

Dysregulation of the calpain-calpastatin system plays a role in the development of cerulein-induced acute pancreatitis in the rat

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Weber, Heike, Ludwig Jonas, Saskia Hühns, and Peter Schuff-Werner. Dysregulation of the calpain-calpastatin system plays a role in the development of cerulein-induced acute pancreatitis in the rat. *Am J Physiol Gastrointest Liver Physiol* 286: G932–G941, 2004; 10.1152/ajpgi.00406.2003.—Calpain, a calcium-dependent cytosolic cysteine protease, is implicated in a multitude of cellular functions but also plays a role in cell death. Recently, we have shown that two ubiquitous isoforms, termed μ -calpain and m-calpain, are expressed in rat pancreatic acinar cells and that calcium ionophore-induced calpain activation leads to acinar cell injury. On the basis of these observations, we have now investigated the role of both calpain forms and the endogenous calpain inhibitor calpastatin in acute pancreatitis. After treatment of rats either without or with calpain inhibitor Z-Val-Phe methyl ester (ZVP; 60 mg/kg ip), pancreatitis was induced by cerulein injections (10 μ g/kg ip; 5 times at hourly intervals). Calpain activation and calpastatin expression in the pancreatic tissue were studied by Western blot analysis. Pancreatic injury was assessed by plasma amylase activity, pancreatic wet/dry weight ratio (edema), histological and electron-microscopic analyses, as well as fluorescence labeling of actin filaments. Cerulein caused an activation of both μ -calpain and m-calpain, accompanied by degradation of calpastatin. Prophylactic administration of ZVP reduced the cerulein-induced calpain activation but had no effect on calpastatin alterations. In correlation to the diminished calpain activity, the severity of pancreatitis decreased as indicated by a decline in amylase activity ($P < 0.01$), pancreatic edema formation ($P < 0.05$), histological score for eight parameters ($P < 0.01$), and actin filament alterations. Our findings support the hypothesis that dysregulation of the calpain-calpastatin system may play a role in the onset of acute pancreatitis.

protease activation; actin microfilaments; cytoprotection; Z-Val-Phe methyl ester

A DISORDERED CALCIUM (Ca^{2+}) homeostasis, particularly a large and sustained cytosolic increase, appears to be an important early and general event in initiation and development of cell injury. There is increasing evidence that an impaired cytosolic Ca^{2+} concentration may also play a key role in the pathogenesis of acute pancreatitis (30, 42, 44). The mechanisms of Ca^{2+} -mediated pancreatic acinar cell damage, however, remain to be elucidated. The activation of Ca^{2+} -dependent proteases such as the serine protease trypsinogen seems to be an early feature in this process (34, 44). Calpains, a family of nonlysosomal, neutral cysteine proteases, are also Ca^{2+} regulated and thus may be activated as well. 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 identified, requiring micro- and milli-

molar Ca^{2+} concentrations, respectively, for their in vitro activation (6). Both ubiquitous calpains are heterodimers, composed of one large 80-kDa catalytic subunit and one small 30-kDa regulatory subunit (6). The large subunits of μ - and m-calpain have different amino acid sequences encoded by different genes, whereas the small subunits are common to both isozymes (15, 37). The activation of calpains involves Ca^{2+} -induced conformational change and intramolecular autolysis of the NH_2 terminus of both subunits. During this conversion, the 80-kDa subunit is processed to a 76-kDa form through a 78-kDa intermediate, which increases the Ca^{2+} sensitivity of the enzymes (21, 32). The autolysis of the small subunit produces an 18-kDa fragment and does not affect the Ca^{2+} requirement (21, 32). After this initial autolysis and in the continued presence of Ca^{2+} , further degradation occurs, mainly of the large subunit, which abolishes calpain activity later on (1, 10, 24). Both the activation and catalytic activity of calpains are strictly regulated by their natural inhibitor named calpastatin. Calpastatin, similar to calpain, is ubiquitously present in most cell types. Whereas only one gene for calpastatin has been identified, several isoforms have been detected, resulting from differential mRNA splicing and posttranslational modifications such as phosphorylation and proteolysis (6, 27, 38).

Calpains modify a variety of proteins including cytoskeletal proteins, membrane proteins (growth factor receptors, adhesion molecules, ion transporters), enzymes (kinases, phospholipases, phosphatases), as well as cytokines and various transcription factors by selective and very limited proteolytic degradation, suggesting that its function is to modulate protein structure and activity rather than to catabolize proteins. Thus regulated proteolytic modification of proteins by calpain may transduce a cellular signal or induce cellular functions such as membrane fusion or cell proliferation and differentiation (40). Uncontrolled activation of calpain has been observed to lead to cell damage, which may be attributed to irreversible alterations and consecutive loss of calpain substrate functions. There is evidence that nonphysiological calpain activation plays a role in cataract development, various neurodegenerative diseases, muscle dystrophy, and pathological apoptosis (5). In addition, calpain has been found to be involved in ischemic-reperfusion injury in different systems including liver, kidney, heart, and brain cells or tissues (8, 16, 17, 26, 29, 46, 47). As a mechanism for increasing calpain activity during this process, proteolytic degradation of calpastatin has been suggested (35, 36).

Recently, we showed for the first time that both calpain isoforms are expressed in rat pancreatic acinar cells. Addition-

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ally, our results revealed that ionomycin-induced cytosolic Ca^{2+} increase leads to calpain activation and acinar cell damage (43). On the basis of these findings, in the present study, we have investigated whether calpain activation plays a role in acute pancreatitis induced by cerulein hyperstimulation that is also known to cause an abnormal, sustained cytosolic Ca^{2+} increase in pancreatic acinar cells (4, 18, 30).

We provide evidence that cerulein induces a decrease in calpastatin, which may contribute to the activation of both calpain isoforms, observed in parallel. Prophylactic administration of the calpain inhibitor Z-Val-Phe methyl ester (ZVP) has no protective effect on the calpastatin alterations but significantly reduces the calpain activation. In correlation to the decreased calpain activity, the severity of cerulein pancreatitis is clearly diminished, thus suggesting a role of calpain in the onset of pancreatitis.

MATERIALS AND METHODS

Antibodies and reagents. For immunoblotting, rabbit polyclonal antibodies against μ -calpain (domain I, pAB1) and m-calpain (domain III, pAB2) were purchased from Calbiochem-Novabiochem (San Diego, CA). Other rabbit polyclonal antibodies against μ -calpain (domain I, NH_2 terminal, pAB3) and m-calpain (domain I, NH_2 terminal, pAB4) were from Sigma-Aldrich (St. Louis, MO). The anti- μ -calpain antibodies showed no cross-reactivity with m-calpain, and the anti-m-calpain antibodies did not cross-react with μ -calpain (data not shown). The mouse monoclonal anti-calpastatin antibody (clone 1F7E3D10; domain IV) was purchased from Sigma-Aldrich and did not cross-react neither with μ -calpain nor m-calpain (data not shown). μ -Calpain (porcine erythrocytes) and m-calpain (porcine kidney) were obtained from Calbiochem-Novabiochem. Horseradish peroxidase-conjugated secondary antibodies, SDS, polyvinylidene difluoride (PVDF) membranes, and Precision Plus protein standards were from Bio-Rad (Hercules, CA). Bodipy FL phalloidin was from Molecular Probes (Eugene, OR). All other reagents, including cerulein and ZVP, were purchased from Sigma.

Animals. Female rats of an inbred Lewis strain (LEW-1W, Department of Laboratory Animal Science, University of Greifswald, Germany) were used. Animals were starved for 18 h before starting the experiments but had free access to water.

Induction of cerulein pancreatitis. The experiments were approved by the ministry of agriculture of the federal state Mecklenburg-Vorpommern to be in accordance with the standards of care and use of laboratory animals.

Animals (180–200 g body wt) were injected intraperitoneally either with ZVP (60 mg/kg dissolved in 0.4 ml of DMSO) or with an equal volume of DMSO alone as previously described ($n = 6$ in each group) (16, 17). After 2.5 h, pancreatitis was induced by hourly injections of 10 $\mu\text{g}/\text{kg}$ cerulein for 5 h. Controls received DMSO and saline in the same manner. One hour after the final injection, animals were killed, blood samples were taken by heart puncture, and pancreata were rapidly removed for further analyses.

Pancreatic acinar cell preparation. Acinar cells were prepared from pancreata taken from animals weighing 150–180 g by collagenase digestion as described previously (43).

Plasma markers of pancreatic tissue injury. Plasma amylase and lipase were used as established markers of acute pancreatitis. The activities were determined with test kits AMYL and LIP, respectively, from Roche Diagnostics (Mannheim, Germany) using an EPOS 5060 analyzer (Eppendorf, Hamburg, Germany).

Preparation of rat pancreatic homogenate. Pancreatic tissue was suspended in 20 mM Tris-HCl buffer (pH 7.4, 4°C) containing (in mM) 5 EDTA, 5 EGTA, 1 DTT, 145 NaCl, and 2 Pefabloc with 10.5 μM leupeptin and disrupted by using a Potter Elvehjem homogenizer. The homogenized tissue was centrifuged at 19,000 g for 30 min (4°C).

The supernatant was again spun at 100,000 g for 90 min (4°C) and stored at -80°C .

Immunoblotting procedures. The protein concentration of homogenates was determined using the Advanced Protein Assay (Cytoskeleton, Denver, CO). Homogenate samples were mixed with sample buffer Roti-Load 1 (Carl Roth, Karlsruhe, Germany) according to the manufacturer's suggested protocol, heated for 5 min at 95°C, and subjected to Laemmli SDS-PAGE using 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 Towbin buffer (125 mM Tris, 95 mM glycine, 0.02% SDS, 20% methanol). Membranes were blocked in PBS with Tween-20 containing 5% BSA and 5% fat-free milk powder for 5 h at room temperature and probed with anti- μ -calpain antibody pAB1 (1:5,000) or pAB3 (1:1,000), with anti-m-calpain antibody pAB2 (1:5,000) or pAB4 (1:1,000), and with anti-calpastatin antibody (1:2,500), respectively, overnight at 4°C. The membranes were washed in PBS with Tween-20 (3×30 min) and incubated with horseradish peroxidase-conjugated secondary antibody (1:3,000) for 60 min. The colorimetric Opti-4CN detection kit (Bio-Rad) or the Amplified Opti-4CN detection kit (Bio-Rad) was used to visualize the immunolabeling.

Characterization of the calpain antibodies. Ten microliters of purified m-procalpain (1 mg/ml) or μ -calpain (0.7 mg/ml) were added to 170 μl buffer (60 mM imidazole, 5 mM cysteine, 2.5 mM 2-mercaptoethanol, pH 7.3, 37°C) and incubated without or with 10 μl casein (20 mg/ml). The calpain activation was induced by addition of 10 μl CaCl_2 (100 mM). After 5 and 30 min, the reaction was quenched by mixing 21- μl sample aliquots with 7 μl SDS-PAGE sample buffer. The proteins were analyzed by SDS-PAGE (10% minigels) and immunoblotted as described above. The anti-calpain antibodies were used at 1:1,000 dilution, and immunoreactivity was visualized by the Opti-4CN detection kit.

Pancreatic water content. For quantification of pancreatic edema, a small piece of tissue was resected, trimmed of fat, blotted dry, and weighed (wet weight). Thereafter, the specimen was desiccated for 48 h at 105°C and weighed again (dry weight). Pancreatic water content was determined by calculating the wet weight/dry weight ratio.

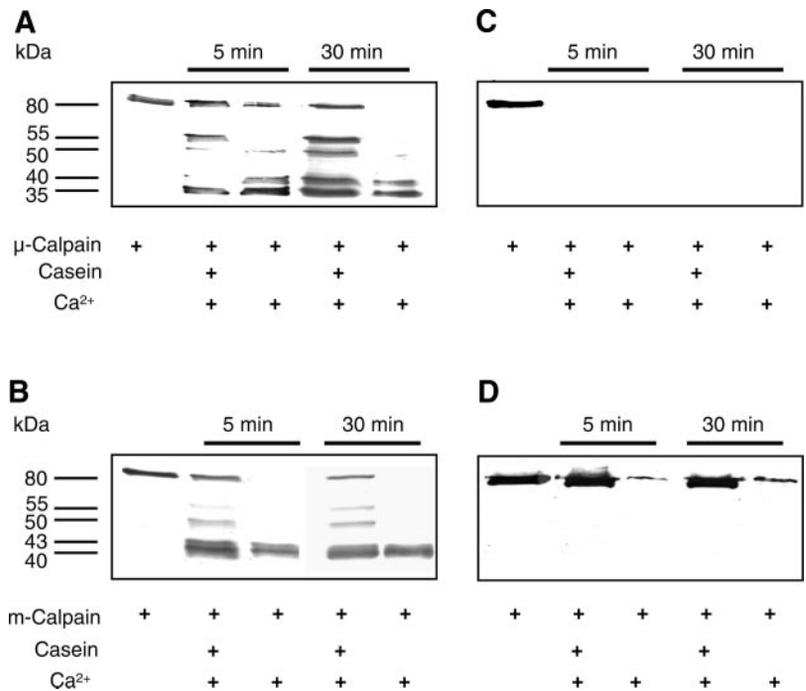
Morphology. Cerulein-induced morphological pancreas damage was assessed by a pathologist who was not aware of the specimens identity.

Histological evaluation. For light microscopy, pancreatic tissue samples were fixed in 4% buffered neutral formaldehyde for 24 h, ethanol dehydrated, and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin and examined with a light microscope Jenamed (Carl Zeiss, Jena, Germany). The extent of acidophilia, vesiculation, and vacuolization of acinar cells as well as eosinophilia, hyperemia, edema, and granulocyte and round cell infiltration of the interstitial space was quantified using a previously described scoring system (45). According to the severity of tissue damage, a score from 0 to 3 was given for each parameter.

Electron-microscopic evaluation. For electron-microscopic investigations, pancreatic tissue was fixed for 1 h in 4% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0), washed in the same buffer, and postfixed in 1% osmium tetroxide. Thereafter, it was embedded in 0.1% liquid agar. Small cubes of the samples 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 (Zeiss, Oberkochen, Germany).

Localization of F-actin in rat pancreatic tissue. Cryosections were prepared with a cryostat CM 3000 (Leica) and fixed with acetone at 4°C for 10 min. After repeated rinsing with PBS and incubating with Dako Protein Block (Dako Diagnostika, Hamburg, Germany) for 30 min, the sections were stained with 100 μl bodipy FL phalloidin (5

Fig. 1. In vitro autolysis of μ -calpain and m-calpain. Porcine μ -calpain (34 μ g/ml) or m-calpain (50 μ g/ml) was incubated without or with casein (1 mg/ml) at 37°C and activated by addition of Ca^{2+} (5 mM) as described in MATERIALS AND METHODS. Aliquots were withdrawn after the indicated times and analyzed by immunoblotting. Representative μ -calpain immunoblots using antibodies directed against the 80-kDa subunit of μ -calpain: antibody pAB1 (A) and antibody pAB3 (C; each with 26 μ g protein on a 10% gel), and representative m-calpain immunoblots directed against the 80-kDa subunit of m-calpain: antibody pAB2 (B) and antibody pAB4 (D; each with 38 μ g protein on a 10% gel). Ca^{2+} quickly induces autoproteolytic cleavage of the corresponding 80-kDa calpain subunit, leading to the formation of various autolysis products.



U/ml) for another 30 min in the dark, following the manufacturer's suggested protocol. Thereafter, the sections were washed twice with PBS and embedded in fluorescence mounting medium (Dako), which was followed by viewing and micrography on a fluorescence microscope Jenaluma (Carl Zeiss, Jena, Germany).

Statistical analysis. Means of normally distributed data were compared by Student's *t*-test. In the case of significant differences in variance, means were compared by Welch's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Cerulein-induced calpain activation in rat pancreatic tissue. The autoproteolytic cleavage of the 80-kDa subunit at the NH_2 terminus of calpain leading to a complex pattern of transient and stable autolysis products has been observed to be associated with calpain activation (10, 21, 24, 28). To investigate whether cerulein hyperstimulation induces activation of calpain in rat pancreatic tissue, we used a set of antibodies directed against the 80-kDa subunit of the corresponding cal-

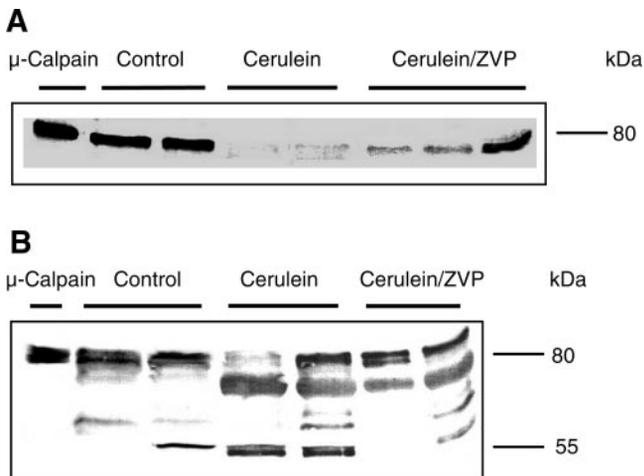


Fig. 2. Cerulein-induced activation of μ -calpain in rat pancreatic tissue. At 2.5 h after treatment either with Z-Val-Phe methyl ester (ZVP; 60 mg/kg ip in 0.4 ml DMSO) or with ZVP solvent DMSO, pancreatitis was induced by injections of 10 μ g/kg ip cerulein (5 times at hourly intervals). One hour after the final injection, the rats were killed. Homogenized pancreatic tissue was subjected to Western blot analysis. Representative μ -calpain immunoblots using antibodies directed against the 80-kDa subunit of μ -calpain: antibody pAB3 (A; 40 μ g protein on a 10% gel) and antibody pAB1 (B; 20 μ g protein on a 7.5% gel). Cerulein hyperstimulation induces activation of μ -calpain as indicated by the decreased 80-kDa calpain subunit and the concomitant increased formation of a cleavage product corresponding to 55 kDa. ZVP preconditioning reduces the cerulein-mediated μ -calpain activation.

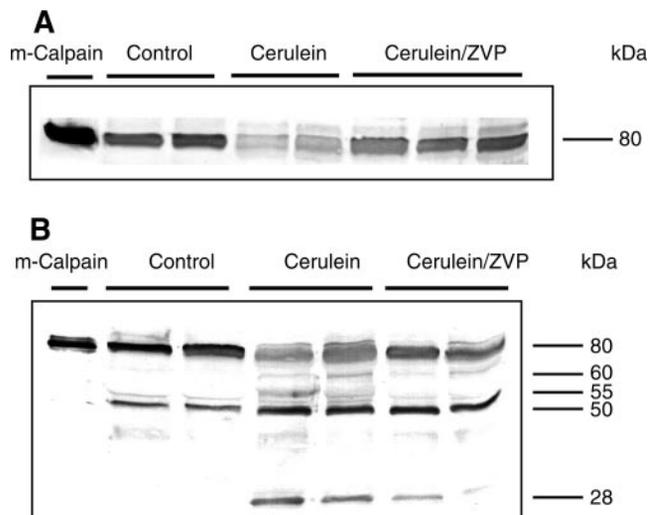


Fig. 3. Cerulein-induced activation of m-calpain in rat pancreatic tissue. Experimental design as in Fig. 2. Representative m-calpain immunoblots using antibodies directed against the 80-kDa subunit of m-calpain: antibody pAB4 (A) and antibody pAB2 (B; each with 40 μ g protein on a 10% gel). Cerulein hyperstimulation induces activation of m-calpain as indicated by the decreased 80-kDa calpain subunit and the concomitant increased formation of different autolysis products including fragments corresponding to 50 and 28 kDa. ZVP preconditioning reduces the cerulein-mediated m-calpain activation.

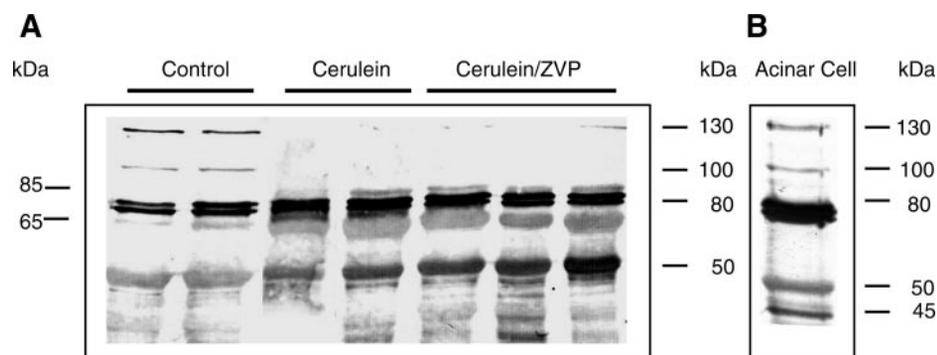


Fig. 4. Effect of cerulein hyperstimulation on calpastatin. Experimental design as in Fig. 2. *A*: representative immunoblot for calpastatin (20 μ g protein on an 8% gel) showing an altered calpastatin expression pattern in response to cerulein hyperstimulation that is not affected by ZVP preconditioning. *B*: calpastatin expression pattern in untreated isolated rat pancreatic acinar cells (20 μ g protein on an 8% gel).

pan form on Western blots. In pilot in vitro experiments, we tested whether the selected antibodies are capable of detecting the calpain activation. Immunoblot analyses using anti- μ -calpain antibody pAB1 and anti-m-calpain antibody pAB2 showed that both purified μ -calpain and m-calpain autolyzed rapidly when incubated with Ca^{2+} in the presence of the calpain substrate casein. After only 5 min, the 80-kDa band had significantly decreased, accompanied by the formation of immunologically reactive fragments (Fig. 1, *A* and *B*; each with *lane 2*). After 30 min, both antibodies clearly identified cleavage products corresponding to apparent molecular weights of 55, 50, and 40 kDa (Fig. 1, *A* and *B*; each with *lane 4*). In addition, pAB1 detected a further μ -calpain fragment of ~ 35 kDa, whereas pAB2 reacted with an m-calpain autolysis product of ~ 43 kDa. When the calpains were activated with Ca^{2+} in the absence of casein, the autolysis was still faster than in the presence of the substrate, leading to a complete disappearance of the 80-kDa band after 30 min (Fig. 1, *A* and *B*; each with

lanes 3 and *5*). The 55- and 50-kDa cleavage products were likewise disappeared, suggesting that they are merely transient products, whereas the other low molecular weight fragments observed seem to be more resistant to autolytic proteolysis.

Anti- μ -calpain antibody pAB3 and anti-m-calpain antibody pAB4 detected the Ca^{2+} -induced processing of the 80-kDa subunit as well but were not able to react with calpain fragments (Fig. 1, *C* and *D*; each with *lanes 2–4*). Thus our results indicate that all antibodies tested can be used for the immunochemical detection of calpain activation.

Western blot analysis of both calpain isoforms in homogenized pancreatic tissue of the control group clearly detected the 80-kDa subunit using these antibodies as demonstrated in Figs. 2, *A* and *B*, and 3, *A* and *B* (each with *lanes 2* and *3*). Stimulation of animals with supramaximal doses of cerulein caused a significant decrease in the 80-kDa subunit of both calpain forms compared with the respective control, which may indicate the activation of the proteases (Fig. 2*A*, *lanes*

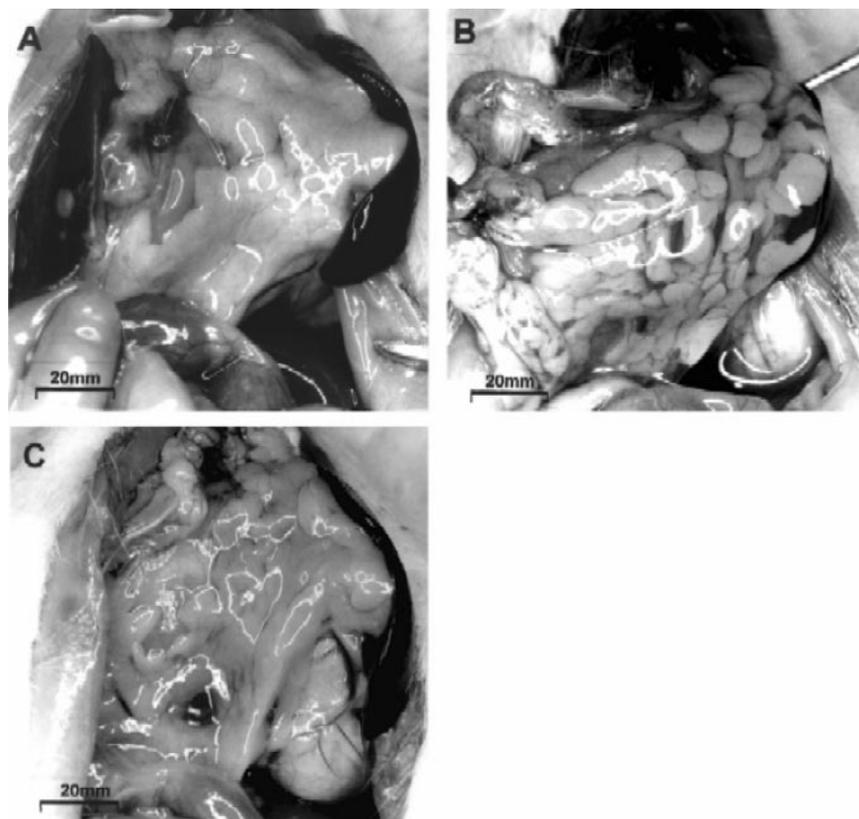


Fig. 5. Effect of ZVP on cerulein-induced macroscopic alterations of rat pancreas. Experimental design as in Fig. 2. Shown are control (*A*) and cerulein administration either without (*B*) or with ZVP preconditioning (*C*). Note that the macroscopically visible edema formation associated with cerulein hyperstimulation is obviously reduced in response to ZVP preconditioning.

4 and 5; Fig. 2B, lane 4; Fig. 3, A and B; each with lanes 4 and 5). A further indication for cerulein-induced calpain activation may be the concomitant appearance of cleavage products possessing the same molecular weights as the calpain autolysis fragments generated after calpain activation *in vitro*. Thus immunoblot analysis of μ -calpain using antibody pAB1 showed the increased formation of a \sim 55-kDa product compared with the control (Fig. 2B, lanes 4 and 5). With the use of anti-m-calpain antibody pAB2, enhanced concentrations of immunologically reactive cleavage products corresponding to \sim 50 and \sim 55 kDa were observed. In addition, both a minor fragment of \sim 60 kDa and a prominent fragment of \sim 28 kDa appeared (Fig. 3B, lanes 4 and 5). These fragments were not observed in the present activation experiment with purified m-calpain but have been described by others (10, 24).

Treatment of animals with the calpain inhibitor ZVP before exposure to cerulein clearly diminished the cerulein-induced activation of both calpain isoforms. This was reflected by the decreased processing of the corresponding 80-kDa subunit in the cerulein group pretreated with ZVP compared with the cerulein group without inhibitor preconditioning. Admittedly,

the efficiency of ZVP was different. In some cases, the inhibitor nearly completely prevented the decrease in the 80-kDa subunit (Figs. 2A and 3A; each with lane 8; Fig. 2B, lanes 6 and 7), whereas in others, this effect was less pronounced (Figs. 2A and 3, A and B; each with lanes 6 and 7).

Cerulein-induced decrease in the calpastatin protein expression in rat pancreatic tissue. The calpain activation as well as the calpain activity are regulated by the endogenous inhibitor calpastatin. Therefore, we investigated whether cerulein-mediated calpain activation may be associated with a decrease in the pancreatic calpastatin level. Western blot analysis of calpastatin in the pancreatic tissue of the control animals detected two minor high molecular weight proteins of \sim 130 and 100 kDa and two major low molecular weight proteins corresponding to \sim 80 kDa (Fig. 4A, lanes 1 and 2). In addition, several bands of lower molecular weights were also recognized by the antibody. To further confirm these findings, similar immunoblot analysis was performed with untreated isolated rat pancreatic acinar cells. A representative immunoblot is demonstrated in Fig. 4B and reveals a comparable band pattern.

Cerulein hyperstimulation led to clear changes in the calpastatin expression pattern (Fig. 4A, lanes 3 and 4). Thus both

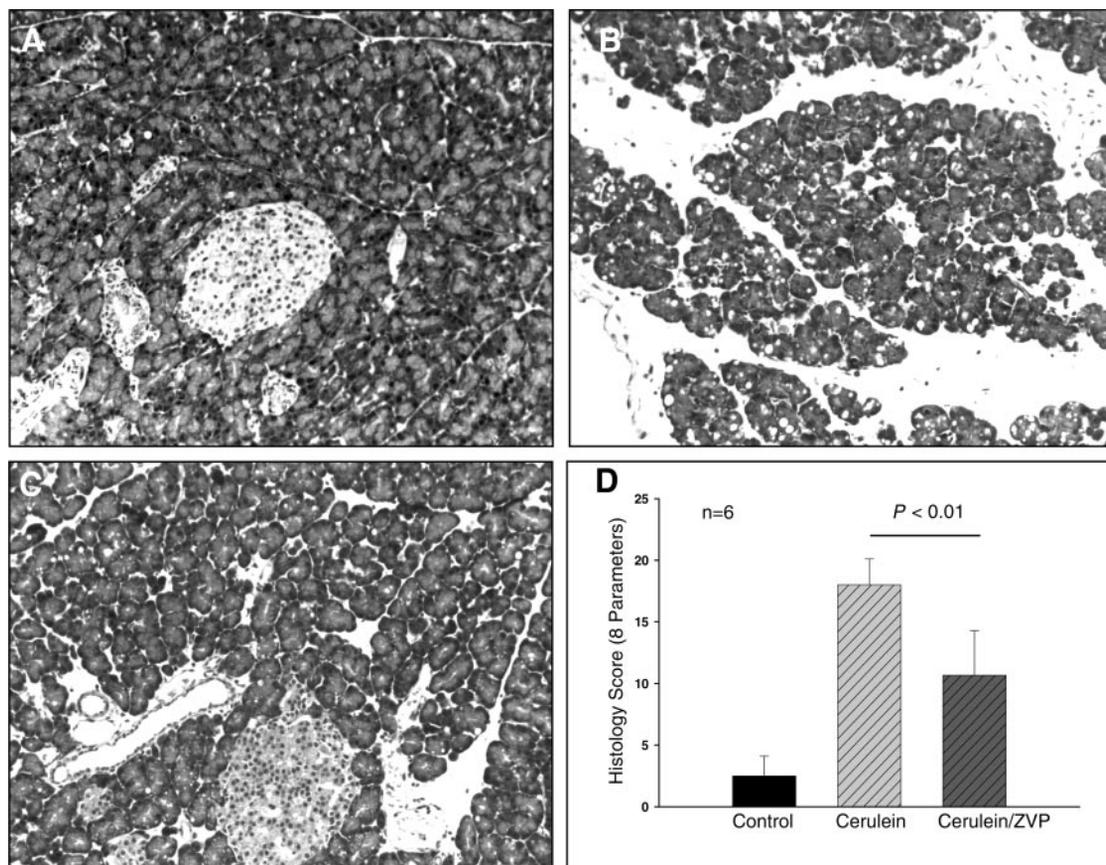


Fig. 6. Effect of ZVP on cerulein-induced histological alterations of rat pancreas. Experimental design as in Fig. 2. Pancreata from control rats (A) and from rats either without (B) or with ZVP treatment before cerulein application (C) were cut and stained with hematoxylin and eosin for light-microscopic evaluation. The extent of acidophilia, vesiculation, and vacuolization of acinar cells, hyperemia, edema, and granulocyte and round cell infiltration of the interstitial space was quantitated by a scoring system (range 0–3). The results are expressed as means \pm SD; *P* values were calculated by *t*-test. The evaluation was performed by a pathologist blinded to the experimental protocol. The photomicrographs demonstrate that ZVP preconditioning impairs cerulein-induced pancreas alterations, particularly the marked interstitial edema and the extensive acinar cell vacuolization (compare B and C; original magnification \times 148). This observation is supported by the significant decrease in the average score of the inhibitor-pretreated cerulein group compared with the cerulein-only-treated group (D).

high molecular weight proteins became undetectable, accompanied by the appearance of immunologically reactive bands of ~85 and 65 kDa compared with the control, probably representing calpastatin cleavage products. In addition, a significant increase in the intensity of the ~50-kDa band was observed. The magnitude of both the 65- and the ~50-kDa band suggests that a fragmentation of the 80-kDa native calpastatin forms may occur as well.

Our results further suggest that treatment of rats with ZVP before cerulein hyperstimulation does not affect the cerulein-mediated alterations of calpastatin (Fig. 4A, lanes 5–7).

Effect of ZVP on the severity of cerulein-induced acute pancreatitis. To gain insight into the possibility that calpain is involved in cerulein-induced pancreatic damage, we investigated whether the attenuation of cerulein-induced pancreatic calpain activation in response to ZVP preconditioning may be associated with protection against cerulein-induced pancreatitis. Our results revealed that ZVP induced a protective effect that was already macroscopically apparent. Thus, whereas cerulein provoked an edematous pancreatitis with pancreata macroscopically appearing swollen and grayish, these changes were greatly diminished when cerulein was given after ZVP preconditioning (Fig. 5). Histologically, the extent of interstitial edema and acinar cell vacuolization, the most prominent histological features of the pancreas in rats suffering from cerulein pancreatitis, was less pronounced compared with the group treated with cerulein only (Fig. 6). Quantifying the histopathological changes by our scoring system, a decline of

the average score for all morphological criteria investigated was found (data not shown). Altogether, the histological score decreased by 41% ($P < 0.01$) in the inhibitor-pretreated cerulein group compared with the only cerulein-treated group as demonstrated in Fig. 6D.

The beneficial effect of ZVP treatment before cerulein administration was also evident at the ultrastructural level. In particular, the large cytoplasmic vacuoles that also characterize cerulein pancreatitis were strongly reduced in size and number compared with the cerulein group without ZVP preconditioning (Fig. 7, A–C). Furthermore, the pronounced loss of microvilli and disappearance of the terminal microfilaments likewise seen with cerulein alone were clearly smaller (Fig. 7, D–F). Thus the apical region in acinar cells of ZVP-pretreated animals resembled, at least in part, that visible in control animals. Staining of the actin filaments with bodipy FL phalloidide also reflected the protective effect of ZVP on the actin cytoskeleton, consequently further supporting the electron-microscopical findings. Whereas cerulein hyperstimulation resulted in a nearly absent fluorescence along the luminal and lateral membrane of the acinar cells compared with the control, the loss of actin labeling was less vigorous when cerulein was injected after ZVP treatment (Fig. 8).

Finally, the protective effect of ZVP against cerulein pancreatitis noted in the morphological studies was also apparent on biochemical criteria that characterize this model, such as hyperamylasemia, hyperlipasemia, and increased tissue water

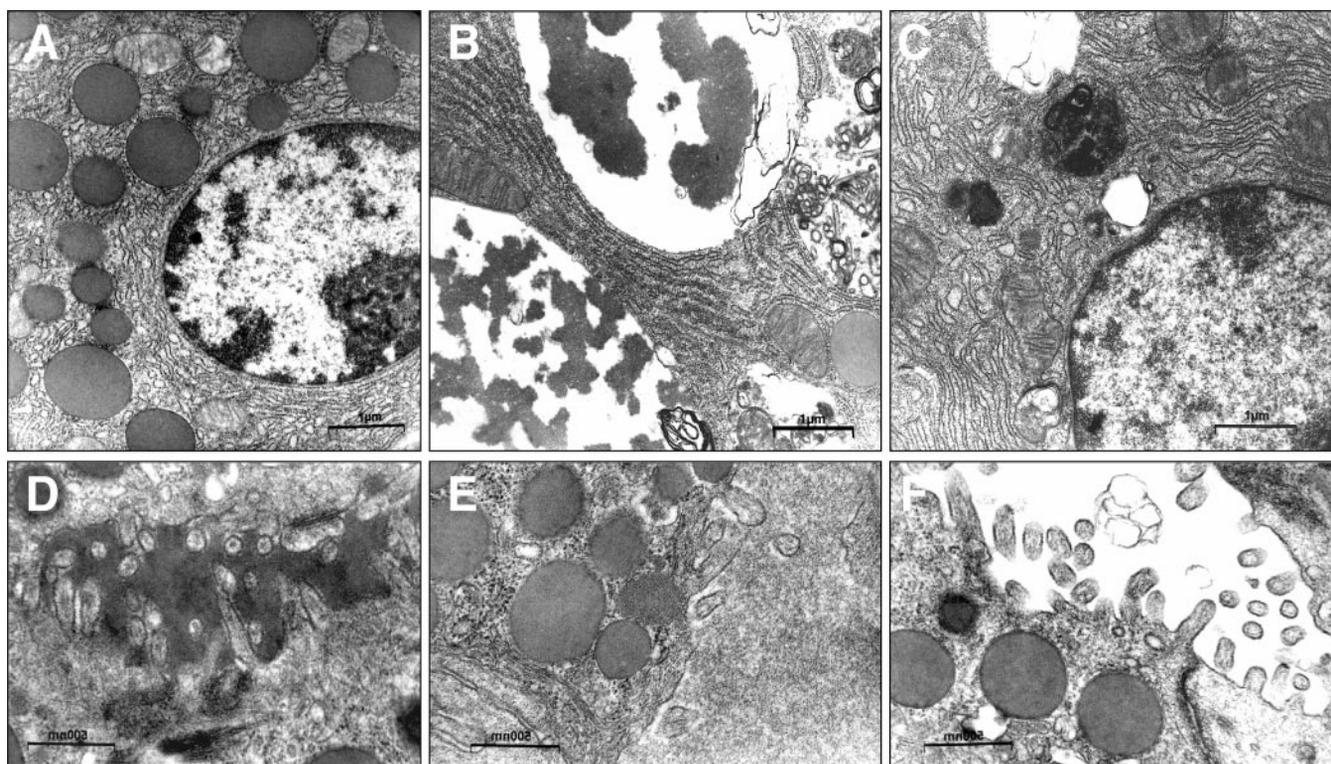


Fig. 7. Effect of ZVP preconditioning on cerulein-induced ultrastructural alterations of rat pancreatic acinar cells. Experimental design as in Fig. 2. The evaluation was performed by a pathologist blinded to the experimental protocol. Representative electron micrographs of pancreatic acinar cells from control rats (A, D) and from cerulein-treated rats either without (B, E) or with ZVP preconditioning (C, F). Note cytoplasmic vacuole formation in response to cerulein hyperstimulation that is markedly reduced when ZVP was given before cerulein (compare B and C). Additionally, changes in the apical cytoskeletal elements associated with cerulein hyperstimulation are less pronounced following ZVP preconditioning (compare E and F).

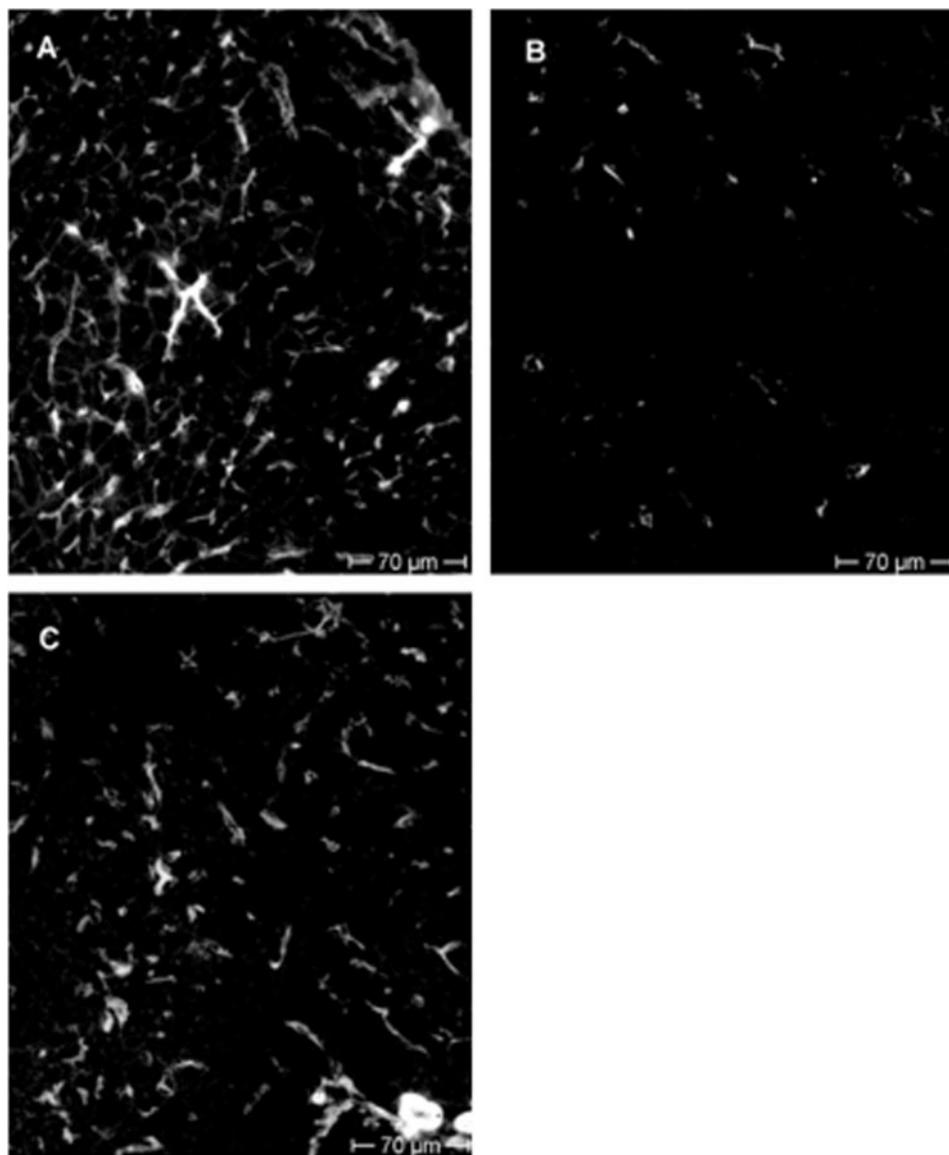


Fig. 8. Effect of ZVP preconditioning on cerulein-induced changes in the actin cytoskeleton of rat pancreatic acinar cells. Experimental design as in Fig. 2. The evaluation was performed by a pathologist blinded to the experimental protocol. Bodipy FL phalloidine staining of the actin microfilaments in pancreatic acinar cells of control rats (A) and of cerulein-stimulated rats pretreated either without (B) or with ZVP (C) showing that the loss of actin labeling associated with cerulein hyperstimulation is less pronounced when ZVP was given before cerulein hyperstimulation.

content, which was considered to represent pancreatic edema. In fact, the activities of amylase and lipase decreased by 46 ($P < 0.01$) and 62% ($P < 0.01$), respectively, when cerulein was given after ZVP treatment (Fig. 9, A and B). The pancreatic edema was reduced by 60% ($P < 0.05$), thus confirming our morphological observations (Fig. 9C).

DISCUSSION

In the present study, we investigated the role of calpain activation in acute cerulein pancreatitis. To specifically explore which of the calpain isoforms is activated in pancreatic tissue in response to cerulein hyperstimulation, we performed Western blot analysis. In preliminary experiments with purified porcine μ -calpain and m-calpain, we found that the antibodies chosen for immunoblotting reacted specifically with the corresponding form of calpain and not with the other (data not shown). We also found that the antibodies can be used for the immunochemical detection of calpain activation. Specifically, anti- μ -calpain antibody pAB1 and anti-m-calpain antibody pAB2 have been observed to be good tools. Unlike pAB3 and

pAB4, these antibodies identified not only the autolysis of the respective 80-kDa subunit associated with calpain activation but also recognized different autolysis products of the large subunit (10, 24). With the use of these antibodies, our results clearly indicate that both calpain isoforms are constitutively expressed in rat pancreatic tissue, thus reconfirming our recent immunohistological findings (43). In addition, our data indeed provide evidence that stimulation of rats with supramaximal doses of cerulein leads to activation of both μ -calpain and m-calpain. This is further supported by the observation that administration of calpain inhibitor ZVP before cerulein treatment clearly reduces the activation of both calpains. ZVP is a hydrophobic cell-permeable dipeptidyl methyl ester, containing a benzyloxycarbonyl residue at the NH_2 terminus. Studies by others (8, 16, 17) using this low molecular weight inhibitor have shown that it is relatively specific for calpain and highly potent, causing sustained inactivation of the protease. The mechanism of action probably involves a covalent interaction between an electrophilic center of the inhibitor and the thiol group of the active site cysteine of calpain (31).

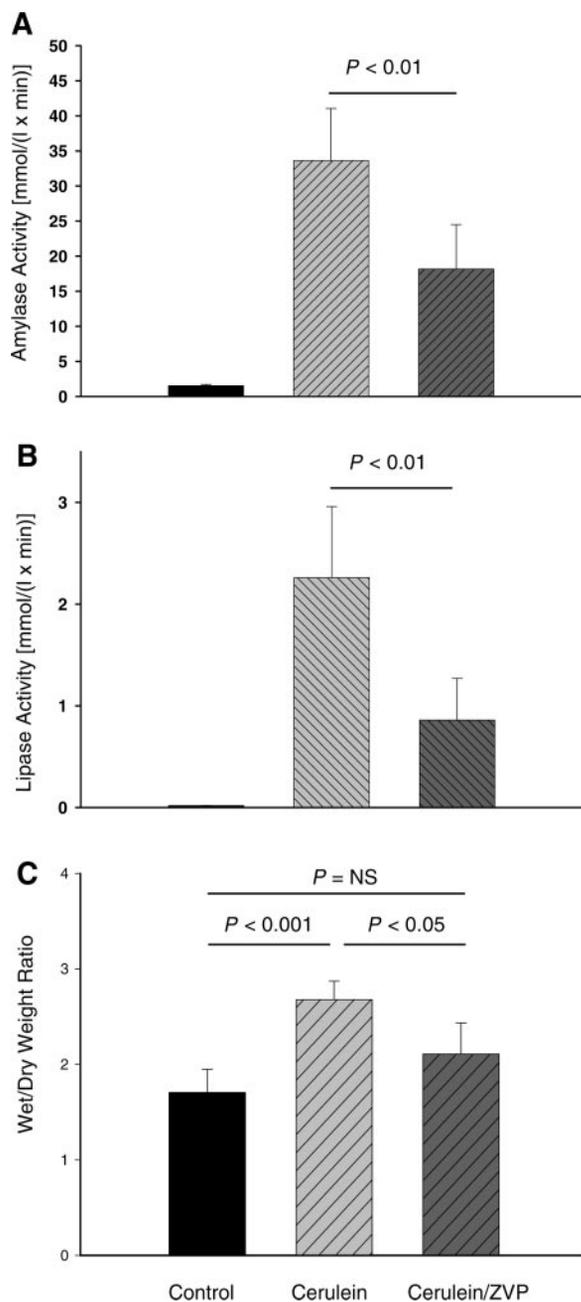


Fig. 9. Effect of ZVP on plasma amylase (A) and lipase activities (B) and pancreatic edema (C) in cerulein-induced pancreatitis. Experimental design as in Fig. 2. Note ZVP pretreatment significantly reduces the extent of cerulein-induced hyperamylasemia, hyperlipasemia, and edema formation. The results are expressed as means + SD; *P* values were calculated by *t*-test. NS, not significant.

The activation as well as the activity of calpain are Ca^{2+} -dependently regulated by their endogenous inhibitor calpastatin (22). Thus calpain activation requires both increased cytosolic Ca^{2+} and inadequate calpastatin activity. A sustained cytosolic Ca^{2+} increase has previously been reported to occur in response to cerulein hyperstimulation (4, 30). Consequently, here we investigated whether cerulein-induced calpain activation may be associated with a calpastatin downregulation. Our immunoblot analyses reveal for the first time that rat pancreatic tissue contains multiple calpastatin forms. In isolated rat pancreatic acinar cells, we observed a similar protein pattern,

further confirming the findings in the pancreatic tissue. Consistent with our observation, calpastatin has been found to be present in different multiple forms in different tissues and mammalian species, likely resulting from alternative splicing, mutations, or posttranslational modifications such as phosphorylation and dephosphorylation (19, 20, 38). In addition, low molecular weight calpastatins can be produced by conservative proteolytic degradation of a high molecular precursor (7). The expression of multiple calpastatin forms is supposed to be relevant for the specific regulation of different calpain isoforms normally present in a single cell type (20).

Cerulein hyperstimulation of rats caused marked alterations of the pancreatic calpastatin pattern, possibly leading to loss of inhibitory activity. Similarly, a downregulation of calpastatin accompanied by a decline in calpastatin activity has been observed in various injury models, such as in rat hearts and renal cortex during ischemia-reperfusion (35, 36) and in erythrocytes of hypertensive rats (7). There is evidence that the decrease in calpastatin is caused by nonspecific proteolytic degradation induced by different mechanisms. In erythrocytes of hypertensive rats, during rat cerebral hypoxia-ischemia, and TNF-induced apoptosis in U937 cells, calpain by itself was responsible for this finding (3, 7, 27). In contrast, during rat renal ischemia-reperfusion and in several cellular apoptotic systems, members of another family of cysteine proteases, the caspases, were identified to cleave the calpain inhibitor (27, 35, 41). Our results showing a cerulein-mediated fragmentation of the pancreatic calpastatin may indicate a proteolytic process as well. However, on the basis of our finding that ZVP has no effect on cerulein-induced calpastatin alterations, we conclude that proteases other than calpain have to be responsible for this phenomenon. Whether caspases provoked the calpastatin degradation, as observed in other systems, or other proteases activated during acute pancreatitis remains to be elucidated.

The decrease in calpastatin may represent critical situations under which calpain activity cannot be regulated within physiological ranges, consequently leading to cell damage. Consistent with this assumption, our data reveal that cerulein-induced pancreatic injury requires activation of calpainlike protease. Thus inhibition of the calpain activity by treatment of rats with calpain inhibitor ZVP before cerulein administration indeed resulted in less-severe pancreatic damage. In particular, the pancreatic edema, the acinar cell vacuolization, as well as cytoskeletal alterations, characteristic histological features of cerulein-induced pancreatitis, were obviously reduced (11, 14). Certainly, we cannot rule out that proteases other than calpain account for some of the ZVP effects reported here. However, previous studies (8, 16, 17) showing that ZVP reduced the calpain activity to a great extent in hypoxic isolated renal cells and during ischemia-reperfusion in the liver, resulting in marked attenuation of cell damage, support our conclusion. Further support is provided by the observation that ZVP has only minimal inhibitory effects on other cysteine proteases such as cathepsin B and several caspases such as caspase-1, -2, -3, or -7 (8, 17). Finally, in our own in vitro preliminary experiments, ZVP was found to only minimally inhibit caspase-8 and the serine proteases trypsin and chymotrypsin (data not shown).

The fact that ZVP induced merely a partially protective effect suggests that, apart from calpainlike proteases, other enzymes or factors concomitantly or independently contribute

to morphological pancreas damage evoked by cerulein. In this regard, a similar protection was found by using the synthetic inhibitor E3123, which blocks trypsin, plasmin, elastase, and phospholipase A2 activities (25). Accordingly, the activation of digestive zymogens such as trypsinogen, procarboxypeptidase A1, procarboxypeptidase B, and chymotrypsinogen in pancreatic acini as a consequence of supramaximal secretagogue stimulation has been demonstrated (12, 30, 34).

Regarding the mechanisms underlying calpain-mediated pancreatic damage, we can only speculate at this time. Some of the many calpain substrates include various actin-associated proteins such as α -actinin, spectrin, talin, and ankyrin that are involved in maintaining and regulating of cytoskeletal structures and functions (6, 33). There is increasing evidence that nonphysiological calpain activation is coincident with degradation of cytoskeletal proteins. In particular, calpain activated during ischemia or hypoxia in different systems was observed to cleave spectrin and ankyrin (2, 13, 35, 47). In isolated hepatocytes exposed to oxidative stress, a close relationship between calpain activation and degradation of talin and α -actinin was proven (23). Additionally, calpain inhibitors attenuated cleavage of actin and actin-associated proteins, resulting in cytoprotection against toxic injury, ischemia, oxidative stress, as well as apoptosis (2, 9, 23, 39). Accordingly, our ultrastructural findings showing a beneficial effect of ZVP against cerulein-mediated alterations of the actin cytoskeleton suggest that a similar injurious mechanism may also be important in our system. However, because calpain modifies a wide variety of substrates, in particular those playing a role in signal transduction, one can expect that further calpain-induced mechanisms may likewise contribute to cerulein-mediated acinar cell injury.

In summary, our study demonstrates that cerulein hyperstimulation causes a decrease in the calpastatin level, which may contribute to the activation of both calpain isoforms observed in parallel. Prophylactic administration of calpain inhibitor ZVP has no protective effect on calpastatin alterations but significantly reduces calpain activation. In correlation to the decreased calpain activity, the severity of cerulein pancreatitis was clearly diminished, implicating a role of calpain in the onset of acute pancreatitis. Consequently, prophylactic application of calpain inhibitors may be a promising strategy for alleviating the pancreatic damage occurring during pancreas transplantation including organ preservation. Whether ZVP exerts a beneficial effect on preestablished pancreatitis remains to be investigated. We believe that the inhibitor is likely to prove ineffective, because the protease activation has already occurred.

GRANTS

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