Subcellular kinetics of early trypsinogen activation in acute rodent pancreatitis

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Mithöfer, Kai, Carlos Fernández-Del Castillo, David Rattner, and Andrew L. Warshaw. Subcellular kinetics of early trypsinogen activation in acute rodent pancreatitis. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G71–G79, 1998.—To investigate the debated role of intracellular trypsinogen activation and its relation to lysosomal enzyme redistribution in the pathogenesis of acute pancreatitis, rats were infused with the cholecystokinin analog caerulein at 5 µg·kg⁻¹·h⁻¹ for intervals up to 3 h, and the changes were contrasted with those in animals receiving saline or 0.25 µg·kg⁻¹·h⁻¹ caerulein. Saline or 0.25 µg·kg⁻¹·h⁻¹ caerulein did not induce significant changes. In contrast, 5 µg·kg⁻¹·h⁻¹ caerulein caused significant hyperamylasemia and pancreatic edema within 30 min. Pancreatic content of trypsinogen activation peptide (TAP) increased continuously (significant within 15 min). TAP generation was predominantly located in the zymogen fraction during the first hour but expanded to other intracellular compartments thereafter. Cathepsin B activity in the zymogen compartment increased continuously throughout the experiments and correlated significantly with TAP generation in the same compartment. Total trypsinogen content increased to 143% with marked interstitial trypsinogen accumulation after 3 h. Supramaximal caerulein stimulation causes trypsinogen activation by 15 min that originates in the zymogen compartment and is associated with increasing cathepsin B activity in this subcellular compartment. However, a much larger pool of trypsinogen survives and accumulates in the extracellular space and may become critical in the evolution of necrotizing pancreatitis.

Pancreas; protease activation; acinar cell; experimental; cathepsin B

One hundred years ago Chiari (5) suggested that autocidigestion by premature extraintestinal activation of the digestive enzyme precursors is responsible for the histopathological changes in acute pancreatitis. Only recently has proof of intrapancreatic protease activation been found in both human (12) and experimental pancreatitis of different etiologies (3, 28, 33, 34, 58). However, the events leading to this extraintestinal activation are still obscure. Multiple biochemical and morphological abnormalities have been detected within the acinar cell in models of acute pancreatitis, including alterations of enzyme sorting and compartmentation (1, 23, 42–45, 54, 55), and current investigations are focusing on possible intracellular trigger mechanisms for premature zymogen activation (1, 14, 23, 26, 28, 42–44, 54–56). Immunocytochemical studies have described subcellular colocalization of digestive enzyme precursors and lysosomal enzymes in crinophagic autophagic vacuoles in several experimental models of acute pancreatitis (1, 45, 54, 55). Subcellular fractionation techniques demonstrated a coincident increase of lysosomal enzyme activity in the zymogen compartment, which includes crinophagic vacuoles (29, 43, 56). Because previous studies have demonstrated the capability of the lysosomal enzyme cathepsin B to activate trypsinogen in vitro (11, 15), it has been suggested that such an intracellular admixture of cathepsin B and trypsinogen may cause intracellular activation of trypsinogen (43, 49, 50, 54). Inasmuch as trypsin triggers the activation of the pancreatic enzyme cascade (40), it is speculated that cathepsin B-induced intracellular trypsinogen activation may be an early event in the pathophysiological processes leading to autodigestion of the gland (43, 50). However, evidence of trypsinogen activation in association with the subcellular changes of cathepsin B activity has never been demonstrated, partly because of the technical difficulty of reliably quantitating subcellular trypsin activity (17). Moreover, because colocalization of zymogens and lysosomal enzymes has recently been demonstrated in normal pancreatic cells (29, 45) and because the extent of this phenomenon does not seem to correlate with the severity of the disease (29), its relevance in the development of acute pancreatitis and its progression to a panandular autodigestive disease is currently a matter of debate (13, 17, 51).

The conversion of trypsinogen to active trypsin occurs by cleavage of a small trypsinogen activation peptide (TAP), which is easily assayed and provides a quantitative index of active trypsin generation (10, 19). Using TAP as a stable, equimolar measure of trypsinogen activation in nanomolar quantities, we studied the kinetics and subcellular localization of early pancreatic trypsinogen activation, its possible association with the intracellular changes of lysosomal enzyme activity, and its relationship to intrapancreatic trypsinogen distribution in experimental pancreatitis induced by supramaximal secretagogue stimulation.

In this study of acute edematous pancreatitis induced by supramaximal caerulein stimulation, we report that there is very early ectopic activation of trypsinogen that originates in the zymogen compartment and that may be caused by the increasing cathepsin B activity in the same subcellular compartment. However, a large pool of not yet activated trypsinogen survives and accumulates in the extracellular compartment. Our observations suggest that interstitial rather than intracellular protease activation may be critical in the evolution from the mild edematous pancreatitis of this model to severe necrotizing pancreatitis.
MATERIALS AND METHODS

Materials. H-arginyI-arginine-β-naphthylamide was purchased from Bachem Biosciences (Philadelphia, PA). Bovine pancreatic trypsin inhibitor (BPTI) (N-9389) were obtained from Sigma (St. Louis, MO). Rabbit labeled extravidin (E-2636), and goat anti-rabbit immunoglobulin G (B-9642), phosphatase-conjugated rabbit anti-(Asp)p-Lys antiserum were kindly provided by J. Herman-Taylor. Caerulein was obtained from Farmitalia.

Experimental protocol. The study was approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital and performed according to the Guide for the Care and Use of Laboratory Animals, (Department of Health and Human Services Publication No. (NIH) 85–23).

Materials were prepared and diluted in Tris buffer (100 mM; pH 7.4) and supplemented with soybean trypsin inhibitor (100 µg/ml) to prevent artificial protease activation. All other chemicals were of reagent grade and purchased from Sigma.

Subcellular fractionation. Isolation of subcellular acinar fractions was achieved by differential centrifugation as described by Tartakoff and Amieson (52) and DeLisle et al. (6) with slight modifications. Briefly, samples were homogenized in a Brinkman Polytron (Brinkman Instruments, Westbury, NY) for 30 s. After subsequent centrifugation (1,500 rpm, 10 min, 4°C), the resulting supernatants were coded and stored at −70°C until assayed. For measurements of TAP in the isolated pancreatic fractions, samples were obtained from each individual fraction diluted in Tris buffer (100 mM; 1:5, vol/vol) and boiled, coded, and stored as above. Aliquots were simultaneously assayed for TAP by competitive enzyme-linked immunosorbent assay. Briefly, samples were added into microtiter plates, coated for 12 h (4°C) with albumin-(Asp)p-Lys conjugate, and incubated with rabbit anti-(Asp)p-Lys antiserum (1:400) at 25°C for 60 min. Plates were washed three times and then incubated with biotin-labeled goat anti-rabbit immunoglobulin G (1:1,000) at 25°C for 30 min. After additional washing, plates were incubated with alkaline phosphatase-extravidin conjugate (1:750) for 30 min. With p-nitrophenyl phosphate used as a substrate, color development was followed for 30 min; absorption at 405 nm was measured in a Dynatech MR 5000 microplate reader (Dynatech Laboratories, Chantilly, VA) and compared with a standard curve. As previously described, this assay has a lower detection limit of TAP of 5 × 10⁻¹² M (10, 16, 19, 35).

Pancreatic trypsinogen. For determination of subcellular changes of pancreatic trypsinogen, samples from isolated subcellular fractions were incubated with excess enterokinase (10 U/l) in Tris-HCl buffer containing 7.5 mM CaCl₂ at pH 8.0 (25°C) for 60 min, allowing for complete activation of trypsinogen contained within the sample (35). The concentration of the released TAP was then assayed as described, and trypsinogen content was calculated by subtracting spontaneous TAP levels from the value measured after enterokinase activation.

Cathespin B. Fractional activity of cathespin B was measured according to the fluorometric technique described by McDonald and Ellis (31), using H-arginyl-arginine-β-naphthylamide as substrate and defined concentrations of unconjugated fluorescent β-naphthylamine for fluorometric calibration. The enzyme was allowed to activate in 50 mM Tris-HCl buffer containing 10 mM diethyrtol and 10 mM EDTA (37°C, pH 6.5) for 5 min. After addition of the substrate, changes of fluorescence intensity were continuously monitored in a SPEX-Flurolog 2 spectrofluorometer system (SPEX Industries, Edison, NJ) using emission and excitation maxima of 410 and 335 nm, respectively (2). Rates of hydrolysis were established by comparing the increase of fluorescence to the standard curve relating fluorescence intensity to β-naphthylamine concentration.
Interstitial trypsinogen/TAP ratio. The edematous interstitial fluid that had accumulated in five animals receiving caerulein stimulation at 5 µg·kg⁻¹·h⁻¹ for 3 h was collected by fine-needle aspiration (ID 0.7 mm) after microdissection of the pancreatic interstitial septae with careful sparing of the pancreatic acinar tissue. The aspirated fluid was immersed in Tris-HCl buffer as above (1:1, vol/vol), and the interstitial trypsinogen and TAP concentration was measured as described earlier.

Data analysis. The data are presented as means ± SE of at least four separate experiments in each group at each time point. Statistical significance was determined using Student’s t-test or Bonferroni two-tailed analysis of variance. Association between variables was tested using linear regression and correlation analysis. Significance was assumed for *P < 0.05. Deviation bars in the figures indicate SE values, whereas their absence indicates a SE value too small for illustration.

RESULTS

Serum amylase activity. No significant changes of serum amylase activity were observed in animals receiving saline infusion (31.4 ± 4.7 U/l). A transient increase of serum amylase was observed until 30 min of low-dose caerulein infusion, but levels returned to baseline values after 3 h (35.0 ± 3.0 U/l) (Fig. 1). In comparison, significant hyperamylasemia developed within 30 min of supramaximal caerulein stimulation (122.7 ± 19.8 U/l, *P < 0.05), increasing even further to 157.0 ± 21.3 U/l (*P < 0.01) at the end of the observation period (Fig. 1).

Pancreatic edema formation. Neither saline infusion nor 0.25 µg·kg⁻¹·h⁻¹ caerulein caused significant pancreatic edema (wet-to-dry weight ratio; 2.58 ± 0.2 and 2.75 ± 0.2, respectively). In contrast, pancreatic water content increased continuously in animals receiving caerulein at 5 µg·kg⁻¹·h⁻¹. The difference became significant by 30 min (3.67 ± 0.24, *P < 0.001) and increased significantly further up to 3 h (6.14 ± 0.40, *P < 0.001, 30 min vs. 3 h). At this time the accumulation of interstitial fluid was clearly visible, with up to an 8.5-fold increase of pancreatic wet-to-dry weight ratio (Fig. 2).

Pancreatic trypsinogen content and distribution. At baseline 51.7 ± 1.8% of total pancreatic trypsinogen was localized in the zymogen-rich compartment. The remaining distribution was 12.1 ± 3.5% in the LRF, 3.9 ± 1.1% in the microsomal fraction, and 28.4 ± 1.4% in the soluble fraction. This distribution pattern confirmed the quality of the subcellular fractionation procedure, as high levels of trypsinogen are expected to be physiologically present in the zymogen compartment (ZRF) and lumina of pancreatic ducts (PMF). No significant changes of pancreatic trypsinogen status were observed in animals infused with saline or 0.25 µg·kg⁻¹·h⁻¹ caerulein. At 3 h of physiological caerulein stimulation, the trypsinogen distribution was 49.4 ± 7% (ZRF), 9.0 ± 3.2% (LRF), 4.9 ± 2.4% (MRF), and 36.5 ± 5.2% (PMF), perhaps reflecting a slight increase in intraluminal trypsinogen secondary to stimulated exocrine secretion. Supramaximal caerulein stimulation increased overall pancreatic trypsinogen content to 143 ± 53% (not significant [NS]) of baseline values at 3 h. The main increase was observed in the PMF, which contained 44.6 ± 9.5% (NS) of the total fractional trypsinogen at 3 h (Fig. 3), whereas zymogen-rich (49.8 ± 7.9%), lysosome-rich (4.3 ± 1.4%), and microsomal fractions (1.8 ± 0.9%) did not show a relative increase.

Pancreatic TAP content and distribution. Pancreatic TAP content in control animals was 279 ± 31 nmol·l⁻¹·g⁻¹. Values were not significantly different after low-dose caerulein infusion (308 ± 35 nmol·l⁻¹·g⁻¹). There were no significant changes in the distribution of TAP among the individual fractions in the control or low-dose caerulein group. However, pancreatic TAP content in animals infused with 5 µg·kg⁻¹·h⁻¹ caerulein increased to 822 ± 128 nmol·l⁻¹·g⁻¹ within 15 min (P < 0.01) and to 5,081 ± 960 nmol·l⁻¹·g⁻¹ at 3 h.
Starting from an almost equal distribution of TAP between the isolated fractions at baseline, an increase of TAP generation, reflecting trypsinogen activation, was observed in all isolated fractions of animals treated with supramaximal caerulein (Fig. 5A). An immediate increase was observed in the ZRF, which became significant after 30 min (294 ± 61%, P < 0.05) and continued throughout the experimental period (539 ± 102% at 3 h, P < 0.01). Although changes of TAP levels were minor in the PMF within the first hour, a significant increase to 395 ± 63% (P < 0.01) was observed during the subsequent experimental period. The biphasic character of the changes of fractional TAP distribution within the 3 h of supramaximal caerulein stimulation became more obvious when the data were plotted for relative changes. As Fig. 5B demonstrates, early TAP generation predominantly occurred in the ZRF, reaching significance after 30 min (P < 0.05) and continuing until 1 h, when 50.6 ± 4.1% of all TAP were localized in the ZRF (P < 0.01). However, whereas TAP generation in the zymogen compartment continued after this early phase, TAP concentrations increased most rapidly in the PMF between 1 and 3 h (16.0 ± 3.2% vs. 31.5 ± 4.0%) (P < 0.05, 1 vs. 3 h).

Subcellular cathepsin B activity. The pattern of subcellular distribution of cathepsin B activity at baseline was 25 ± 3.8% (ZRF), 55 ± 2.5% (LRF), 8.5 ± 2.2% (MRF), and 12 ± 2.9% (PMF) and did not significantly change in animals receiving infusion of 0.9% NaCl or caerulein at 0.25 µg·kg⁻¹·h⁻¹. In contrast, supramaximal caerulein stimulation caused a relative increase of cathepsin B activity in the ZRF to 50 ± 5.2% (P < 0.01) and in the PMF to 21 ± 2.3% (P < 0.05) of total pancreatic activity, changes paralleled by a significant relative decrease of enzyme activity to 26 ± 2.7% (P < 0.01) in the LRF after 3 h (Fig. 6). When the relative increase of cathepsin B activity in the ZRF was plotted against the relative increase of TAP concentration in the same compartment, a positive correlation between both values was obtained (r = 0.87, P < 0.001; Fig. 7).
Interstitial trypsinogen-to-TAP ratio. Only minute amounts of free TAP were detected in the edema fluid, which accumulated in the pancreatic interstitium after 3 h of supramaximal caerulein stimulation (7.5 ± 2.8 nmol/l). Subsequent addition of enterokinase to the aspirated fluid resulted in a massive increase of TAP to 1,807 ± 740 nmol/l, indicating the presence of a large quantity of inert trypsinogen in the extracellular space in acute edematous pancreatitis (Fig. 8). Inasmuch as the edema fluid in these circumstances constitutes most of the tissue weight, the total interstitial trypsinogen content is very substantial.

**DISCUSSION**

Pancreatitis induced by supramaximal exocrine stimulation with the synthetic CCK analog caerulein, first described by Lampel and Kern (25), is characterized by marked interstitial edema, leukocyte infiltration, hyperamylasemia, and severe disturbances of acinar cell morphology and the secretory process. Similar changes are seen in human pancreatitis, which in the great majority of cases presents as an edematous inflammatory process that regresses without development of fulminant necrotizing disease (8). Because of its self-limited character, caerulein-induced pancreatitis offers the attractive possibility to observe the sequence of events that may lead to and cause premature protease activation before widespread autodigestion prevents any systematic investigation.

At baseline ~80% of pancreatic trypsinogen was contained within the zymogen-rich compartment (52%) and the PMF (28%), which includes zymogens already secreted into the lumen. This physiological distribution pattern confirms the validity and quality of our subcellular fractionation procedure. The increase of overall pancreatic trypsinogen content we observed in the pancreatitis model has been previously described and likely reflects in part the caerulein-induced blockage of apical exocytosis in the face of continuing protein synthesis within the acinar cell (42, 44). Although trypsinogen levels increased also in the ZRF, the most prominent increase was observed in the soluble PMF after 3 h. Using the same fractionation technique, Saluja et al. (44) also observed a progressive accumulation of [3H]phenylalanine pulse-labeled proteins and amylase in the soluble final supernatant after 3.5 h of supramaximal caerulein infusion. Because the final supernatant fraction reflects the combined changes in the cytosol, acinar lumina, and interstitium, they posited that the observed accumulation could be located 1)
in the extracellular space (interstitium or intraluminal), 2) the cytosol as a result of intracellular discharge from intact organelles, or 3) inside intact but fragile intracellular organelles that rupture during the homogenization procedure. Because the authors did not observe basolateral exocytosis by radioautography after 2.5 h of supramaximal caerulein infusion and detected pulse-labeled proteins only in zymogen granules and large cytoplasmic vacuoles, they concluded that the recovery of zymogens in the PMF would result from accumulation within fragile intracellular organelles and seemingly excluded interstitial or cytosolic zymogen accumulation. However, a recent study from our laboratory suggested that a significant interstitial trypsino- gen pool exists in caerulein pancreatitis and that this repository of trypsigen can be activated by enterokinase to produce lethal necrohemorrhagic pancreatitis (10). Because the interstitial pool of trypsigen may thus represent a critical component in the progression from acute edematous pancreatitis to fulminant necrotizing pancreatitis, as seen in about 15% of human cases (8), we wished to distinguish between the trypsion and trypsigen contents of the components of the PMF, that is, the cytosolic and interstitial components. This analysis was only possible at 3 h when edema was marked enough for safe collection of interstitial fluid without acinar cell damage (as confirmed by the minute amounts of TAP in the interstitial aspirates), but this is probably the time point of greatest importance. Our findings clearly demonstrate that large amounts of native (uncleaved) trypsion are present in the intercellular space in acute pancreatitis at 3 h. This finding suggests that much of the observed increase of the zymogen in the PMF was caused by its interstitial accumulation.

Although this finding might seem to conflict with the cited work of Saluja et al. (44), a later study from the same group (42) demonstrated that maturation of condensing vacuoles into zymogen granules and intracellular protein transport is impaired during caerulein hyperstimulation, thereby delaying the appearance of newly synthesized, labeled zymogens in the mature zymogen granules. Their observation that newly synthesized proteins were still contained within maturing or cistophagic organelles at 3 h may explain their failure to detect labeled proteins in the interstitium after 2.5 h in the earlier study (44). In addition, previously stored unlabeled zymogens could have already accumulated in the interstitium at 2.5 h and would not be detected without detection by radioautography. By 3 h Saito et al. (42) had begun to detect labeled proteins in the interstitium.

Supramaximal stimulation of the pancreatic acinar cell with caerulein induces a block of the normal secretion of zymogens from the cell into the duct lumen but does not, as noted, prevent the ongoing synthesis of zymogens. Because blocked normotopic discharge with persisting zymogen synthesis cannot indefinitely continue without overwhelming the storage capacity of the acinar cell, abnormal export or leakage of the accumulated zymogens is predictable. Basolateral exocytosis of zymogen granules or vacuoles derived from intracellular zymogen fusion has in fact been described as early as 10–15 min after the start of caerulein hyperstimulation (1, 47) and offers a plausible mechanism for the high interstitial trypsigen concentration found in the present study. Such interstitial discharge might also provide a morphological basis for the increase of serum amylase levels consistently observed by us and others during excessive secretagogue infusion (1, 10, 30, 42–44). Demonstration of increasing serum levels of the zymogen granule protein GP2 in caerulein-induced pancreatitis further supports this interpretation (27). Alternatively, increased ductal permeability secondary to structural changes in the pancreatic duct tight junction seal (7) with consequent leakage of intraluminal content back into the interstitium could also contribute to the observed trypsigen accumulation and may thus explain the early absence of secretory products in the luminal space (25, 47).

Although our finding of large quantities of uncleaved trypsigen in the interstitium is potentially of great importance in the pathogenesis of necrotizing pancreatitis, this latter development occurs only in a minority of human cases and is likely to be a second-tier phenomenon. Much evidence has accumulated that ectopic intracellular trypsogen activation plays a role in the early evolution of pancreatic cell injury. Activation of serine proteases in caerulein pancreatitis has been previously described (3, 28, 56, 58). Protease activity in these earlier studies was measured indirectly by radioimmunoassay or spectrophotometry of colorimetric or fluorometric products liberated by proteolysis. However, under assay conditions, which included delays of up to 4 h between sampling and measurements, trypsion autodegradation occurs readily (31, 41) and has in fact been observed (3). That problem may well explain the failure to detect trypsion activity after CCK stimulation in vitro by Leach et al. (26). Also, the presence of other active proteases or intrinsic protease inhibitors in pancreatic tissue samples affects the sensitivity of these assay techniques and impairs their ability to provide accurate quantitative data (20). We therefore sought an alternative trypsion-specific method for investigating the subcellular events of pancreatic trypsogen activation in caerulein pancreatitis.

The activation of trypsogen occurs by highly specific cleavage after the P1 lysine residue of the amino terminal (Asp)_{11}Lys recognition sequence of trypsogen, with subsequent release of an activation peptide (TAP), and active conformation of trypsion (19, 20). Measurement of the small and stable activation peptide, which does not influence the activation process (22), by a well-established enzyme-linked immunosorbent assay (10) provides a reliable, equimolar index of subcellular changes of trypsogen activation at nanomolar quantities. This method is free of the shortcomings inherent to the previously used techniques (9, 19, 33, 34).

A small amount of TAP was found in both pancreatic tissue homogenates and fractions at baseline. As in previous studies (10, 33, 34, 36), these levels remained
unchanged in control and low-dose caerulein animals throughout the experimental period and may reflect the small amount of limited physiological activation that occurs in the normal pancreas (3, 14, 28, 58). A significant increase of trypsinogen activation was seen in pancreatic tissue homogenates as early as 15 min after the start of supramaximal caerulein infusion, and TAP levels increased progressively until the end of the experiments. The early and rapid increase of pancreatic TAP content supports the hypothesis that premature intrapancreatic activation of digestive enzymes may be an early or even an initiating event in the evolution of secretagogue-induced experimental pancreatitis (49, 56).

Within the first hour TAP generation was largely confined to the ZRF, reaching significance at 30 min. These results are supported by the study of Bialek et al. (3), who also detected an increase of serine protease activity in their subcellular fraction of secretory granules and vacuoles in caerulein pancreatitis. Although TAP generation in the ZRF continued until the end of the experiments, a proportionally greater increase of TAP was observed in the soluble PMF between 1 and 3 h. As the analysis of the interstitial aspirates at 3 h shows, this fractional TAP increase reflects an intracellular process because TAP levels in the interstitial component of the PMF were negligible. Thus TAP could either be located in the cytosol after discharge or spillage from degrading organelles or still contained inside intracellular organelles that become increasingly fragile and rupture during the fractionation process. Diffuse staining of the cytoplasm with antizymogen immunofluorescence during exocrine hyperstimulation (54) favors the first option, whereas the latter possibility is supported by the finding of increasing membrane fragility of crinophagic organelles with their rapid disruption during homogenization in caerulein pancreatitis (44). The present study cannot answer this question. However, the protection afforded by supplemental protease inhibitors, which reduce hyperamylasemia, edema, and the changes of intracellular organelle integrity, lysosomal enzyme distribution, and the secretory pathway (18, 37, 38, 57), supports the likelihood of free cytosolic proteases. These are known to promote cytoskeletal disassembly and cellular dysfunction by proteolytic depolymerization of intracellular microtubules and microfilaments (21, 53).

The question remains as to how the activation of trypsinogen is initiated. Previous investigators have speculated that intracellular colocalization of lysosomal enzymes and trypsinogen may be the initiating event because previous studies have demonstrated the capability of cathepsin B to activate trypsinogen in vitro (11, 15). In accordance with other reports, no significant changes of enzyme distribution were induced by infusion of saline or 0.25 µg·kg⁻¹·h⁻¹ caerulein (43), but during caerulein hyperstimulation there was a continuous increase of cathepsin B activity in the ZRF and PMF in parallel with a relative decrease of its activity in the LRF. Comparable distribution changes have been reported by others (24, 43).

Although the hypothesis that exposure of trypsinogen to cathepsin B is responsible for activation of the former logically should require that increased cathepsin B activity be demonstrable before or at least coincident with TAP generation, that temporal relationship is not delineated precisely in the present experiments. Both cathepsin B and TAP were increased in the ZRF at 30 min, although the change in cathepsin B did not achieve statistical significance until the following determination. There was a high baseline activity of cathepsin B (25%) in the ZRF, which is the product both of the well-recognized limitation of the ultracentrifugation technique for separating "heavy" lysosomes from zymogen granules (18, 24, 43, 45, 52) and of the physiological colocalization of zymogens and lysosomal enzymes (56). Whatever the explanation of the high baseline cathepsin B, it did not cause trypsinogen activation or TAP generation in either the saline or physiological caerulein controls. The data, supported by the correlation depicted in Fig. 7, show that only the further increase above baseline is associated with trypsinogen cleavage in the zymogen granules, although the high baseline limits the sensitivity of the method for detecting initial small increments in cathepsin B activity that might be meaningful.

The finding that preincubation of isolated acinar cells with the cysteine protease inhibitor E-64 did not prevent CCK-induced activation of procarboxypeptidase has previously raised doubts that cathepsin B is the agent of protease activation (26). However, a recent in vitro study has demonstrated that E-64 achieves only incomplete inhibition of cathepsin B. Complete inhibition by the more potent agent E-64d prevented protease activation induced by caerulein hyperstimulation of pancreatic acinar cells (46).

Despite the intracellular trypsinogen activation documented in this study, pancreatic autodigestion did not occur in caerulein pancreatitis by 3 h. Because the observation period is relatively short, one could conclude that at this early point the activation process is still contained within the acinar cell (49). However, in caerulein pancreatitis no progression to necrotizing disease occurs even later. In fact, edema is completely reabsorbed, and only minimal single-cell necrosis develops (25). It therefore appears that intracellular trypsinogen activation in this model does not reach the level that will cause panglandular autodigestion. Thus, even if there is a true causal relationship between cathepsin B redistribution and trypsinogen activation, the phenomenon is not powerful enough to cause fulminant necrotizing pancreatitis, as previously proposed (51). Our interpretation is also consistent with the lack of correlation between the extent of cathepsin B redistribution and disease severity (29). The observation that ischemia superimposed on hyperstimulation causes progression to severe autodigestion (39) whereas ischemia alone does not cause such extensive damage (33), may suggest that intracellular derangements such as those induced by caerulein hyperstimulation sensitize and predispose the gland to necrosis. In this setting the accumulation of trypsinogen in the interstitium may
assumed critical pathophysiological significance because its activation has been shown to result in the development of massive pancreatic necrosis (10), but the trigger for the activation of the interstitial pool of trypsinogen remains unknown. If the second injury fails to occur within a limited time frame, the interstitial zymogens are presumably washed out by the increased pancreatic capillary perfusion in edematous pancreatitis (48).

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REFERENCES


