Low Extracellular pH Induces Damage in the Pancreatic Acinar Cell by Enhancing Calcium Signaling*[S]

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Low extracellular pH (pHe) occurs in a number of clinical conditions and sensitizes to the development of pancreatitis. The mechanisms responsible for this sensitization are unknown. Because abnormal Ca^{2+} signaling underlies many of the early steps in the pathogenesis of pancreatitis, we evaluated the effect of decreasing pHe from 7.4 to 7.0 on Ca^{2+} signals in the acinar cell. Low pHe significantly increased the amplitude of cerulein-induced Ca^{2+} signals. The enhancement in amplitude was localized to the basolateral region of the acinar cell and was reduced by pretreatment with ryanodine receptor (RYR) inhibitors. Because basolateral RYRs also have been implicated in the pathogenesis of pancreatitis, we evaluated the effects of RYR inhibitors on pancreatitis responses in acidic conditions. RYR inhibitors significantly reduced the sensitizing effects of low pHe on zymogen activation and cellular injury. These findings suggest that enhanced RYR-mediated Ca^{2+} signaling in the basolateral region of the acinar cell is responsible for the injurious effects of low pHe on the exocrine pancreas.

Acute pancreatitis is a common, inflammatory disease of the exocrine pancreas, often caused by alcohol or gallstone disease (1). Clinical conditions associated with an acute acid load, including diabetic ketoacidosis (2), propionic acidemia (3), and lactic acidosis (4–7), are associated with an increased risk or severity of pancreatitis. These disease states can be associated with a systemic pH of 7.0 or lower (8). Reducing extracellular pH (pHe)[2] from 7.4 to 7.0 sensitizes acinar cells to pancreatitis responses both *in vitro* and *in vivo* in a cerulein model of pancreatitis (9). Other findings also suggest a relationship between acidic conditions and acute pancreatitis. Tissue damage seen with supramaximal stimulation has been linked to luminal acidification that occurs as a result of protons co-released during enzyme secretion (10). Further, when intracellular pH is increased by the weak base, chloroquine, pathologic intraacinar zymogen activation and acinar cell injury are ameliorated, and survival improves in several pancreatitis models (11, 12). Additionally, pathologic zymogen activation observed in hyperstimulation models of pancreatitis requires the activity of a specific proton pump that acidifies intracellular compartments (13). However, the mechanism responsible for the sensitizing effect of low pH is unknown.

Abnormal Ca^{2+} signaling has been linked to most of the early pathologic acinar cell responses in acute pancreatitis, including premature zymogen activation, inhibition of secretion, and necrosis (14–18). Two distinct Ca^{2+} release channels, the apical inositol 1,4,5-trisphosphate receptor (IP3R) and the basolateral ryanodine receptor (RYR), generate increases in cytosolic Ca^{2+} and have been implicated in both physiologic acinar cell responses and in the pathogenesis of acute pancreatitis (16, 19–22). In the context of pancreatitis, the effects of pathophysiologically relevant decreases in pHe on Ca^{2+} signaling in the acinar cell are unknown. Therefore, we investigated whether the injurious effects of low pHe on the acinar cell are mediated through changes in Ca^{2+} signaling.

MATERIALS AND METHODS

Preparation and Stimulation of Pancreatic Acini—Acini were isolated from rat pancreas as described (23). Briefly, male Sprague-Dawley rats, ~50 g, were killed by carbon dioxide narcosis. Acinar medium contained 10 mM HEPES (pH 7.4), 95 mM NaCl, 4.7 mM KCl, 0.6 mM $MgCl_2$, 1 mM NaH_2PO_4, 10 mM glucose, 2 mM glutamine, plus 0.1% bovine serum albumin, 1 × minimal essential medium amino acids (Invitrogen), and 1.3 mM $CaCl_2$. The pancreas was collected in 15 ml of Ca^{2+}-free acinar medium. The pancreas was then minced in Ca^{2+}-free medium for 5 min and washed three times with Ca^{2+}-free medium. The minced tissue was then placed into a 50-ml flask with 12 ml of acinar medium containing 100–200 units/ml type 4 collagenase (Worthington, Freehold, NJ) for 60 min at 37 °C with shaking (120 rpm). The digest was then washed three times with buffer and manually shaken vigorously to isolate acini (groups of 5–15 acinar cells) for Ca^{2+} signaling experiments.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[2] The abbreviations used are: pHe, extracellular pH; BCECF-AM; 2′,7′)-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescin, acetomethyl ester; IP3R inositol, 1,4,5-trisphosphate receptor; LDH, lactate dehydrogenase; pHi, intracellular pH; RYR, ryanodine receptor.

For zymogen activation and lactate dehydrogenase (LDH) assays, larger acini (20–200 cells) were isolated by filtration through a 300–400-μm mesh (Sefar American, Depew, NY). Acini were recovered for 120 min at 37 °C under constant O_2 with shaking (90 rpm). Medium was changed at 60 min, and pH was adjusted at 105 min. At 120 min, acini were treated with physiologic cerulein (10–100 pM), supramaximal cerulein (100 nM), or carbachol (100 nM) in the presence or absence of 75 μM dantrolene or 100 μM ryanodine. Samples were collected, placed in 1.5-ml centrifuge tubes (USA Scientific, Waltham, MA), and centrifuged for 1 min at 30× g. Fifty μl of the resulting cell-free supernatant was removed to a 0.5-ml microcentrifuge tube to assay for LDH. The remaining 450 μl of cells and medium was retained for zymogen activation assays and determination of total LDH. All samples were stored at −80 °C.

Detection of Cellular Ca²⁺ Signals—Acini were incubated for 30 min with a Ca^{2+}-sensing dye, either fluo-5F/AM or fluo-4/AM (both at 6 μM; Molecular Probes). Acinar cells were then perfused with cerulein (10 pM or 100 nM) or carbachol (0.1 μM) after a 2- min pretreatment with buffer at pH 7.4 or 7.0. A Zeiss LSM 510 NLO confocal microscope (Thornwood, NY) was employed to visualize Ca^{2+} signals, using either a 20 × or 63 × 1.4 numerical aperture. An argon laser was used to excite the dye at 488-nm wavelength, and emission signals >515 nm were collected.

Fluorescence Measurement of Intracellular pH—Pancreatic acinar cells were loaded with the pH-sensitive fluoroprobe BCECF-AM (2 μM) (Molecular Probes) by incubation for 30 min at room temperature and allowed to settle and adhere to a coverslip. The coverslips were mounted in a perfusion chamber on the stage of an inverted microscope (Zeiss Axiovert 100). This was equipped with a video imaging system (Visitron, Munich, Germany). Experiments were performed at room temperature. Cells were then alternately excited at 440 and 490 nm by using a DG-4 filter changer (Sutter Instruments, Novato, CA) every 10 s, and emitted fluorescence was captured at 530 nm by using a Cooke Sensicam (Cooke, Auburn Hills, MI) 12-bit frame transfer digital camera. A ratio of the fluorescence at 490 *versus* 440 nm was computed. Intracellular pH (pHi) was then estimated by using an *in situ* calibration (24), where external pH was changed in the presence of high K^+ and the ionophore nigericin (5 μM). The high K^+ solution used to calibrate ratios into pH values contained 105 mM KCl, 32.8 mM N-methyl-D-glucamine, 1.2 mM $MgCl_2$, 1.0 mM $CaCl_2$, 32.2 mM HEPES, 5 mM mannitol, and 0.005 mM nigericin, and the pH was adjusted to between 6.5 and 7.5. The fluorescence ratios computed for pH solutions over the physiological range of 6.5–7.5 were linear. All data are representative of four or more experimental runs.

Enzymatic Activity Assays—Samples containing 450 μl of cells and medium were thawed on ice, homogenized in a conical 1-ml Eppendorf tube with a pestle, and centrifuged at 1,000 × g for 1 min. From the resulting postnuclear supernatant, 100 μl was added to wells of a 24-well tissue culture plate containing 350 μl of trypsin assay buffer (50 mM Tris (pH 8.1), 150 mM NaCl, 1 mM $CaCl_2$, 0.01% BSA). Finally, 50 μl of 400 μM enzyme substrate (trypsin 3135; Peptides Inter-

national, Louisville, KY, and chymotrypsin; Calbiochem) diluted in trypsin assay buffer (40 μM final) was added to each well. The plate was read by using a fluorometric microtiter plate reader (model HTS 7000; Perkin-Elmer Analytical Instruments, Shelton, CT) using a 380-nm excitation wavelength and 440-nm emission for 20 reads over 10 min. The slope of the line, which represents enzyme activity of the homogenate, was normalized to total amylase activity and expressed as relative fluorescence units per second per microgram of total amylase.

LDH Assay—LDH activity was measured using the commercially available Cytotox 96 nonradioactive cytotoxicity assay kit (Promega). In brief, acinar cells were treated with physiologic (100 pM) or supramaximal (100 nM) cerulein alone or in the presence of either 100 μM ryanodine or 75 μM dantrolene for 2 h at pH 7.4 or 7.0. Samples were collected and centrifuged at 30 × g for 30 s. A 50-μl aliquot of medium was then removed to measure LDH release from the cells. A manufacturer-provided lysis reagent was then added to the remaining 450 μl of cells and medium to determine total LDH. Both cell and medium samples were assayed. The results are expressed as the percent LDH released into the medium.

Statistical Analysis—Data represent means ± S.E. of at least three individual experiments unless otherwise noted, with each experiment being performed in at least duplicate. A Student's t test analysis was used to determine statistical significance and p values of <0.05 were assigned significance.

RESULTS

Low pHe Alters Ca²⁺ Signals—To determine the effects of low pHe, isolated acinar cells were perfused with either pH 7.4 or 7.0 HEPES buffer for 2 min. Consistent with the work of others (25, 26), we found that pHi was directly related to pHe under these conditions (supplemental Fig. S1). To examine Ca^{2+} signals, acini were treated with a physiologic concentration of cerulein (10 pM). Cytosolic Ca^{2+} measurements were obtained once per second by time lapse confocal microscopy (Fig. 1*A*) and normalized to base-line fluorescence for each individual cell (Fig. 1, *B* and *C*). Changing pHe from 7.4 to 7.0 in the absence of cerulein had no effect on base-line Ca^{2+} signals, demonstrating that the Ca^{2+} dye used was unaffected by pH values in this range (data not shown). Low pHe effects on frequency and amplitude occurred over 2 min (data not shown) and were reversible (Fig. 1*H*).

Fig. 1*F* demonstrates that the frequency of cerulein-induced Ca^{2+} oscillations significantly decreased under low pH conditions (1.03 oscillations/min at pH 7.4 compared with 0.68 oscillations/min at pH 7.0; $p < 0.05$). Fig. 1*G* shows that cells treated at pH 7.0 conditions also exhibited higher amplitude Ca^{2+} signals than those treated at pH 7.4 (512% base line *versus* 352% base line, respectively; $p < 0.05$). Similar effects on amplitude and frequency were seen when the pHe was decreased further to 6.8. There were no significant changes in Ca^{2+} signals when the pH was increased from 7.4 to 7.6 (supplemental Fig. S2). To ensure that these responses were not limited to Ca^{2+} signals induced by a specific G protein-coupled receptor, the effects of low pHe on muscarinic agonist (carbachol)-induced Ca^{2+} oscillations were also examined
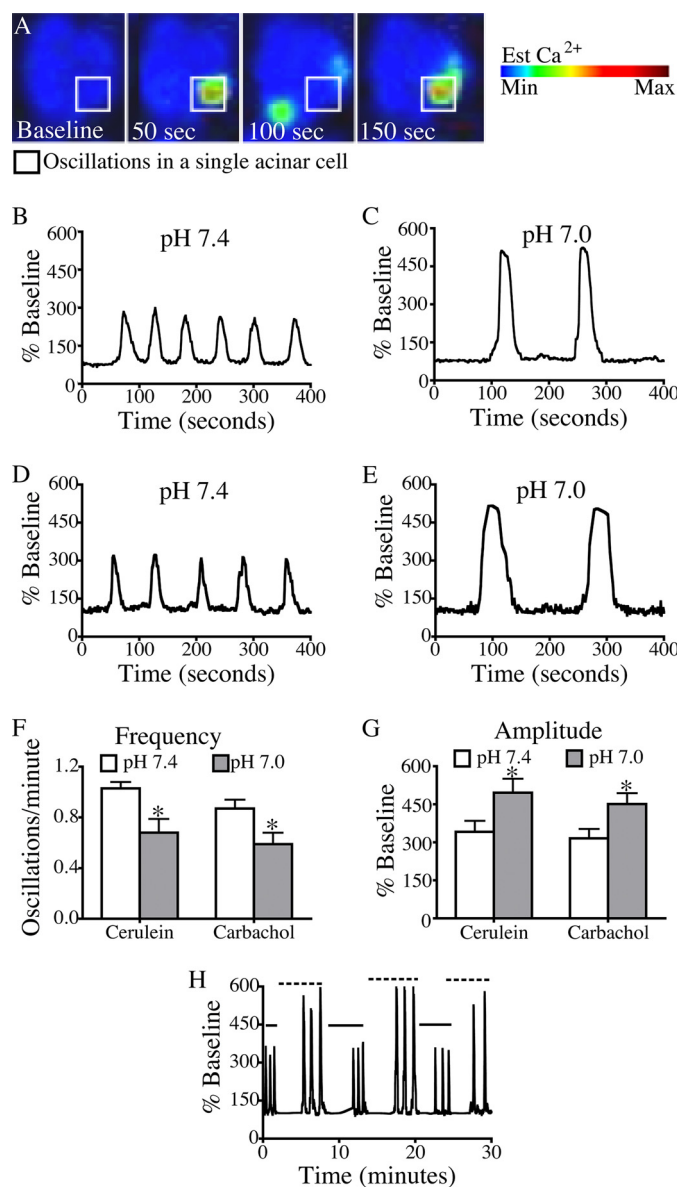
FIGURE 1. **Reducing pHe from 7. 4 to 7.0 is associated with a reversible decrease in the frequency and an increase in the amplitude of Ca²⁺ oscillations.** *A*, changes in whole cell cytosolic Ca²⁺ were measured once per second by time lapse confocal microscopy using the Ca²⁺ dye fluo-4/AM. *B–E*, representative plots of fluorescence over time were recorded from a single cell treated with 10 pM cerulein at pH 7.4 (*B*) and 7.0 (*C*) and 100 nM carbachol at pH 7.4 (*D*) and pH 7.0 (*E*). *F* and *G*, the pH-dependent changes in frequency (*F*) and amplitude (*G*) were quantified in cells treated with 10 pM cerulein or 100 nM carbachol. *H*, the pH-dependent changes in frequency and amplitude were reversible when pH was alternated between pH 7.4 (*solid line*) and pH 7.0 (*dashed line*). The percentage of cells responding per treatment condition was 70% for cerulein (pH 7.4), 73% for cerulein (pH 7.0), 63% for carbachol (pH 7.4), and 57% for carbachol (pH 7.0). There were 30 cells/treatment group; mean ± S.E. (*error bars*) for three separate experiments is shown. *, *p* < 0.05 *versus* pH 7.4.

(Fig. 1, *D* and *E*). A similar pattern of decreased oscillation frequency and increased amplitude was observed (Fig. 1, *F* and *G*) with 100 nM carbachol at pH 7.0.

Because low pHe also enhances zymogen activation and cellular injury induced by supramaximal secretagogue stimulation (9), the effects of low pH on calcium signals induced by supraphysiologic (100 nM) cerulein were also examined. With high dose cerulein stimulation at pH 7.4 and 7.0, a peak pla-
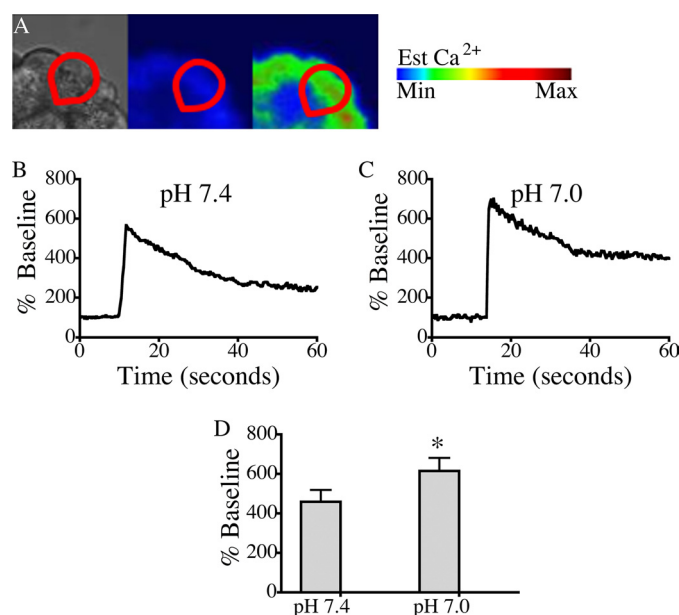

FIGURE 2. **Reducing pHe from 7. 4 to 7.0 is associated with an enhancement in Ca²⁺ signal amplitude in acini treated with supramaximal cerulein.** *A*, changes in whole cell (*red outline*) cytosolic Ca²⁺ were measured by time lapse confocal microscopy using the Ca²⁺ dye fluo-5F/AM. *B* and *C*, representative plots of fluorescence over time were recorded from a single cell treated with 100 nM cerulein at pH 7.4 (*B*) and 7.0 (*C*). *D*, the pH-dependent changes in amplitude were quantified in three separate experiments. The percentage of cells responding per treatment condition was: 94% for cerulein (pH 7.4) and 88% for cerulein (pH 7.0). There were 18 cells/treatment group; mean ± S.E. (*error bars*) is shown. *, *p* < 0.05 *versus* pH 7.4.

teau Ca²⁺ signal was observed (Fig. 2, *A–C*). Fig. 2*D* demonstrates that at pH 7.0, acinar cells responded with significantly higher peak amplitude increasing from 459% base line to 615% base line (*p* < 0.05). These findings establish that lowering pHe significantly enhances agonist-induced Ca²⁺ signals in pancreatic acinar cells.

Low pHe Modulates RYR-mediated Ca²⁺ Release—Several studies were performed to determine the source of Ca²⁺ responsible for the effects of lowering pHe. To determine the role of influx of extracellular Ca²⁺, cells were perfused with Ca²⁺-free medium containing 0.5 mM EGTA (Fig. 3). This medium did not alter the signaling responses to low pH, suggesting that these effects are mediated by intracellular Ca²⁺ channels rather than Ca²⁺ entry. To determine which intracellular Ca²⁺ release channel is responsible for the changes in Ca²⁺ signals seen at low pHe, cells were treated with inhibitors of IP3R and RYR, the two principal channels in acinar cells (21, 27, 28). When cells were treated with the IP3R antagonist, 2-aminoethoxydiphenyl borate (100 μM), Ca²⁺ oscillations at either pH 7.4 or 7.0 ceased (supplemental Fig. S3). These findings are consistent with previous observations that Ca²⁺ signals in the acinar cell are initiated by Ca²⁺ release from IP3Rs (29, 30), which are concentrated in the apical region of the cell (20, 31, 32). Fig. 4 shows that when cells were pretreated with the RYR inhibitors, dantrolene (75 μM) and ryanodine (100 μM), there was a significant reduction in oscillation amplitude, but no change in oscillation frequency. These data suggest that Ca²⁺ signals at pHe 7.4 or 7.0 require activation of IP3R, but the effects of low pHe on oscillation amplitude are mediated by RYR.

Low pHe Enhances Calcium Signaling in the Acinar Cell

To confirm that the effects of low pHe are mediated by the basolaterally localized RYR, subcellular changes in cytosolic Ca^{2+} were measured. Work by others in acinar cells suggests that low affinity dyes are more sensitive in characterizing subcellular differences in cytosolic Ca^{2+} (33). Therefore, the low affinity Ca^{2+} dye, fluo-5 ($K_d = 2.3\ \mu M$) was used to determine whether low pHe enhances Ca^{2+} signals in a particular subcellular region. Fig. 5 shows that there was a significant increase in the amplitude of basolateral Ca^{2+} signals when pHe was reduced from 7.4 to 7.0 ($p < 0.05$), but there was no sig-
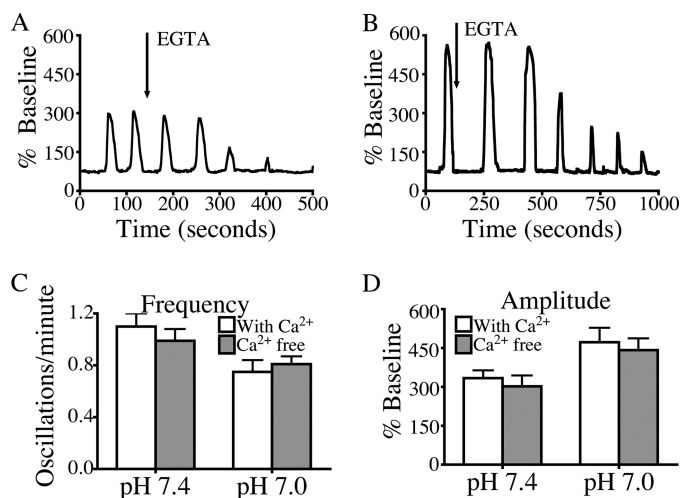
nificant difference in apical Ca^{2+} signaling. The increase in basolateral Ca^{2+} at low pHe was abrogated with dantrolene pretreatment ($p < 0.05$). The previously identified role of RYR in propagating Ca^{2+} waves from the apical to basolateral subcellular regions (22, 34) was not evaluated in this study.

The role of RYR in mediating the effects of low pH in acini treated with supramaximal cerulein was also evaluated. As previously reported, supramaximal agonist stimulation enhances basolateral Ca^{2+} signaling via RYR (21). Fig. 6 shows that low pH further enhances basolateral Ca^{2+} signal amplitude. The enhancement in basolateral Ca^{2+} signaling at pH 7.0 was significantly reduced by dantrolene pretreatment. In acini treated with dantrolene, there was no significant difference between basolateral signal amplitude at pH 7.4 and 7.0. These results further support the hypothesis that low pHe enhances Ca^{2+} signaling through the RYR.

Low pHe Promotes Pancreatitis Responses through Its Effects on the RYR—Low pHe sensitizes the acinar cell to key measures of pancreatitis *in vitro*: zymogen activation and cellular injury (9). Given our observation that low pHe effects on Ca^{2+} signaling are mediated by RYR, we hypothesized that the sensitizing effects of low pHe on pancreatitis responses would be decreased by pretreatment with RYR inhibitors, dantrolene and ryanodine. This was tested in three ways: 1) by measuring premature intracellular activation of zymogens (35), 2) by determining LDH release as a marker of loss of cell integrity (23), and 3) by examining morphological changes associated with acinar cell damage (36). Fig. 7 shows that the sensitizing effects of acid on trypsin and chymotrypsin activation are significantly reduced by pretreatment with RYR inhibitors ($p < 0.05$).

Fig. 8*A* shows that sensitizing effects of acid on cellular injury are also ameliorated by RYR inhibitors, as demonstrated by a decrease in LDH release in cells treated with 100 pM cerulein at pH 7.0 from 10.2% in control cells to 5.2% and 6.0% in cells pretreated with dantrolene and ryanodine, respectively

FIGURE 3. **Calcium-free medium does not alter low pH effects.** *A* and *B*, representative plots of fluorescence over time are recorded from a single cell treated with 10 pM cerulein at pH 7.4 (*A*) and 7.0 (*B*), with a change to Ca^{2+}-free medium containing 0.5 mmol EGTA denoted by an *arrow*. *C* and *D*, frequency and amplitude of the first two oscillations after the change to Ca^{2+} free medium are quantified and were not significantly different from controls treated with Ca^{2+}-containing buffer. Given that oscillations diminish as intracellular Ca^{2+} is depleted, only the first two oscillations after the change to Ca^{2+} free medium were quantified in three separate experiments. The percentage of cells responding per treatment condition was 64% for cerulein (pH 7.4) and 67% for cerulein (pH 7.0) for cells treated with Ca^{2+}-free medium. There were 30 cells/treatment group; mean ± S.E. (*error bars*) is shown. *, $p < 0.05$.
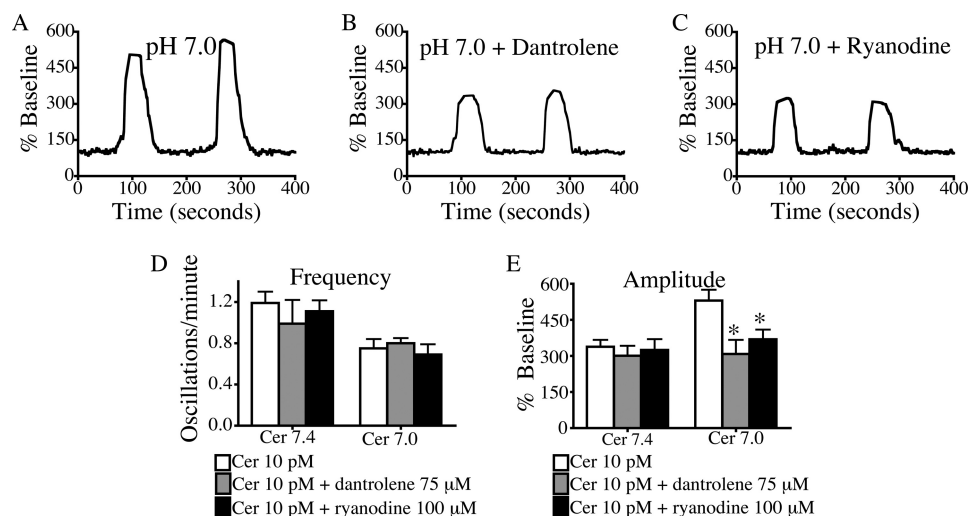

FIGURE 4. **RYR inhibitors reduce low pH effects on amplitude but do not affect frequency.** Cells were pretreated for 30 min with 75 μM dantrolene or 100 μM ryanodine and then perfused with 10 pM cerulein (*Cer*). *A–C*, representative plots of fluorescence over time from a single cell treated with 10 pM cerulein at pH 7.0 (*A*) and with 10 pM cerulein at pH 7.0 in the presence of dantrolene (*B*) and ryanodine (*C*) are shown. *D* and *E*, changes in (*D*) oscillation frequency and (*E*) amplitude of Ca^{2+} signals were quantified in three separate experiments under each condition. The percentage of cells responding per treatment condition was 67% for cells treated with dantrolene and 73% for cells treated with ryanodine. There were 30 cells/treatment group; mean ± S.E. (*error bars*) is shown. *, $p < 0.05$ *versus* 10 pM cerulein.
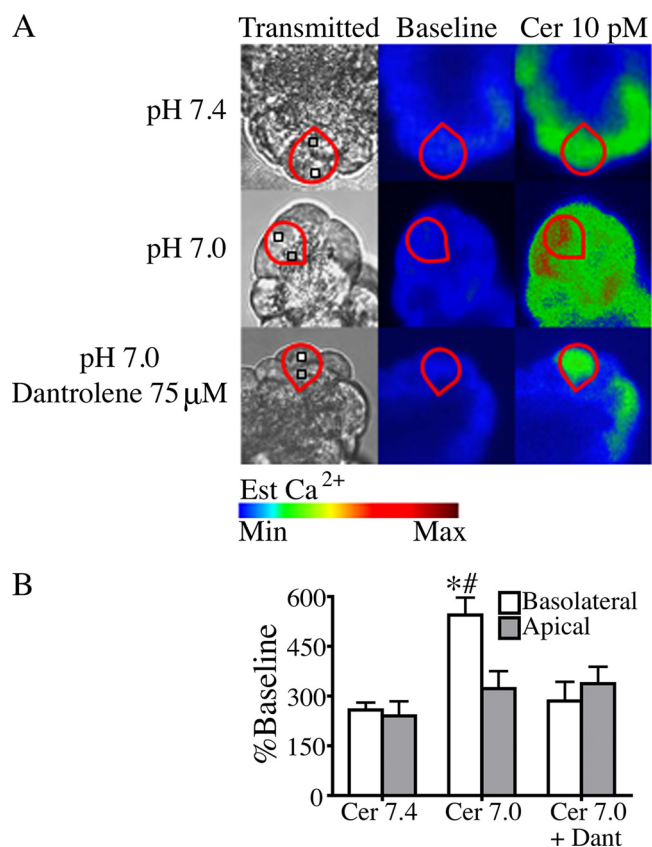
FIGURE 5. **Low pHe selectively enhances basolateral Ca^{2+} signals in cells treated with 10 pM cerulein.** *A*, cerulein (*Cer*)-induced (10 pM) changes in subcellular cytosolic Ca^{2+} at pH 7.4 and 7.0 were measured using the low affinity Ca^{2+} dye fluo-5F/AM and observed by time lapse confocal microscopy. Regions of interest in the basolateral (*white box*) and apical (*gray box*) region were analyzed for each individual cell (*red outline*). *B*, a significant increase in basolateral Ca^{2+} signaling was seen at pH 7.0 but was inhibited by pretreatment with 75 μM dantrolene (*Dant*). There were no significant changes in apical Ca^{2+} signals (three separate experiments with 18 cells/treatment group; mean $\pm$ S.E. (*error bars*); *, $p < 0.05$ *versus* cerulein 7.4; #, $p < 0.05$ *versus* cerulein 7.0 + dantrolene).

($p < 0.05$). RYR inhibitors had no effect on zymogen activation or LDH at pH 7.4 with 10 pM cerulein (data not shown). To evaluate the effects of RYR inhibition on cellular injury further, morphological indicators of injury were examined (Fig. 8*C*). First, the appearance of large cytoplasmic vacuoles, a hallmark of acute pancreatitis, was evaluated. Whereas untreated control cells or those exposed to physiological concentrations of cerulein at pH 7.4 did not exhibit this feature, cells treated with low dose cerulein at pH 7.0 demonstrated prominent vacuoles. This effect was reduced significantly by pretreatment with RYR inhibitors. Blebbing of the basolateral membranes, a characteristic of injury in isolated acini, similarly was seen in cells treated with 100 pM cerulein at pH 7.0, and this marker of injury was also eliminated by RYR inhibitors. Cellular injury in acini treated with supramaximal cerulein was assessed by examining LDH release (Fig. 8*B*). Pretreatment with dantrolene reduced LDH release in cells treated with 100 nM cerulein at pH 7.0 from 16.9% to 6.6% ($p < 0.05$). Taken together, these complementary measures of acinar cell injury provide further support that RYR mediates the injurious effects of cerulein combined with low pHe on the acinar cell.
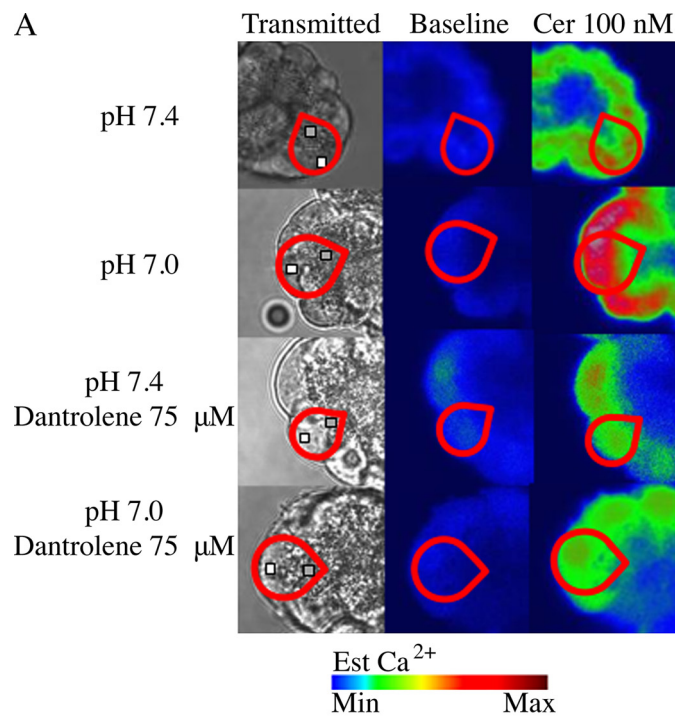
FIGURE 6. **Low pHe selectively enhances basolateral Ca^{2+} signals in cells treated with 100 nM cerulein.** *A*, cerulein (*Cer*)-induced (10 pM) changes in subcellular cytosolic Ca^{2+} at pH 7.4 and 7.0 were measured using the low affinity Ca^{2+} dye fluo-5F/AM and observed by time lapse confocal microscopy. Regions of interest in the basolateral (*white box*) and apical (*gray box*) region were analyzed for each individual cell (*red outline*). *B*, supramaximal cerulein at pH 7.4 increases basolateral Ca^{2+} signaling. A further increase in basolateral Ca^{2+} signaling was seen at pH 7.0. Basolateral Ca^{2+} signals at pH 7.4 and 7.0 were inhibited by pretreatment with 75 μM dantrolene (*Dant*). There were no significant changes in apical Ca^{2+} signals between treatments. The percentage of cells responding per treatment condition was 94% for cerulein (pH 7.4) and 100% for cerulein (pH 7.0), 88% for cerulein with dantrolene (pH 7.4), and 94% for cerulein with dantrolene (pH 7.0). There were three separate experiments with 18 cells/treatment group; mean $\pm$ S.E. (*error bars*) for three separate experiments; *, $p < 0.05$ *versus* cerulein (pH 7.4); #, $p < 0.05$ *versus* cerulein (pH 7.0) + dantrolene.

DISCUSSION

A number of studies have provided evidence that the early events in pancreatitis are mediated by pathologic Ca^{2+} signaling (14–18). In other nonexcitable cell types, low pHe has been shown to enhance Ca^{2+} signals (37). However, the injurious effects of low pHe on the acinar cell have not previously been linked to abnormal Ca^{2+} signaling patterns. Our data show that a pathophysiologically relevant decrease in pHe from 7.4 to 7.0 enhances the amplitude of Ca^{2+} signals. Furthermore, when the effects of low pHe on Ca^{2+} signal amplitude are inhibited by RYR antagonists, the sensitizing effects of low pHe on intracellular zymogen activation and cellular injury are eliminated.
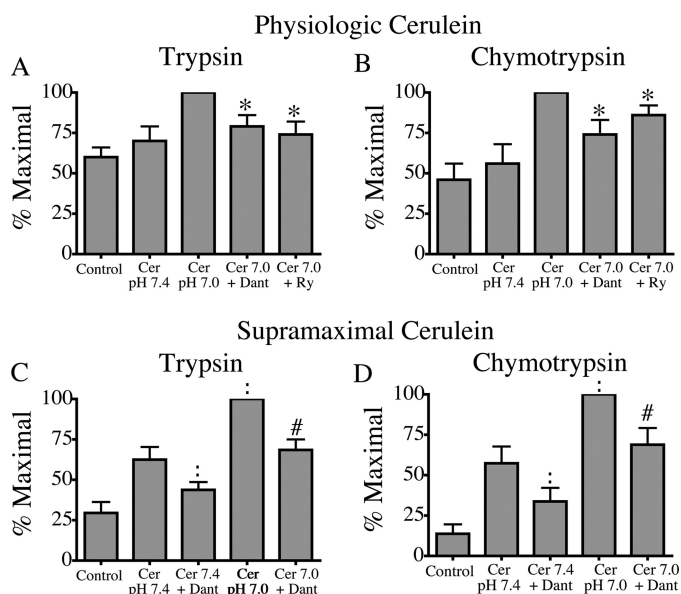
FIGURE 7. **RYR inhibitors reduce the sensitizing effects of low pHe on premature zymogen activation.** Acini were incubated with or without 75 μM dantrolene (*Dant*) or 100 μM ryanodine (*Ry*) in the presence of physiologic (100 pM) and supramaximal (100 nM) cerulein (*Cer*). Samples were assayed for trypsin (*A* and *C*) and chymotrypsin (*B* and *D*) activity. Results of three separate experiments are expressed as a percentage of the treatment that produced maximum zymogen activation (mean $\pm$ S.E.; *, $p < 0.05$ *versus* 100 pM cerulein at pH 7.0; :, $p < 0.05$ for 100 nM at pH 7.4; #, $p < 0.05$ for 100 nM cerulein at pH 7.0).

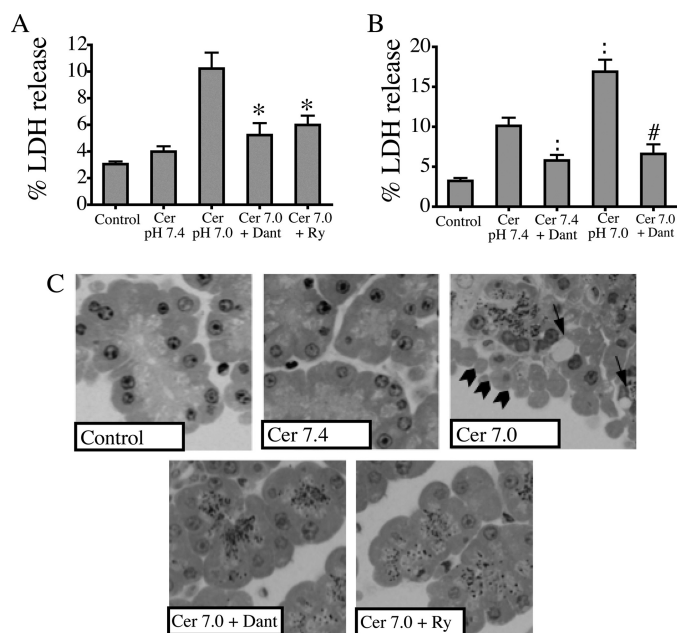

FIGURE 8. **RYR inhibitors ameliorate the injurious effects of low pHe.** Acini were incubated with or without 75 μM dantrolene (*Dant*) or 100 μM ryanodine (*Ry*) in the presence of 100 pM or 100 nM cerulein (*Cer*) for 2 h. *A* and *B*, samples were assayed for LDH release. %LDH release was quantified in three separate experiments. *C*, morphological indicators demonstrate that RYR antagonists ameliorate injury caused by low pHe in these representative photographs showing basolateral blebbing (*arrowheads*) and vacuolization (*arrows*) (mean $\pm$ S.E. (*error bars*); *, $p < 0.05$ *versus* 100 pM cerulein at pH 7.0; :, $p < 0.05$ for 100 nM cerulein at pH 7.4; #, $p < 0.05$ for 100 nM cerulein at pH 7.0).

One possible explanation for the observed effects of low pHe on Ca^{2+} signaling in the acinar cell is that acidic conditions change ligand binding properties. For cholecystokinin

receptors, the optimum pH for ligand binding is slightly acidic at 6.0–7.0 (38). In contrast, ligand binding to muscarinic receptors is very modestly inhibited at pH 7.0, compared with pH 7.4 (39, 40). Given that ligand binding to cholecystokinin and muscarinic receptors is affected in opposite ways by low pH, the consistent effects of low pH on carbachol- and cerulein-induced oscillations suggest that the effects of low pH are not mediated by changes in ligand binding.

A key and previously undescribed finding of this study is that the effects of low pHe are mediated through RYR. Our study identifies RYR as the mediator of low pHe-related Ca^{2+} release in two ways: 1) using RYR inhibitors and 2) selectively evaluating basolateral Ca^{2+} signals. RYR is concentrated in the basolateral region of the acinar cell (21, 41, 42). This subcellular localization overlaps with the supranuclear vesicular compartment where initial zymogen activation occurs, and RYR-mediated Ca^{2+} release has been implicated in the pathogenesis of acute pancreatitis *in vitro* and *in vivo* (21, 43–45). Our data provide the first evidence that low pH can sensitize to RYR-mediated Ca^{2+} release and injury under conditions of physiologic and supramaximal agonist stimulation. Further, because RYR inhibitors had no effect on the frequency or amplitude of Ca^{2+} signals produced by low dose cerulein at pH 7.4, this study also suggests that RYR-mediated Ca^{2+} release is not essential for the production of physiologic Ca^{2+} oscillations.

Work in other cell types has shown that low pHe can sensitize to RYR-mediated Ca^{2+} release (46), but the mechanism underlying this effect is unknown. Single channel studies have shown that RYR open probability is modestly inhibited by low pH (47), which suggests that the effects of low pHe on RYR-sensitive Ca^{2+} stores are indirect and not the result of receptor protonation. RYR activity is known to be affected by post-translational modifications, including phosphorylation (48–54), oxidation (55–57), and nitrosylation (58–60). Furthermore, in addition to kinases and phosphatases, RYR interacts with a number of accessory proteins, most notably calmodulin (61, 62), which could be a direct target of low pH. Determining which, if any, of these mechanisms is responsible for the effects of low pH on RYR-mediated Ca^{2+} release will require further study.

Our study demonstrates that low pHe also has a RYR-independent effect on the frequency of Ca^{2+} oscillations. The slowed frequency of oscillations may be caused by an effect on the IP3R. IP3 binding to IP3R and the rate of IP3-induced Ca^{2+} release are inhibited by acidic pH in several cell types (63–65). The pacemaker function that initiates and propagates acinar cell Ca^{2+} signals is IP3R-dependent as well (29, 66). Patch clamp studies have shown that low pH can affect IP3R spike duration (10). In our study, IP3R inhibition caused cessation of all Ca^{2+} signaling at both pH 7.4 and pH 7.0, making it difficult to conclude whether low pHe may be having a subtle effect on pacemaker function. However, it appears that the effects of low pHe on signal amplitude, rather than the effects on signal frequency, are responsible for the enhanced zymogen activation and cellular injury seen at pHe 7.0.

Other studies have implicated additional mechanisms to explain the effects of low pH on the acinar cell. In a model of

ERCP pancreatitis, low pH contrast injected into the pancreatic duct mediated injurious effects through the pH-sensitive cation channel TRPV1 (67). However, this model of pancreatitis based on elevated intraductal pressure may not be relevant to systemic acidosis. Furthermore, the most pronounced TRPV1-mediated effects were seen at pH 6.0, which is not a clinically relevant systemic pH. Unlike our study, which found no relationship between low pHe and Ca^{2+} entry, a previous study showed that decreasing pHe inhibited Ca^{2+} uptake into the cell (68). However, a significantly lower pHe of 6.4 was used in those experiments, which may explain the discrepancy. Several studies have shown that low pHi, achieved through the application of weak acids, can enhance acinar cell Ca^{2+} signals through effects on intracellular stores (69–71). However, none of these studies examined the effects of low pHe, and the magnitude of pH changes often was not quantified, making it difficult to extrapolate such findings to the sensitizing effects of acidosis *in vivo*. Furthermore, the identity of putative pH-sensitive intracellular Ca^{2+} stores has not previously been identified.

In summary, the current work provides evidence that decreasing pHe significantly increases the amplitude of secretagogue-dependent Ca^{2+} signaling in the acinar cell. The enhancement in amplitude is mediated by RYR and is responsible for the enhanced pancreatitis responses including zymogen activation and cellular injury seen with low pHe. Further work will be needed to determine whether the risk of pancreatitis can be ameliorated by pharmacologic inhibition of RYR in patients with clinical conditions that are associated with acidosis.

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