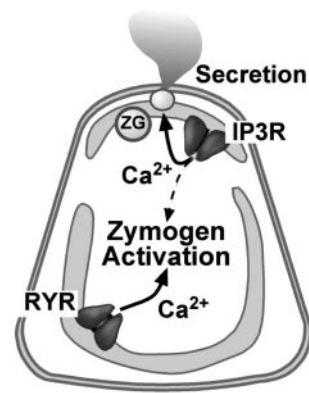


Fig. 6. Model for the regulation of subcellular  $\text{Ca}^{2+}$  pools by RYR and IP3R in pancreatic acinar cells. Pathologic zymogen activation is primarily mediated by  $\text{Ca}^{2+}$  release from RYR-regulated pools. Pancreatic acinar cell secretion is mediated by  $\text{Ca}^{2+}$  release through IP3R, but not RYR. Note that some  $\text{Ca}^{2+}$  released through IP3R or other mechanisms may contribute to zymogen activation. ZG, zymogen granule.

of the acinar cell. RYR  $\text{Ca}^{2+}$  release is largely responsible for this distinct intracellular pattern of  $\text{Ca}^{2+}$  signaling.

The distinct spatial patterns of  $\text{Ca}^{2+}$  signaling are relevant to both pancreatic enzyme secretion and pathologic zymogen activation. Physiologic agonist stimulation is associated with maximal enzyme secretion and minimal zymogen activation; supraphysiologic stimulation reduces secretion and causes pathologic zymogen activation (38). Inhibiting RYR  $\text{Ca}^{2+}$  release during physiologic stimulation has been shown to diminish the basolateral component of the  $\text{Ca}^{2+}$  wave but does not affect its capacity to elicit maximal enzyme secretion (36). In our studies, inhibiting RYR  $\text{Ca}^{2+}$  release during supraphysiologic stimulation diminished the  $\text{Ca}^{2+}$  peak in the basolateral region but did not significantly change the apical  $\text{Ca}^{2+}$  peak (Fig. 2I). Furthermore, RYR inhibition reduced pathologic zymogen activation but did not affect secretion (Fig. 3 D and E and Table 1). Together, these data suggest that RYR  $\text{Ca}^{2+}$  release mediates zymogen activation but not secretion.

RYR inhibition by dantrolene, ryanodine, or depletion of RYR-sensitive  $\text{Ca}^{2+}$  stores by caffeine incompletely inhibited zymogen activation. Further, dantrolene reduced the peak  $\text{Ca}^{2+}$  signal in the basolateral region but did not eliminate it. There are several explanations for these results. The effects on RYR inhibition or RYR  $\text{Ca}^{2+}$  pool depletion may have been incomplete. Alternatively, RYR-independent pathways, such as those mediated by the IP3R, may contribute to the  $\text{Ca}^{2+}$  signal in the basolateral region that regulates zymogen activation (Fig. 6).

The finding that RYR  $\text{Ca}^{2+}$  release modulates zymogen activation needs further study in other models of pancreatitis. It is intriguing, for example, that recent data suggest that pancreatic duct

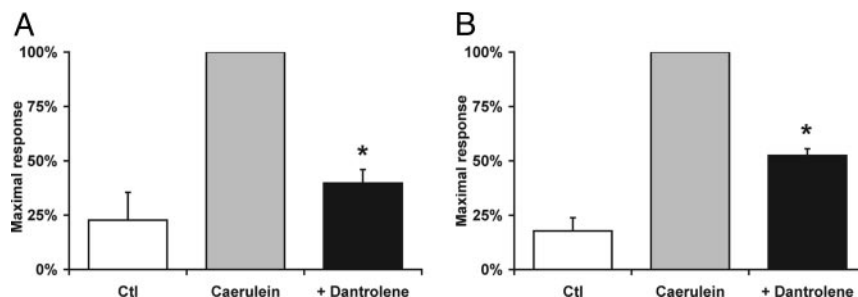


Fig. 5. RYR inhibition reduces zymogen activation induced by *in vivo* caerulein hyperstimulation. Chymotrypsin (A) and trypsin (B) activities are shown for rats injected with either normal saline control (Ctl), caerulein hyperstimulation alone, or with dantrolene pretreatment ( $n = 3$ ). \*,  $P < 0.05$  with respect to the maximal response.



obstruction, a cause of pancreatitis, affects acinar cell  $\text{Ca}^{2+}$  signaling (39). We found that the premature zymogen activation was inhibited by dantrolene pretreatment for 1 h, but not 4 h, after caerulein hyperstimulation *in vivo*. In addition, edema and histological pancreatitis severity were not affected. The lack of a dantrolene effect on longer durations of pancreatitis might be due to pharmacologic factors that reduce RYR inhibition over time. Furthermore, IP3R-regulated  $\text{Ca}^{2+}$  release may independently contribute by permitting a threshold amount of zymogen activation required to cause pancreatitis.

The findings of this study raise issues relating to both the physiologic and pathologic importance of RYR in acinar cells. In this context, we have observed that even physiologic CCK concentrations cause a modest increase in zymogen activation (40). However, unlike suprathysiologic treatment, during physiologic treatment the active enzymes are secreted from the cells (41). One possible role for secreting active enzymes under physiologic conditions may be to coordinate enzyme secretion with fluid secretion in a paracrine fashion, possibly by activating protease-activated receptors such as PAR-2 on duct and acinar cells (42). Thus,  $\text{Ca}^{2+}$  release by RYR may regulate zymogen activation under both physiologic and pathologic conditions.

Although aberrant  $\text{Ca}^{2+}$  signaling is a central feature of acute pancreatitis (43), this work reports a previously undescribed link between RYR and pancreatitis. RYR dysfunction, often caused by RYR mutations, is associated with disordered channel function and

disease. Mutations in RYR1, the major isoform expressed in skeletal muscle, lead to abnormal ( $\text{Ca}^{2+}$ )<sub>i</sub> release and cause malignant hyperthermia and central core disease (44). Notably, dantrolene is used as first-line therapy for malignant hyperthermia (23). Mutations in RYR2, the major isoform expressed in heart, increase channel activity during exercise and are associated with cardiac arrhythmia (45). Polymorphisms in RYR that affect its function could potentially contribute to the pathogenesis of acute pancreatitis. Furthermore, some forms of pancreatitis, such as that caused by ethanol, seem to be initiated by factors that sensitize the pancreas to harmful stimuli (26, 46). Factors that enhance the functioning of RYR might contribute to such sensitization. For example, two that sensitize acinar cells to zymogen activation, increased cellular cAMP (14, 27) or free fatty acids (47), also increase RYR channel opening (48, 49). Further understanding of the factors causing RYR-mediated zymogen activation may help elucidate the mechanisms causing pancreatitis.

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