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Calpain activation contributes to oxidative stress-induced pancreatic acinar cell injury

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Abstract

Oxygen radicals have been implicated as mediators in the pathogenesis of pancreatic acinar cell necrosis. However, the sequence of events between the oxidative insult and cell damage remains unclear. In the current study, we investigated whether the Ca^{2+}-regulated cytosolic cysteine protease calpain is activated by oxidative stress and contributes to oxidant-induced acinar cell damage. Isolated rat pancreatic acinar cells were exposed to hydrogen peroxide (H_2O_2)-generated oxidative stress in the presence or absence of the Ca^{2+} chelator 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM) and different calpain inhibitors including benzyloxycarbonyl-valyl-phenylalanine methyl ester. Calpain activation was studied by fluorescence spectrophotometry and immunoblotting. Cell injury was assessed by lactate dehydrogenase (LDH) release and characterization of the cellular ultrastructure including fluorescence-labeled actin filaments. Exposure of acinar cells to H_2O_2 provoked a time- and dose-dependent increase in calpain proteolytic activity involving the ubiquitous isoforms μ- and m-calpain. The activation of calpain reflected the time course of developing cytotoxicity as demonstrated by increased LDH release. Inhibition of oxidant-induced calpain activity by BAPTA-AM and various calpain inhibitors provoked a decline in oxidant-induced cell injury. In particular, changes in the actin filament organization characterized by an increase in the basolateral actin and by a detachment of actin from the cell membrane in the region of membrane blebs were clearly reduced. In summary, our findings suggest that acinar cell damage through oxidative stress requires activation of calpain and that the actin cytoskeleton belongs to the cellular targets of the protease. The results support the hypothesis that calpain activation may play a role in the development of acute pancreatitis.

Keywords: Calpain; Calcium-mediated cell damage; Actin cytoskeleton; Phospholipase A_2; Necrosis

1. Introduction

Reactive oxygen species are regularly generated during oxidative cellular metabolism and are eliminated by a system of enzymatic and non-enzymatic antioxidants. Accumulation of oxygen species (oxidative stress) caused by increased formation and/or insufficient antioxidant defense mechanisms leads to cell injury and death [1]. Various experimental and clinical studies suggest that oxidative stress may play a crucial role in the pathogenesis of acute pancreatitis, but the sequence of events between the oxidative insult and pancreatic acinar cell damage remains to be defined [2,3]. Previously, our working group and others have found that pancreatic acinar cells respond to oxidative stress with a disturbance of intracellular Ca^{2+} homeostasis leading to a sustained increase in cytosolic Ca^{2+} concentration that precedes morphological and functional damage [4–6]. One consequence of altered Ca^{2+} homeostasis may be the activation of calpains, a family of Ca^{2+}-dependent neutral cysteine proteases. Apart from several tissue-specific isoforms (n-calpains), two well-defined ubiquitous isozymes, termed μ-calpain (calpain 1) and m-calpain (calpain 2), have been described, that differ in their Ca^{2+} requirement for in vitro activation [7]. The relative proportion of μ- and m-calpain in different cells varies widely. Thus, in some cells both forms are found, whereas others may express only one form [8]. In rat

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pancreatic acinar cells, we recently identified both calpain isoforms [9]. The ubiquitous calpains are heterodimers, composed of one large 80 kDa catalytic subunit and one small 30 kDa regulatory subunit [7]. The large subunits have different amino acid sequences and are encoded by different genes, whereas the small subunits are common to both isozymes [10,11]. The activation of calpains involves Ca^{2+}-induced conformational change and autolysis of the NH_2 terminus of both subunits. During this conversion, the 80 kDa subunit is processed to an active 76 kDa form through a 78 kDa intermediate, which increases the Ca^{2+} sensitivity of the proteases. The autolysis of the small subunit produces an 18 kDa fragment and does not affect the Ca^{2+} requirement [12,13]. Following this initial autolysis and in the continued presence of Ca^{2+}, further degradation occurs, mainly of the large subunit that abolishes calpain activity later on [14–16]. In addition, calpains are also regulated by various phospholipid degradation products lowering its Ca^{2+} requirement for autolysis, and by their natural inhibitor protein named calpastatin [7,17].

Calpain has been proposed to act in signal transduction processes by modifying a wide variety of proteins including cytoskeletal proteins, membrane proteins, enzymes and transcription factors [18]. The ability of calpain to cleave a growing number of substrates suggests a potentially important role for this protease in the regulation of cell injury and death. We previously showed that calpain activated by Ca^{2+} ionophore ionomycin affects the plasma membrane of rat pancreatic acinar cells [19]. There is also evidence for a role of calpain in the mechanism of cell damage induced by oxidative stress in hepatocytes and neuronal cells [20–23] and in the pathogenesis of diseases attributed to oxidative stress, including ischemic injury and Alzheimer's disease [24,25]. However, whether calpain may also be an important mediator of oxidant damage to pancreatic acinar cells have not yet been evaluated to date. This subject, therefore, was examined in the current study.

We demonstrate that both μ- and m-calpain are activated by oxidative stress and that inhibition of calpain activity clearly reduces oxidant-induced ultrastructural alterations, including changes in the actin cytoskeleton. Our data suggest that calpain activation may be one of the mechanisms underlying oxidative stress-induced damage to pancreatic acinar cells.

2. Material and methods

2.1. Antibodies

For immunoblotting, rabbit polyclonal antibodies that react to the propeptide region (domain I) of μ-calpain (pAB3) and m-calpain (pAB4), respectively, were purchased from Sigma–Aldrich (Deisenhofen, Germany). Another rabbit polyclonal antibody against domain III of m-calpain (pAB2) was from Calbiochem-Novabiochem (San Diego, CA). Testing these antibodies in preliminary experiments with purified porcine μ- and m-calpain, we found that they react specifically with the corresponding form of calpain and not with the other (data not shown). We also found that anti-μ-calpain antibody pAB3 and anti-m-calpain antibody pAB4 detect the corresponding 80 kDa subunit, but are not able to react with calpain autolysis products including the 76 kDa fragment [9]. The anti-m-calpain antibody pAB2 not only recognizes the autolysis of the latent 80 kDa subunit but also different cleavage products corresponding to apparent molecular weights of 55, 50 and 40 kDa [9]. However, using antibody pAB2 in the present study, we only observed non-specific bands in the molecular weight regions where we had found these fragments when analyzing pancreatic homogenates [9].

2.2. Reagents

μ-Calpain (porcine erythrocytes), m-calpain (porcine kidney), benzyloxycarbonyl-leucyl-norleucinal (calpeptin, Calp) and lactacystin (Lact) were purchased from Calbiochem-Novabiochem. SDS, PVDF membranes and Precision Plus Protein Standard were obtained from Bio-Rad (Munich, Germany). All cell culture material was from GIBCO Laboratories (Eggenstein, Germany). Fluphenazine, 7-amido-4-methylcoumarin (AMC), benzyloxycarbonyl-valyl-phenylalanine methyl ester (ZVF), 3-aminopropyltriethoxysilane and 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pefabloc) were purchased from Sigma–Aldrich. 1,2-Bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM) was from BIOMOL (Hamburg, Germany). Succinyl-leucyl-leucyl-valyl-tyrosyl-7-amido-4-methylcoumarin (SucLLVY-AMC) and benzyloxycarbonyl-leucyl-leucyl-tyrosyl-diazomethyl ketone (ZLLY) were purchased from BACHEM (Heidelberg, Germany). Quinine sulfate was from Serva (Heidelberg, Germany). Bodipy FL phallacidine was obtained from Molecular Probes (Eugene, OR). Most other chemicals used were from Sigma.

2.3. Pancreatic acinar cell preparation

Acinar cells were isolated from pancreata of 18 h starved female rats of an inbred Lewis strain (LEW 1W, Institute of Pathology, University of Rostock, Rostock, Germany; 150–180 g body weight) according to a standard procedure using collagenase A (Roche, Mannheim, Germany). Finally, the cells were suspended in Krebs–Ringer buffer (KRB) (pH 7.4, 37 °C) [26].

2.4. Exposure of rat pancreatic acinar cells to H_2O_2

All investigations were carried out between 8 and 12 a.m. to avoid any potential circadian effects. Aliquots of 10^6 cells/ml KRB were incubated for 30 min with 20 μM BAPTA-AM, 12.5 μM fluphenazine, 10 μM lactacystin or

500 μM Pefabloc or for 60 min with 100 μM ZLLY, 100 μM ZVF or 50 μM calpeptin. BAPTA, ZLLY, ZVF and calpeptin were dissolved in DMSO (final DMSO concentration, 1%). Thereafter, the cells were exposed to H_2O_2 to give final concentrations of 0.5 and 1 mM. Other aliquots of 10^6 cells/ml were incubated with vehicle in the presence or absence of H_2O_2. Samples were taken at 0, 30, 60, 90 and 120 min after starting the experiments by H_2O_2 application.

2.5. Analysis of calpains by western blotting

Cells were pelleted and washed twice with ice cold PBS. The pellet was lysed in buffer containing 50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 25 mM NaF, 25 mM β-glycerophosphate, 2 mM Pefabloc, 0.011 mM Leupeptin, 0.2% Triton X-100 and 0.3% NP40, kept on ice for 10 min and stored at −80 °C until use. For immunoblotting, samples were defrosted on ice, sonified and centrifuged for 20 min at 4 °C. The protein concentration in the supernatant was determined using the Advanced Protein Assay (Cytoskeleton, Denver, CO, USA). Thereafter, 20 μg of protein were mixed with sample buffer Roti-Load 1 (Carl Roth, Karlsruhe, Germany) according to the manufacturer's suggested protocol, heated for 4 min at 95 °C and subjected to Laemmli SDS-PAGE using 10% Tris–glycine minigels. The fractionated proteins were either stained with Coomassie blue to confirm the uniformity of protein loading or electrophoretically transferred onto PVDF membranes using Towbins buffer (125 mM Tris, 95 mM glycine, 0.02% SDS, 20% methanol). Membranes were blocked in PBS-T containing 5% BSA and 5% fatfree milk powder for 5 h at room temperature and probed with the respective anti-calpain antibody (dilution 1:5000) overnight at 4 °C. Labeled proteins were visualized by enhanced chemiluminescence, following the manufacturer's suggested protocol, using horseradish peroxidase-conjugated secondary antibody supplied with the kit at a 1:25,000 dilution for 60 min (Amersham, Freiburg, Germany). Densitometric analyses of immunoblots were performed using an electronic camera and the EASY program (Herolab, Wiesloch, Germany). To adjust for differences in protein loading and western transfer efficiency, the efficiency of batches of antibodies used, and the time of exposure, appropriate control samples were included in each gel.

2.6. Measurement of calpain activity in situ

Calpain activity was determined in intact acinar cells by measuring the release of the fluorescent product AMC from the cell-permeable protease substrate SucLLVY-AMC according to a modified method of Bronk and Gores [27] as we have previously described [19]. Briefly, 2×10^6 cells suspended in 1 ml KRH buffer containing 115 mM NaCl, 1 mM KH_2PO_4, 2 mM $CaCl_2$, 5 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na-HEPES and 0.2% bovine serum albumin (pH 7.4) were incubated with 50 μM SucLLVY-AMC. After 30 min incubation at 37 °C, the reaction was terminated by addition of 0.4N HCl. The concentration of AMC correlating with protease activity was measured using the spectrophotometer Specord M 40 (Carl Zeiss Jena, Jena, Germany; 360 nm excitation and 363 nm emission filter). Standard curves were generated with AMC. Calpain-like activity was expressed as pmol AMC released per minute of incubation time per 10^6 cells.

For measuring the calpain activity in BAPTA-AM-preloaded cells, the incubation with substrate was performed in Ca^{2+} free KRH buffer to prevent further Ca^{2+} influx.

2.7. Determination of cell membrane damage

Cell damage was assessed by calculating the ratio of lactate dehydrogenase (LDH) activity in the supernatant to total activity containing in the lysed cell pellet and in the supernatant using LDH test kit and Synchron LX 20 analyzer (Beckman Coulter, Krefeld, Germany). To separate the supernatant from cells, a sample of cell suspension was centrifuged through a 2:1 mixture (v/v) of dibutyl phthalate and bis (3,5,5-trimethylhexyl) phthalate. Total LDH activity was determined after lysis of cells in buffer containing 130 mM Tris, 75 mM NaCl, 10 mM $CaCl_2$ and 0.2% Triton X-100, pH 8.0.

2.8. Electron-microscopic evaluation

For electron-microscopic investigations, cells were fixed for 1 h in 4% glutaraldehyde containing 0.1 M phosphate buffer, washed in the same buffer and postfixed in 1% osmium tetroxide. Thereafter, the cells were embedded in 0.1% liquid agar. Small cubes of the agar were dehydrated in a graded series of ethanolic solutions and embedded in Durcupan ACM. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and examined with an electron microscope EM 902 A (Carl Zeiss, Oberkochen, Germany).

2.9. Localization of F-actin

F-actin staining was performed as we have recently described [28]. Briefly, acinar cells were fixed for 30 min with 4% paraformaldehyde dissolved in HEPES buffer (in mM: 200 HEPES, 10 EGTA, 4 $MgCl_2$, pH 7.7). The fixed cells were incubated with 50% acetone and 1% BSA each for 10 min. Thereafter, they were treated for 5 min with 1% Triton X-100 in imidazole buffer (in mM: 20 imidazole, 2 $MgCl_2$, 80 KCl) containing 2 mM EGTA and for another 5 min with imidazole buffer containing 1 mM EGTA. The last two steps were performed on ice. F-actin was stained with Bodipy FL phallacidine (5 U/ml) for

20 min in the dark following the manufacturer's suggested protocol. The cells were washed four times with PBS and once with water, centrifuged on 3-aminopropyltriethoxysilane-coated coverslips and embedded in fluorescence mounting medium (Dako Diagnostika, Hamburg, Germany). Actin filament distribution was visualized using a confocal laser scanning microscope LSM 410 (Carl Zeiss, Jena, Germany) equipped with a 488 nm argon-ion laser, an emission filter LP 515 and a 63× objective (Plan-Neofluar). The images show representative results from four independent experiments.

2.10. Statistical methods

One-way analysis of variance (ANOVA) with subsequent post hoc comparison by Student Newman–Keuls test was performed using the statistical software package SigmaStat from Jandel Corporation (Erkrath, Germany). A $P < 0.05$ was considered as statistically significant.

3. Results

3.1. H_2O_2 induces activation of calpain in rat pancreatic acinar cells

To induce oxidative stress, we chose H_2O_2 because it is known to be generated by granulocytes and macrophages during inflammatory processes. Toxic concentrations of H_2O_2 vary with the cell density, components in incubation medium, and cell type studied [29,30]. Exposure to low doses of H_2O_2 (0.01–0.1 mM) has been reported to induce apoptosis in a variety of cell types including AR4-2J pancreatic acinar cells, whereas high doses lead to necrosis [29]. Therefore, to investigate the role of calpain in the mechanism of oxidative stress-initiated necrotic cell death, rat pancreatic acinar cells were incubated with 0.5 or 1 mM H_2O_2 as described in Section 2. H_2O_2 led to a time- and dose-dependent protease activation in rat pancreatic acinar cells compared to the control. Thus, 60 min after administration of 0.5 mM H_2O_2, proteolytic activity began to rise, reaching a maximum level of ∼1.5-fold over baseline at 90 min ($P < 0.05$), and tended to decline thereafter (Fig. 1A). Exposure of cells to 1 mM H_2O_2 already significantly enhanced activity at 60 min ($P < 0.05$). Similar to the experiments with 0.5 mM H_2O_2, the activity further increased, peaked at ∼3-fold over baseline at 90 min ($P < 0.05$) and showed a decreasing trend thereafter (Fig. 1A).

To support that the proteolytic activity induced by H_2O_2 is truly due to calpain, we investigated whether it depends on Ca^{2+} and can be blocked by a calpain inhibitor. Quenching of cytosolic Ca^{2+} by preincubation of cells with the intracellular Ca^{2+} chelator BAPTA-AM decreased the proteolytic activity induced by both 0.5 and 1 mM H_2O_2 to nearly normal levels, when examined

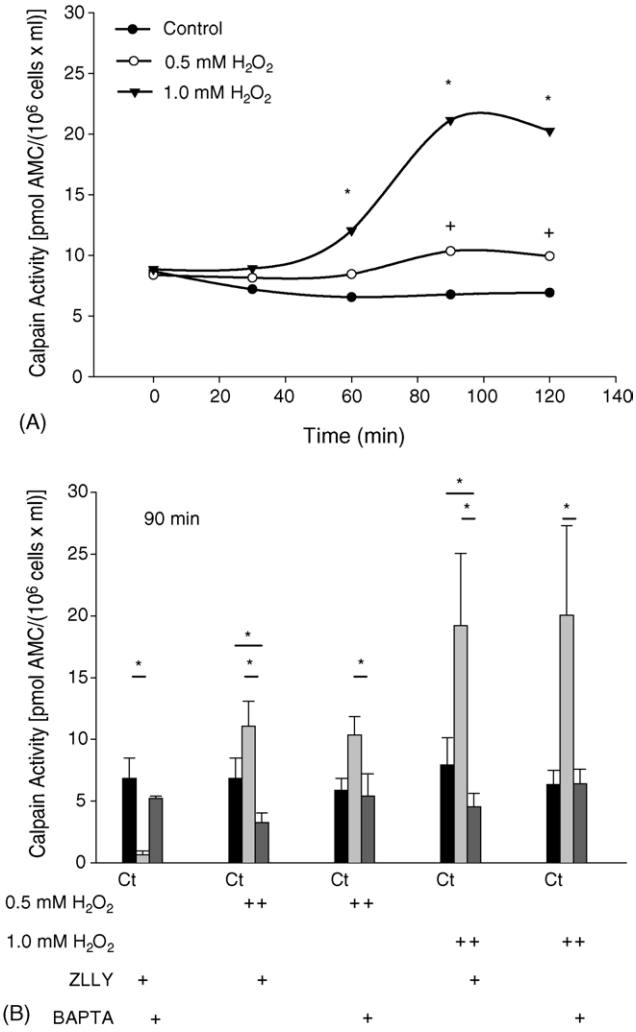

Fig. 1. H_2O_2 induces activation of calpain in rat pancreatic acinar cells. 10^6 cells suspended in 1 ml KRB were exposed to 0.5 and 1 mM H_2O_2 in the presence or absence of Ca^{2+} chelator BAPTA-AM (20 μM) or calpain inhibitor ZLLY (100 μM). At times indicated, 2×10^6 cells were resuspended in 1 ml KRH buffer and calpain activity was determined by adding the fluorescent calpain substrate SucLLVY-AMC and measuring free AMC generation by fluorescence spectrophotometry after 30 min. (A) H_2O_2 causes calpain activation in a time- and dose-dependent manner. The results are expressed as mean; statistical comparisons were performed using ANOVA followed by Student Newman–Keuls test; $^{+}P < 0.05$, untreated cells vs. cells exposed to 0.5 mM H_2O_2; $^{*}P < 0.05$, untreated cells vs. cells exposed to 1 mM H_2O_2 ($n = 4$–7). (B) BAPTA-AM and ZLLY prevent H_2O_2-induced calpain activation. The results are expressed as mean + S.D. Statistical comparisons were performed using ANOVA followed by Student Newman–Keuls test ($n = 4$–6).

at 90 min. Indeed, there were no significant differences between cells exposed to H_2O_2 in the presence of BAPTA-AM and control cells (Fig. 1B). Preconditioning of cells with the competitive irreversible calpain inhibitor ZLLY not only led to a nearly complete inhibition of the basal proteolytic activity ($P < 0.05$), but also significantly suppressed the activity in cells exposed to 0.5 or 1 mM H_2O_2 below the control level (Fig. 1B).

To further rule out the possibility that H_2O_2-induced proteolytic activity was caused by Ca^{2+}-dependent serine

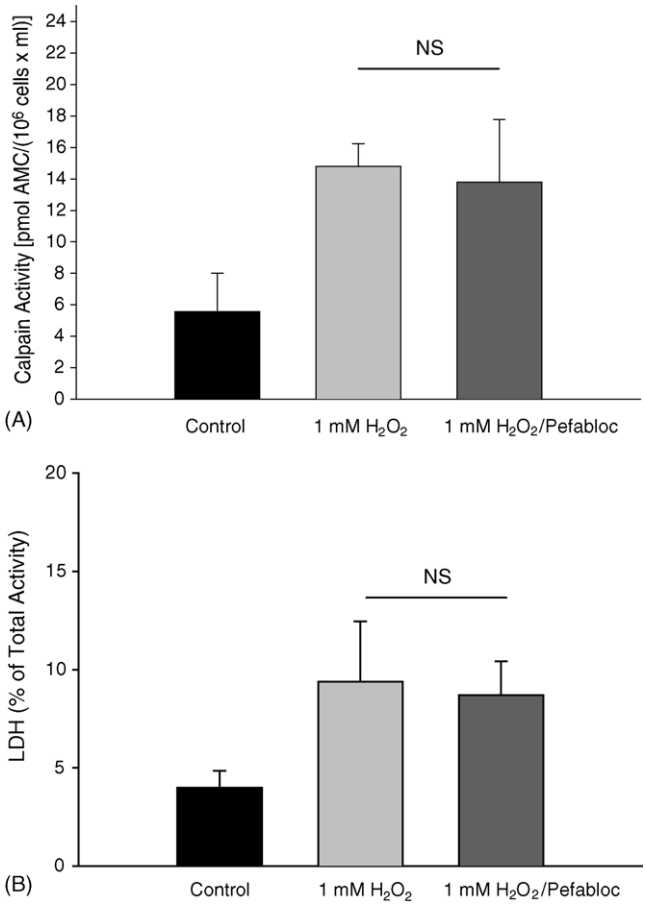

Fig. 2. Serine protease inhibitor Pefabloc does not afford any inhibitory effect on H_2O_2-induced proteolytic activity and does not prevent H_2O_2 toxicity in rat pancreatic acinar cells. 10^6 cells suspended in 1 ml KRB were exposed to 1 mM H_2O_2 in the presence or absence of 500 μM Pefabloc. After 90 min, calpain activity was measured as described in Fig. 1 and the percentage of total cellular LDH released into the incubation medium was measured spectrophotometrically ($n = 4$–7). (A) Effect of Pefabloc on H_2O_2-induced proteolytic activity. (B) Effect of Pefabloc on H_2O_2-induced LDH release. The results are expressed as mean + S.D. Statistical comparisons were performed using ANOVA followed by Student Newman–Keuls test.

proteases, we used the irreversible potent serine protease inhibitor Pefabloc. As demonstrated in Fig. 2A, Pefabloc exerted no inhibitory effect on protease activity induced by 1 mM H_2O_2 at 90 min. The results showed no difference in the proteolytic activity between cells treated with H_2O_2 in the presence or absence of Pefabloc.

Calpain activation involves autoproteolytic truncation of the large 80 kDa subunit at the NH_2 terminus [12,13]. Therefore, to further confirm calpain activation and to obtain information as to which of the calpain isoforms was activated by stimulation of pancreatic acinar cells with H_2O_2, we used antibodies directed against the 80 kDa catalytic subunit of the corresponding calpain isoform on western blots that only recognize latent but not active calpain as we have recently described [9]. Although the calpain proteolytic activity induced by both 0.5 and 1 mM H_2O_2 peaked out after 90 min (Fig. 1A), the processing of the corresponding large subunit of μ- and m-calpain was insignificant in response to both H_2O_2 concentrations (data not shown). At 120 min after generation of oxidative stress, the further degradation of calpain that has been observed to occur following the initial autolysis [9,12,14–16] approved to detect the calpain activation more easily. Thus, the blots revealed a small decrease in μ-calpain and a stronger decrease in m-calpain after treatment with 0.5 mM H_2O_2, indicating an activation of both isoforms (Fig. 3A and B, each with lane 3; Fig. 3C and D). Stimulation with 1 mM H_2O_2 also led to activation of both calpains (Fig. 4A and B, each with lane 3; Fig. 4C and D). Treatment of cells with the calpain inhibitor ZLLY before exposure to 0.5 or 1 mM H_2O_2 diminished oxidant-induced activity of both calpains as indicated by the decreased processing of the corresponding 80 kDa subunit in the presence of the inhibitor (Fig. 3A and B; each with lane 4; Fig. 3C and D, and Fig. 4A and B each with lane 4; Fig. 4C and D). Similar results were obtained when the cells were incubated with the calpain inhibitor ZVF before exposure to 1 mM H_2O_2 (data not shown).

3.2. Effect of fluphenazine on H_2O_2-stimulated calpain activity

Calpain has been reported to be regulated by small acidic phospholipid degradation products [17,31]. In order to test whether a phospholipid-dependent mechanism may play a role in H_2O_2-induced calpain activation in rat pancreatic acinar cells, calpain activity was measured in cells treated with fluphenazine, a phospholipase inhibitor, before exposure to H_2O_2. As demonstrated in Fig. 5, fluphenazine exerted no inhibitory effect on protease activity caused by 0.5 mM H_2O_2, but significantly decreased the activity induced by 1 mM H_2O_2 when examined after 90 min ($P < 0.05$). Preliminary experiments revealed that fluphenazine used in concentrations as in this study neither alter basal proteolytic activity nor cause significant cell damage (data not shown).

3.3. Calpain contributes to H_2O_2-induced pancreatic acinar cells injury

To gain insight into the possibility that calpain is involved in acinar cell injury provoked by H_2O_2, we investigated whether inhibition of H_2O_2-induced calpain activity by preconditioning of cells with BAPTA-AM and different calpain inhibitors was associated with protection against H_2O_2-induced cell damage that was assessed by the release of LDH into the incubation medium and ultrastructural changes. As evidenced by an increased LDH release, H_2O_2 caused a time- and dose-dependent loss of plasma membrane integrity compared to the control (Fig. 6A), closely related with the observed protease activity (Fig. 1A). Thus, in response to 0.5 mM H_2O_2, a significantly enhanced release in LDH was first noticeable

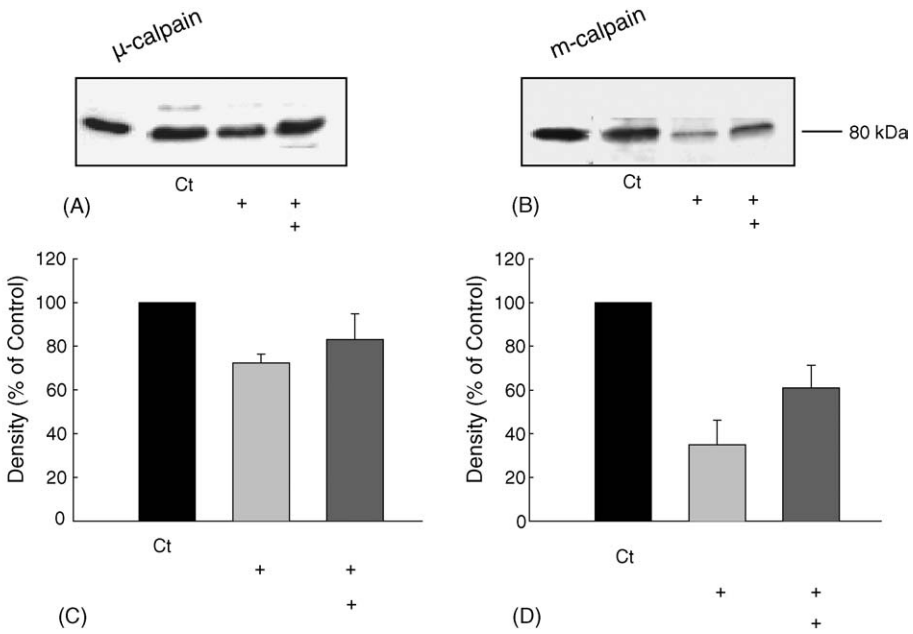

Fig. 3. Stimulation of rat pancreatic acinar cells with 0.5 mM H_2O_2 causes activation of both μ- and m-capain. 10^6 cells suspended in 1 ml KRB were exposed to 0.5 mM H_2O_2 in the presence or absence of 100 μM ZLLY. After 120 min, calpain activation was identified by immunoblotting. (A) Representative μ-calpain immunoblot probed with antibody pAB3 directed against μ-calpain. (B) Representative m-calpain immunoblot probed with pAB4 antibody directed against m-calpain. (C and D) Corresponding densitometric quantification of three experiments.

at 90 min, whereas after stimulation with 1 mM H_2O_2, LDH was already significantly elevated at 60 min. Over time, LDH further increased, reaching ∼1.7-fold ($P < 0.05$) and ∼6-fold elevated levels ($P < 0.05$) in response to 0.5 and 1 mM H_2O_2, respectively, after 120 min. Pretreatment of cells with BAPTA-AM nearly completely prevented LDH release induced by both H_2O_2 doses when examined at 120 min (Fig. 6B). There were no significant differences between H_2O_2-treated cells containing BAPTA and control cells. The protective effect of ZLLY was clearly smaller than those of BAPTA, decreasing the elevation of LDH release induced by 0.5 and 1 mM

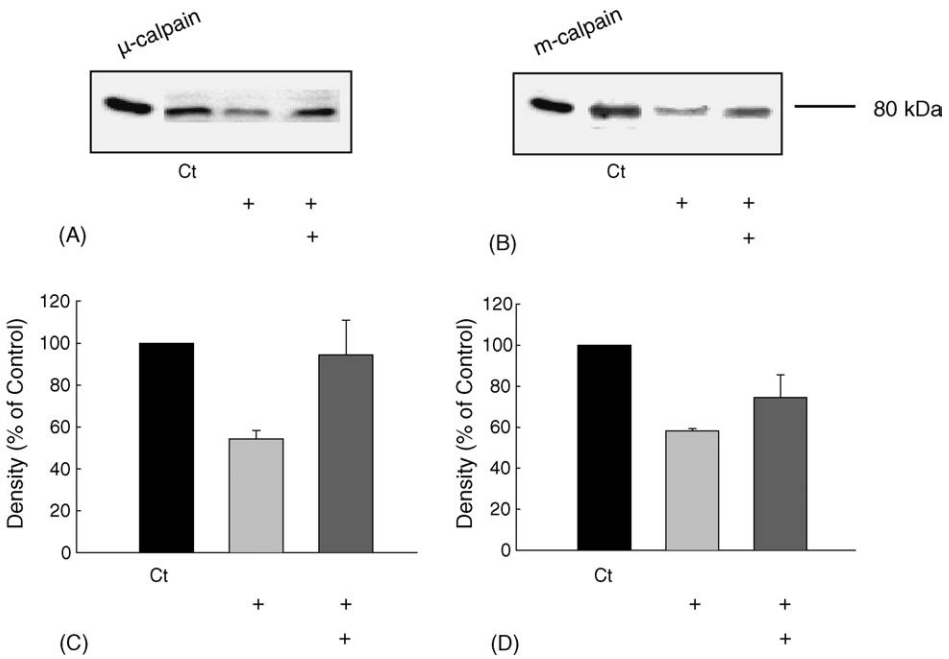

Fig. 4. Stimulation of rat pancreatic acinar cells with 1.0 mM H_2O_2 causes activation of both μ- and m-capain. 10^6 cells suspended in 1 ml KRB were exposed to 1.0 mM H_2O_2 in the presence or absence of 100 μM ZLLY. After 120 min, calpain activation was identified by immunoblotting. (A) Representative μ-calpain immunoblot probed with antibody pAB3 directed against μ-calpain. (B) Representative m-calpain immunoblot probed with antibody pAB2 directed against m-calpain. (C and D) Corresponding densitometric quantification of four experiments.

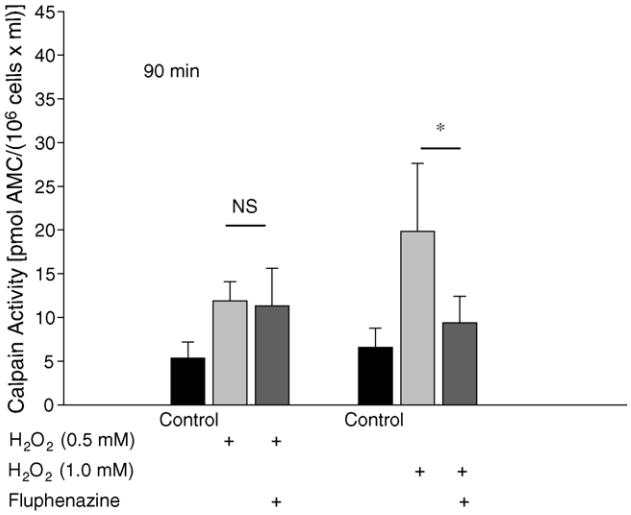

Fig. 5. Effect of phospholipase inhibitor fluphenazine on H_2O_2-induced proteolytic activity. 10^6 cells suspended in 1 ml KRB were exposed to 0.5 and 1 mM H_2O_2 in the presence or absence of 12.5 μM fluphenazine, respectively. After 90 min, calpain activity was measured as described in Fig. 1. Fluphenazine exerts no inhibitory effect on protease activity induced by 0.5 mM H_2O_2, but significantly suppresses the activity induced by 1 mM H_2O_2. The results are expressed as mean + S.D. Statistical comparisons were performed using ANOVA followed by Student Newman–Keuls test; $^*P < 0.05$ ($n = 4$–6).

H_2O_2 by ~50% compared to those induced by H_2O_2 in the absence of the inhibitor (Fig. 6B). In addition, the calpain inhibitors ZVF and calpeptin provoked a comparable protective effect like ZLLY (Fig. 6B). To role out the possibility that proteases other than calpain contribute to H_2O_2 cytotoxicity, we investigated the effect of Pefabloc and proteasome inhibitor lactacystin on LDH release induced by 1 mM H_2O_2. The results showed that both inhibitors did not affect H_2O_2-induced plasma membrane damage (Figs. 2B and 6B). Control experiments revealed that BAPTA-AM and all protease inhibitors employed did not cause significant LDH release (data not shown).

At the ultrastructural level, cell injury was characterized by large cytoplasmic blebs protruding from the basolateral domain, when examined 90 min after application of both 0.5 (images not shown) and 1 mM H_2O_2 (Fig. 7B). These blebs mainly contained fragmented endoplasmic reticulum, but excluded other cytoplasmic organelles, that were localized in the main body of the cell. In addition, marked dilation of the endoplasmic reticulum, small vacuoles and swelling of mitochondria were visible, while the nuclear structures remained intact. At 120 min, there were also numerous lysed cells (images not shown). BAPTA-AM (images not shown) and both ZLLY and ZVF induced a partial beneficial effect against ultrastructural changes caused by 1 mM H_2O_2 (Fig. 7C and D). In particular, the extent of plasma membrane blebbing and mitochondrial swelling was less pronounced compared to the alteration in cells treated with H_2O_2 only, whereas the protective effect on the endoplasmic reticulum was only marginal.

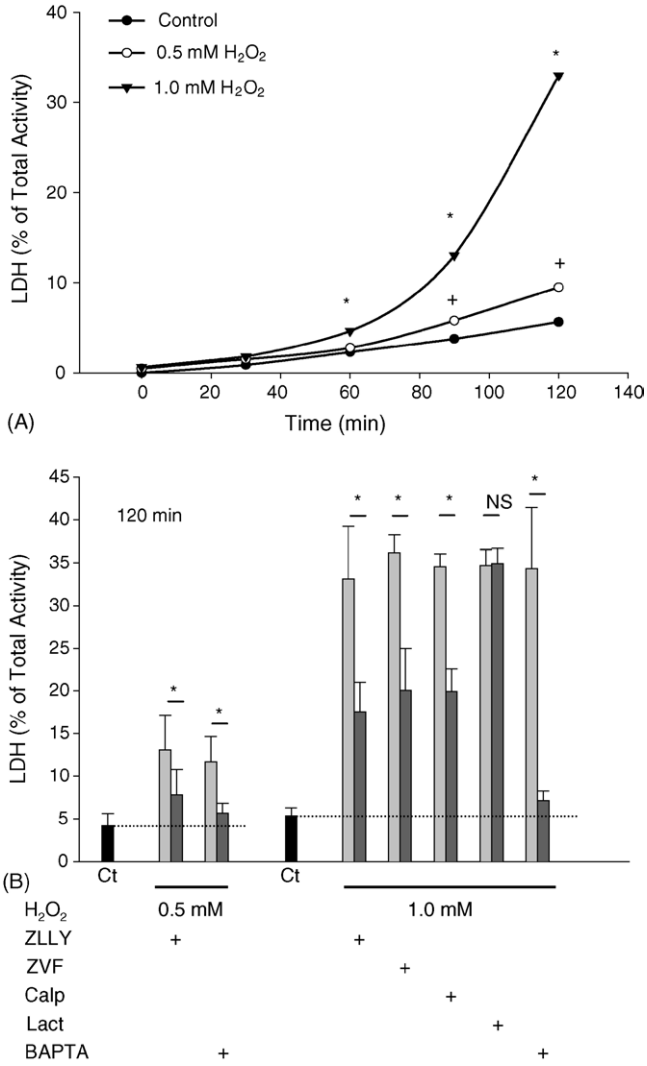

Fig. 6. H_2O_2 induces necrosis in rat pancreatic acinar cells that is mediated by Ca^{2+} and calpain. 10^6 cells suspended in 1 ml KRB were exposed to 0.5 and 1 mM H_2O_2 in the presence or absence of Ca^{2+} chelator BAPTA-AM (20 μM), of calpain inhibitors ZLLY (100 μM), ZVF (100 μM) or calpeptin (50 μM) and of proteasome inhibitor lactacystin (10 μM). At times indicated, LDH release into the incubation medium was measured spectrophotometrically as described in Fig. 2. (A) H_2O_2 dose- and time-dependently induces plasma membrane damage as assessed by LDH release. The results are expressed as mean. Statistical comparisons were performed using ANOVA followed by Student Newman–Keuls test; $^+P < 0.05$, untreated cells vs. cells exposed to 0.5 mM H_2O_2; $^*P < 0.05$, untreated cells vs. cells exposed to 1 mM H_2O_2 ($n = 7$). (B) BAPTA-AM and various calpain inhibitors significantly reduce H_2O_2 cytotoxicity, whereas lactacystin has no protective effect. The results are expressed as mean + S.D. Statistical comparisons were performed using ANOVA followed by Student Newman–Keuls test; $^*P < 0.05$ ($n = 4$–11).

Recent studies on several types of cells have shown that cytoskeletal proteins are substrates for calpain [18]. We, therefore, investigated whether calpain activation induced by 1 mM H_2O_2 was associated with alterations of the actin cytoskeleton. For this purpose, cells were stained with Bodipy FL phallacidine, which is highly specific for filamentous actin, and actin distribution was analyzed by confocal laser scanning microscopy. In resting cells, actin filaments were organized in a thick band surrounding the

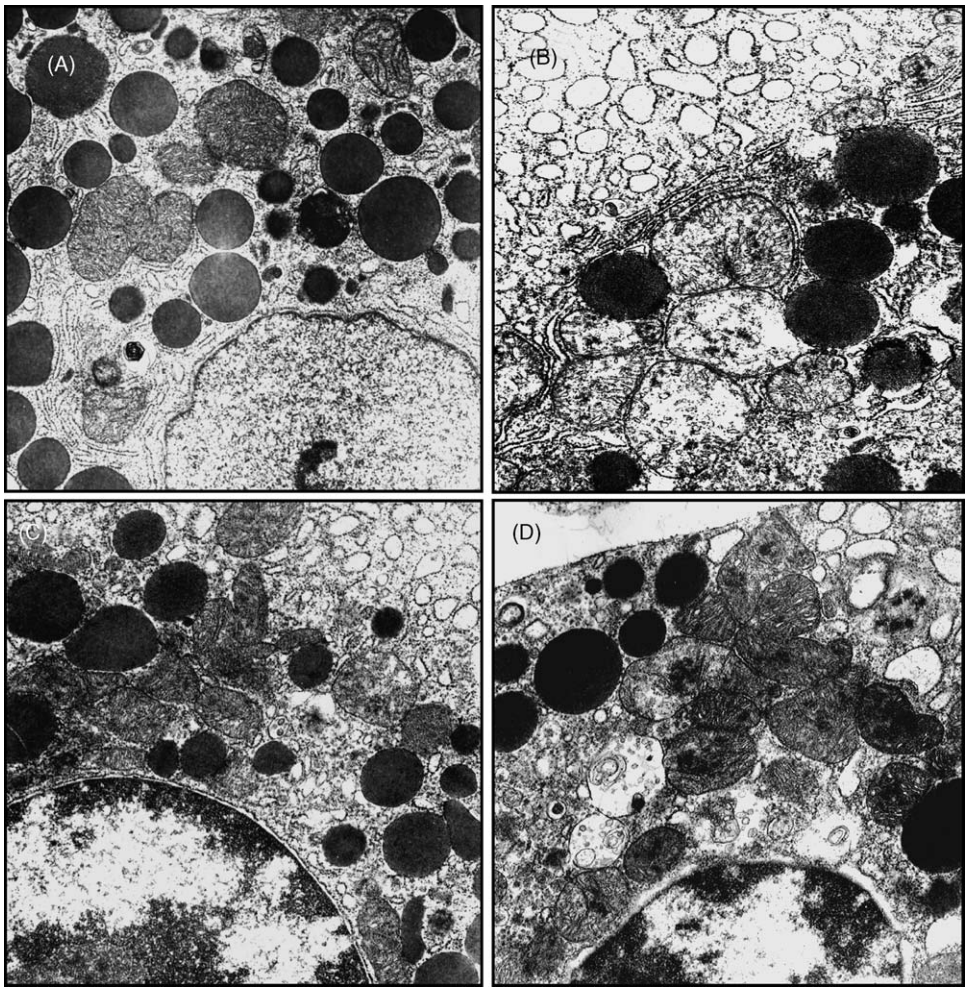

Fig. 7. H_2O_2-induced ultrastructural damage of rat pancreatic acinar cells is mediated by calpain. 10^6 cells suspended in 1 ml KRB were exposed to 1 mM H_2O_2 in the presence or absence of 100 μM ZLLY or 100 μM ZVF. After 90 min, cells were taken for electron-microscopic investigations. The evaluation was performed by a pathologist blinded to the experimental protocol. Representative electron micrographs of a control cell (A) and H_2O_2-stimulated cells pretreated either without (B) or with ZLLY (C) and ZVF (D); original magnification × 21,000. Note dramatic mitochondrial swelling in response to H_2O_2 that is markedly reduced when the inhibitors are given before generation of oxidative stress.

apical membrane and in a narrow band located beneath the basolateral membrane as shown in the overlay image of the fluorescence and the corresponding transmission micrograph (Fig. 8A and A2). The cytoplasm appeared largely unlabeled (Fig. 8A). Following stimulation with H_2O_2, the basolateral actin was increased, whereas the apical actin seemed to be unchanged when examined after 60 min (Fig. 8B). The overlay image reveals that the basolateral actin filaments were detached from the plasma membrane forming a constrictive ring that appeared to prevent organelles from moving into the protrusions. No actin staining was seen within the blebs (Fig. 8B and B2). Preconditioning of cells with BAPTA-AM provoked protection against H_2O_2-mediated microfilament changes. Thus, the actin staining resembled that of the resting cells (Fig. 8C and C2). A reduction of the actin alterations was also found when the cells were incubated with ZVF (Fig. 8D and D2) or ZLLY (Fig. 8E and E2) before exposure to oxidative stress, but the protective effect of both inhibitors was of lower extent than those of BAPTA.

4. Discussion

Oxidative stress can act through a number of important mediators to produce cell injury and death. In the present study, we investigated the role of calpain in the mechanism of oxidative stress-induced toxicity to rat pancreatic acinar cells.

Although numerous other cysteine and serine proteases in pancreatic acinar cells exist, several lines of evidence indicate that oxidative stress generated by H_2O_2 provokes a time- and concentration-dependent activation of calpain. Thus, chelation of intracellular Ca^{2+} by BAPTA-AM led to complete inhibition of H_2O_2-induced proteolytic activity, indicating the Ca^{2+} dependence of the protease activated. Pefabloc, a cell-permeable and potent irreversible serine protease inhibitor, did not block the activity, suggesting that Ca^{2+}-dependent serine proteases do not contribute to this. Consistent with this finding, reactive oxygen species have previously been reported to be unable in activating serine proteases, such as trypsinogen or chymotrypsinogen

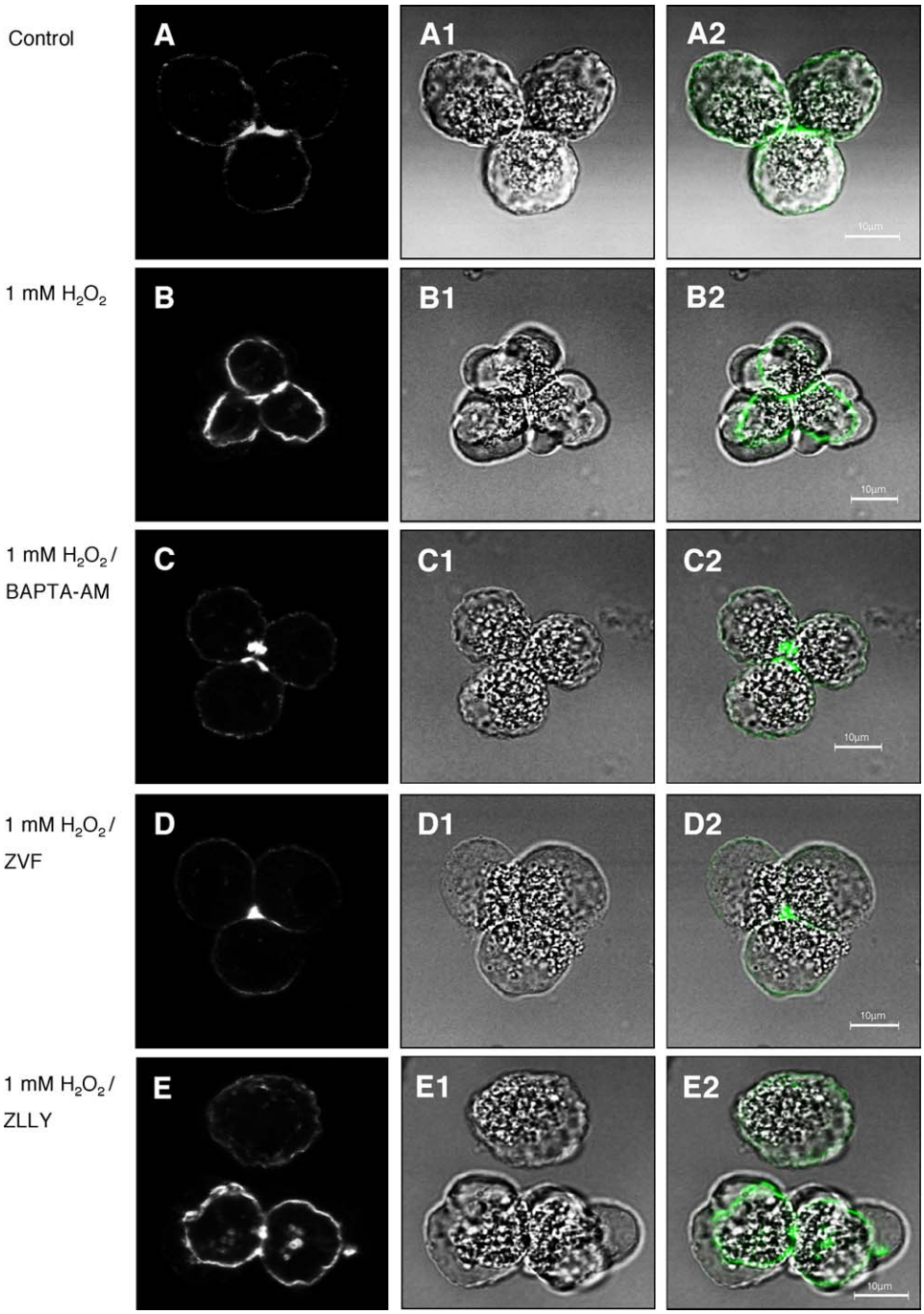

Fig. 8. H_2O_2-induced changes in the actin cytoskeleton of rat pancreatic acinar cells are mediated by Ca^{2+} and calpain. 10^6 cells suspended in 1 ml KRB were exposed to 1 mM H_2O_2 in the presence or absence of 20 μM BAPTA-AM, 100 μM ZVF and 100 μM ZLLY. After 60 min, cells were stained for F-actin with Bodipy FL phallacidine. The fluorescence was detected using confocal laser scanning microscopy. A–E, fluorescence images; A1–E1, transmission micrographs of the corresponding fluorescence images A–E; A2–E2, overlay of the fluorescence images A–E and the corresponding transmission micrographs A1–E1. In resting cells, F-actin is organized in a thick band surrounding the apical membrane (A and A2). Following stimulation with H_2O_2, the basolateral actin is increased and detached from the plasma membrane (B and B2). These changes are less pronounced when BAPTA-AM (C and C2), ZVF (D and D2) and ZLLY (E and E2) are given before H_2O_2.

in human pancreatic juice in vitro [32]. However, ZLLY, a competitive cell-permeable and irreversible active-site-directed calpain inhibitor that has been widely used in other systems [14,27,33,34], completely suppressed proteolytic activity initiated by H_2O_2. This indicates that the Ca^{2+}-dependent protease may be calpain. There is evidence that the inhibitory effects of both BAPTA and ZLLY on protease activity are indeed specific and not a consequence of an oxygen radical elimination by these substances. Thus, by chemiluminescence measurements, we have previously found that BAPTA did not scavenge oxygen species generated by a xanthine oxidase/hypox-

anthine system in pancreatic acinar cells [4]. In addition, in the current study, we observed no protective effect of ZLLY on the marked ATP depletion observed in response to exposure of acinar cells to 1 mM H_2O_2 (data not shown).

Further evidence for H_2O_2-mediated calpain activation is provided by immunoblotting using the autolysis of the 80 kDa catalytic calpain subunit as indicator for the activation. The results suggest that 0.5 mM H_2O_2 particularly may led to activation of m-calpain, whereas 1 mM H_2O_2 seems to activate both calpain isoforms. The data also show that immunochemical detection of calpain activation with antibodies that do not recognize calpain cleavage products requires processing of the 80 kDa calpain subunit to a stronger extent. The observation that administration of ZLLY before induction of oxidative stress reduced the activation of calpain further supports our hypothesis that oxidative stress induces calpain activation in acinar cells. ZLLY, however, merely partially inhibited calpain autolysis, while it completely blocked H_2O_2-mediated calpain proteolytic activity. An explanation for this may be that ZLLY like the endogenous calpain inhibitor calpastatin has only a low affinity for the 80 kDa subunit, but a high affinity for the active 78 and 76 kDa species [12]. Our data are in line with other studies revealing that oxidative stress generated by H_2O_2 or *tert*-butyl hydroperoxide also provokes calpain activation in different neuronal cell lines and hepatocytes [20,22,23,35].

As we have already discussed above, calpain activation mediated by H_2O_2 is Ca^{2+}-dependent. Ca^{2+} induces conformational change and intramolecular autolysis of the NH_2 terminus of both calpain subunits [14–16]. One other mechanism for calpain activation could include small acidic phospholipid degradation products generated by phospholipase A_2 (PLA_2) that have been observed to lower the Ca^{2+} requirement for calpain autolysis in vitro [17], and to activate calpain in anoxic rat hepatocytes [31]. Our data provide first evidence that a PLA_2-dependent mechanism may also be involved in H_2O_2-initiated calpain activation in rat pancreatic acinar cells. PLA_2 activation has been reported to occur in response to H_2O_2-induced oxidative stress in different cell types such as kidney epithelial cells and Her14 fibroblasts [36,37]. Using the phospholipase inhibitor fluphenazine that has been demonstrated to be unable in blocking calpain activity directly [31], we found a clear inhibitory effect on calpain activated by 1 mM H_2O_2. We assume that this effect cannot be the result of antioxidant activity because fluphenazine failed to block proteolytic activity induced by 0.5 mM H_2O_2. This is also supported by the finding that the inhibitor did not prevent H_2O_2-mediated apoptosis of lymphocytes [38]. One explanation for the absent inhibitory effect on proteolytic activity induced by the lower H_2O_2 dose of 0.5 mM may be that only a slight phospholipase activation and consecutive membrane phospholipid degradation was initiated in this case that possibly failed to contribute to H_2O_2-induced calpain activation.

Previously, we have shown that oxidative stress generated by a xanthine oxidase/hypoxanthine system ultimately results in acinar cell necrosis and that prolonged Ca^{2+} elevation is an early and critical event in this pathway [4]. In the present study, oxidative stress induced by H_2O_2 also appears to mainly cause necrotic cell damage as indicated by an early dramatic ATP depletion (data not shown) and by electron-microscopic hallmarks of necrosis such as plasma membrane blebbing and rupture, intracellular organelle swelling and intact nuclear structure [39]. Furthermore, our findings showing that preconditioning of acinar cells with BAPTA-AM reduced the ultrastructural changes to a great extent, suggest that Ca^{2+} may also play a critical role in acinar cell necrosis triggered by H_2O_2-generated oxidative stress. Our assumption is supported by the observation that H_2O_2 led to a prolonged elevation of Ca^{2+} in a wide variety of cell types including mouse pancreatic acinar cells [6]. Based on the following data we assume that calpain may be one molecular event in this Ca^{2+}-dependent stress signaling pathway. Thus, the time course of protease activation and plasma membrane damage appears to be causally related. Additionally, inhibition of protease activity by different calpain inhibitors including calpeptin and ZVF reduced the degree of ultrastructural alterations. Calpeptin has been reported to be a cell-permeable and reversible inhibitor [18] that does not function as antioxidant [40]. ZVF, a hydrophobic cell-permeable inhibitor, was found to be relatively specific for calpain, and highly potent [9,41,42]. ZVF has a very similar chemical structure like calpeptin [25]. We, therefore, assume that it likewise does not scavenge oxygen species. In agreement with our results, calpain has been reported to contribute to oxidative stress-mediated cell injury in neuronal cells and hepatocytes when using calpain inhibitors [20,23] and in HuH7 hepatoma cells in response to calpain antisense [21]. On the other hand, Pefabloc and the irreversible proteasome inhibitor lactacystin [43] had no beneficial effects on H_2O_2-induced cell damage, indicating that serine proteases and proteasome are not involved in this injurious mechanism.

The fact that the calpain inhibitors did not reduce H_2O_2 cytotoxicity to the same extent as BAPTA indicates that other Ca^{2+}-dependent events may contribute to this. In kidney epithelial cells, Ca^{2+}-regulated cytosolic PLA_2 has been identified to be a critical factor in H_2O_2 toxicity [36]. Our observation showing a PLA_2-dependent mechanism of calpain activation with higher H_2O_2 concentrations suggests the possibility that PLA_2 may play a role in Ca^{2+}-mediated damage in our cellular system as well. In addition, there is some evidence that beside destructive hydrolases, an impaired energy metabolism could be another important Ca^{2+}-dependent factor involving in H_2O_2 cytotoxicity. Thus, a sustained increase in Ca^{2+} [6] and a time-dependent dramatic decrease in cellular ATP (data not shown) have been observed to occur when pancreatic acinar cells were stimulated with H_2O_2. Similar results

have also been reported in other cellular systems [44]. Furthermore, in pancreatic acinar cells, Ca^{2+}-dependent decrease in the mitochondrial membrane potential and ATP has likewise been found to contribute to xanthine oxidase/hypoxanthine cytotoxicity [4,5].

To understand the molecular mechanisms underlying calpain-mediated acinar cell necrosis, it is important to identify and characterize critical calpain substrates during cell injury. Some of the primary targets of calpain include various cytoskeletal proteins, such as actin and actin-binding proteins that are important in maintaining and regulating of cytoskeletal structures and functions [7,45–47]. There is increasing evidence that calpain contributes to cell damage through alterations of the cytoskeleton. Thus, calpain inhibitors attenuated cleavage of actin and actin-associated proteins, resulting in cytoprotection against ischemia-reperfusion injury [48,49], toxic injury [45], apoptosis [50] as well as oxidative stress [20] in different systems. Similarly, we recently observed a beneficial effect of the calpain inhibitor ZVF against alterations of the actin cytoskeleton during acute pancreatitis in the rat [9]. Accordingly, the present findings demonstrating that inhibition of calpain activity by preconditioning of acinar cells with BAPTA-AM, ZLLY and ZVF suppressed changes in the actin filament organization provoked by oxidative stress may indicate that the actin cytoskeleton belongs to the cellular targets of calpain in the present system as well. In this regard, another result of our study is noteworthy. We found a close relation between calpain activation, membrane bleb formation and changes in the actin organization in the region of the blebs. Although the pathophysiology of bleb formation is not fully elucidated, it is assumed that a disruption of the membrane lipid bilayer from the underlying cytoskeleton induced by Ca^{2+}-activated proteases among other factors may contribute to this phenomenon [45,50,51]. Studies on hepatocytes exposed to *tert*-butyl hydroperoxide-generated oxidative stress support this assumption showing that degradation of α-actinin and talin, cytoskeletal proteins that connect actin filaments with the plasma membrane, was associated with membrane bleb formation and that calpain was involved in this process [20]. We, therefore, assume that a similar calpain-mediated mechanism may also promote bleb formation in our system.

In summary, our findings suggest that cellular damage through H_2O_2-generated oxidative stress requires activation of Ca^{2+}-dependent enzymes such as the cysteine protease calpain and that the actin cytoskeleton belongs to the cellular targets of calpain in this pathway. It seems conceivable that calpain activation may play a role in the development of acute pancreatitis. Our observations have potential therapeutic implications for ameliorating acinar cell injury. We propose that prophylactic administration of Ca^{2+} modulators supported by calpain inhibitors and/or antioxidants may be a promising strategy for alleviating pancreatic damage occurring during pancreas transplantation, including organ preservation.

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