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Research Article

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Intracellular Activation of Digestive Zymogens in Rat Pancreatic Acini

Stimulation by High Doses of Cholecystokinin

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Abstract

The mechanism by which digestive zymogens become activated during acute pancreatitis remains poorly understood. Given the ability for cholecystokinin (CCK) to induce pancreatitis in vivo, the effects of high dose CCK on preparations of isolated pancreatic acini were examined. Using an immunologic technique for the detection of zymogen activation, CCK was found to stimulate the conversion of procarboxypeptidase A1 to a 35-kD form having the same net charge and electrophoretic mobility as purified recombinant carboxypeptidase A1. This enhanced conversion was proportional to the dose of CCK (maximal at 100 nM), and time dependent. CCK also produced changes in the electrophoretic mobility of procarboxypeptidase B and chymotrypsinogen 2 immunoreactivity, consistent with activation of these zymogens. These events were detectable only within acinar cell pellets and not in the incubation medium, suggesting an intracellular site of conversion. The conversion of procarboxypeptidase A1 to its active form was inhibited by pretreatment with the weak base chloroquine (40 μM) and the protonophore monensin (10 μM). This conversion was also inhibited by pretreatment with the serine protease inhibitor benzamidine (10 mM) but not the cysteine protease inhibitor E64 (100 μM). The results suggest that high dose CCK stimulates the intracellular activation of digestive zymogens within isolated pancreatic acini. This event appears to require an acidic subcellular compartment and serine protease activity. (*J. Clin. Invest.* 1991. 87:362–366.) Key words: pancreas • acinar cell • proteases • pancreatitis • cholecystokinin

Introduction

The exocrine pancreas synthesizes, stores, and secretes a subpopulation of digestive enzymes as inactive precursors or zymogens. The premature activation of these proteolytic and lipolytic zymogens has been assigned a central role in the pathogenesis of acute pancreatitis (1). This concept of "autodigestion" is supported by the detection of activated digestive enzymes within the pancreas in both clinical (2) and experimental forms of pancreatitis (3, 4).

The mechanism and location of pathologic zymogen activation during acute pancreatitis remain unknown. Under normal conditions, pancreatic trypsinogen is converted to trypsin in the intestinal lumen following contact with the brush border enzyme enteropeptidase. Trypsin, in turn, initiates activation of other digestive zymogens. In addition to enteropeptidase, two other enzymatic mechanisms for the conversion of trypsinogen to trypsin have been demonstrated. These include trypsinogen-mediated autoactivation (5, 6) and activation mediated by the lysosomal cysteine protease cathepsin B (6, 7).

Recent investigations have speculated that digestive zymogen activation during acute pancreatitis may occur within the acinar cell itself (8). In particular, the possibility that lysosomal cathepsin B may be responsible for the intracellular generation of activated trypsin has received attention (9). This proposal is based upon the observation that secretory zymogens and lysosomal hydrolases become colocalized within cytoplasmic vacuoles during various experimental forms of pancreatitis (8). In two specific models of experimental pancreatitis, similar vacuoles have been shown to have an acidic internal pH, consistent with the possibility of lysosomal acid hydrolase activity (10).

Further elucidation of the mechanisms responsible for pathologic zymogen activation has been hindered by the lack of a suitable cellular system in which to investigate this process. High doses of cholecystokinin (CCK)[1] or its analogues are capable of initiating pancreatitis in the intact animal (11). To determine if CCK has the ability of directly generate changes in pancreatic acinar cells which might lead to the intracellular activation of digestive zymogens, we exposed isolated pancreatic acini to high concentrations of CCK. This treatment resulted in the rapid (within 15 min) conversion of several zymogens to lower molecular weight species that were consistent with the active forms of each enzyme. Further, this conversion occurred within the cell and required an acidic intracellular compartment.

Methods

Materials. Acridine orange, BSA, benzamidine, chloroquine sulfate, cycloheximide, DTT, E64, and monensin were obtained from Sigma Chemical Co., St. Louis, MO; crude collagenase (type 3) and trypsin-TPCK from Worthington Biochemical Corp., Freehold, NJ; cholecystokinin octapeptide from Squibb Diagnostics, New Brunswick, NJ; *N*-CBZ-arg-arg-4-methoxy-B-naphthylamide from Bachem, Inc., Torrance, CA; ^{125}I-labeled goat anti-rabbit IgG from ICN Biochemi-

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1. *Abbreviation used in this paper:* CCK, cholecystokinin.

cals, Irvine, CA; and Immobilon-P transfer membranes from Millipore/Continental Water Systems, Bedford, MA.

Preparation of isolated pancreatic acini. Male Sprague-Dawley rats (125–150 g) were fasted overnight, anesthetized with ether, and killed by cardiac puncture. Isolated pancreatic acini were prepared by enzymatic digestion and gentle mechanical disruption (12), using collagenase further purified by gel filtration chromatography as previously described (13). Acini were incubated in medium containing 98 mM NaCl, 25 mM Hepes, 4.8 mM KCl, 2.5 mM KH_2PO_4, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.2% BSA, and 0.05% DTT (pH 7.4) under continuous oxygenation at 37°C without exogenous protease inhibitors. After a 30-min equilibration period, various doses of CCK were added. Unstimulated samples received an equivalent volume of medium alone. In selected experiments, pretreatment with specific agents was performed during the equilibration period.

Detection of activated zymogens. At the end of each incubation period, acinar cell proteins were solubilized in 3% SDS, boiled, and subjected to electrophoresis on SDS-polyacrylamide (7.5%) gels (14). In certain experiments, two-dimensional (isoelectric focusing/SDS-PAGE) analysis was performed (15, 16). Following electrophoresis, proteins were transferred to Immobilon membranes, and immunoblotting (17) was performed with one of the three rabbit polyclonal antisera described below. After incubation with the primary antisera, Immobilon-bound immune complexes were labeled with ^{125}I-labeled goat anti-rabbit IgG and visualized by autoradiography. Radiolabeled bands containing the labeled zymogens and their active enzyme forms were excised and quantified by measuring gamma emissions. The fraction of each zymogen converted to its active form was determined by calculating the ratio of counts associated with the active enzyme to their sum, and the effect of specific treatments was expressed as a percentage of unstimulated control values.

The three antisera used in this study were raised in rabbits against dog procarboxypeptidase A1, procarboxypeptidase B, and chymotrypsinogen 2 purified by two-dimensional separation of proteins obtained from a zymogen granule lysate (18). These antisera cross-react with the corresponding rat pancreatic secretory proteins, recognizing with comparable efficiency both the zymogen precursors and the processed, mature forms of the enzymes (designated active enzymes). Unlike trypsinogen, procarboxypeptidase A1 and B undergo cleavage of relatively large fragments during the process of activation (19), facilitating the resolution of each active enzyme from its zymogen precursor by SDS-PAGE. In the case of chymotrypsinogen, activation is accomplished by removal of two different dipeptides, with the three resulting peptide chains joined by disulfide bonds (20).

Manipulation of subcellular pH. To evaluate the role of an acidic subcellular compartment in the activation of zymogen proteins, isolated acini were pretreated for 30 min with either the weak base chloroquine (40 μM) or the protonophore monensin (10 μM) before CCK exposure (21). To confirm effective pH neutralization by these agents, the fluorescent pH marker acridine orange (10) was employed. Acini were incubated for 5 min with 50 μM acridine orange, then washed to remove excess dye. After exposure to either chloroquine or monensin, acridine orange cytofluorescence was examined using an IMT-2 inverted microscope (Olympus Corp. of America, New Hyde Park, NY) equipped with a BP 460–490 excitation filter.

Inhibition of endogenous proteases. To selectively inhibit intracellular protease activity, acini were pretreated for 30 min with specific protease inhibitors before exposure to CCK. These included E-64 (L-*trans*-epoxysuccinyl-leucylamido[4-guanidino]butane; 100 μM), an irreversible inhibitor of cathepsin B and other cysteine proteases (22), and benzamidine (10 mM), an inhibitor of trypsin and other serine proteases (23). In the case of E64, confirmation of complete cathepsin B inhibition was accomplished by serial exchanges of media to remove unincorporated inhibitor, solubilization of acini in 0.1% Triton-X100, and enzymatic determination of cathepsin B activity using the fluorogenic substrate *N*-CBZ-arg-arg-4-methoxy-B-naphthylamide at pH 6.5 (24).

Statistical analysis. All data are expressed as the mean of at least six independent treatment conditions ±SEM. Statistical comparison was performed using nonpaired *t* tests and Bonferroni two-tailed analysis of variance techniques, with differences considered significant at a *P* value of 0.05 or less.

Results

Detection of activated zymogens. The temporal patterns of antiprocarboxypeptidase A1 immunoreactivity as detected on immunoblots of proteins from CCK-treated and control acini are depicted in Fig. 1. In unstimulated controls, immunoreactivity was predominantly localized to a protein with an apparent molecular mass of 46 kD, corresponding to the molecular weight of the inactive zymogen (19). When acini were exposed to 100 nM CCK for up to 60 min, an increased proportion of total immunoreactivity localized to a protein with an apparent molecular mass of 35 kD, corresponding to the molecular mass of the activated enzyme, carboxypeptidase A1 (19). This lower molecular weight form demonstrated considerable lability and required immediate solubilization of treated acini in 3% SDS for its detection. These CCK-induced changes in the electrophoretic mobility of procarboxypeptidase A1 immunoreactivity were indistinguishable from those observed after treatment of a 10% (wt/vol) pancreatic homogenate with 0.1% trypsin (wt/vol) at 37°C. When analyzed by one-dimensional electrophoresis (Fig. 2) exposure to trypsin for 5 s–2 min generated a 35-kD form similar to that observed in CCK-treated acini. The identity of the lower molecular weight forms detected at later time points remains unclear. When subjected to two-dimensional electrophoresis, the 35-kD form generated within isolated acini following exposure to CCK exhibited an isoelectric point identical to that generated within pancreatic homogenate following exposure to trypsin (Fig. 3). In addition, purified recombinant rat carboxypeptidase A1 (supplied by the Hormone Research Institute, University of California, San Francisco) also comigrated with the 35-kD species when resolved by two-dimensional electrophoresis. These findings indicate that the 35-kD immunoreactive species found in CCK-treated acini is carboxypeptidase A1.

The ability of 100 nM CCK to stimulate conversion of procarboxypeptidase A1 to the active enzyme form is quantitated in Fig. 4. Following the addition of CCK, the relative amount of 35-kD immunoreactivity increased threefold. This increase was detectable within 15 min and was maximal by 45 min. In unstimulated acini the relative proportions of the 46-kD and 35-kD bands underwent minimal change during this time pe-

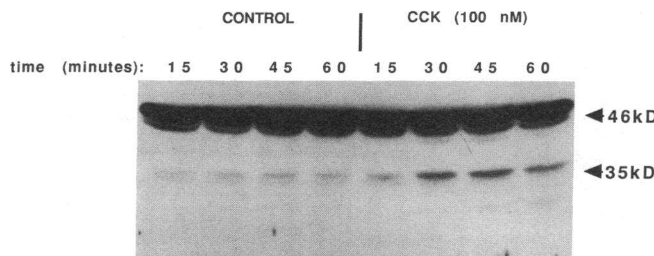

Figure 1. CCK treatment of isolated pancreatic acini stimulates the conversion of procarboxypeptidase A1 to a 35-kD species. After treatment as indicated, acinar cell proteins were separated by SDS-PAGE (7.5%), blotted with antiprocarboxypeptidase A1 antisera, and visualized by autoradiography after application of ^{125}I-labeled goat anti-rabbit IgG. The 46-kD species corresponds to procarboxypeptidase A1. The 35-kD species has the same mobility as carboxypeptidase A1.

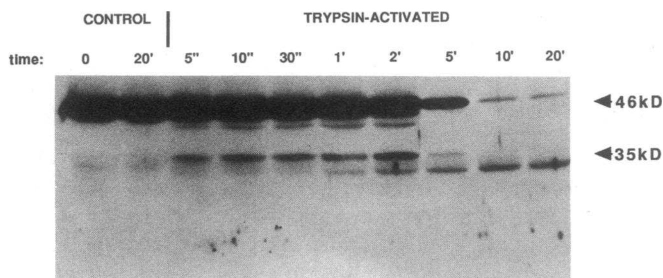

Figure 2. Treatment of pancreatic homogenate with trypsin stimulates the conversion of procarboxypeptidase A1 to 35-kD species. A 10% (wt/vol) homogenate of rat pancreas was incubated with 0.1% (wt/vol) trypsin at 37°C. At various intervals, a fixed amount of the treated homogenate was removed, immediately boiled in 3% SDS, and processed for immunoblotting.

riod. Some interexperiment variability was noted in the amount of zymogen converted to the active form. When the amount converted was expressed as a percentage of the total, a range of 2–6% for unstimulated samples and 4–24% for CCK-treated samples was observed.

To determine if the CCK-stimulated generation of carboxypeptidase A1 represented an intra or extracellular event, separate analysis of isolated acini and incubation media was performed. When acini were separated from the incubation medium by sedimentation, acini stimulated by 100 nM CCK contained both the 46-kD and 35-kD species. In contrast, only the 46-kD zymogen form was detected in the incubation medium.

The concentration-dependence of the CCK-stimulated conversion of procarboxypeptidase A1 to carboxypeptidase A1 is depicted in Fig. 5. Acini exposed to increasing concentrations of CCK for 30 min exhibited increasing amounts of the active enzyme form (35-kD species). This effect was maximal at 100 nM CCK, with nearly a fivefold increase in the amount of zymogen converted to the active form observed during this set of experiments.

To determine if the relative increase in carboxypeptidase A1 immunoreactivity stimulated by CCK represented new synthesis of a truncated form of the zymogen during the treatment period, acini were pretreated with cycloheximide, an inhibitor of protein synthesis. Pretreatment with 10 μM cycloheximide for 30 min, a regimen that inhibited virtually all incorporation

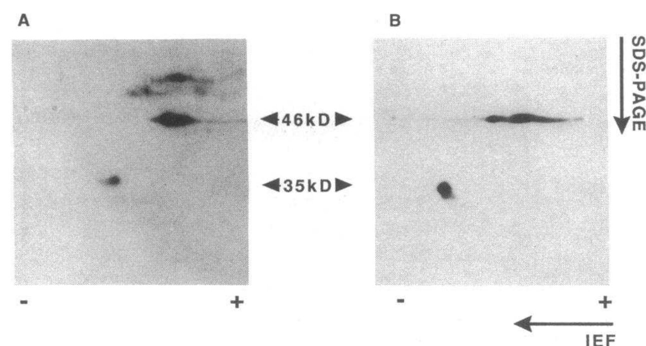

Figure 3. Two-dimensional immunoblot analysis of procarboxypeptidase A1 and 35-kD species in pancreatic acini and pancreatic homogenate. The 35-kD species generated by CCK treatment (100 nM for 30 min) of pancreatic acini (*A*) and by trypsin treatment (0.1% for 30 s) of pancreatic homogenate (*B*) comigrate.

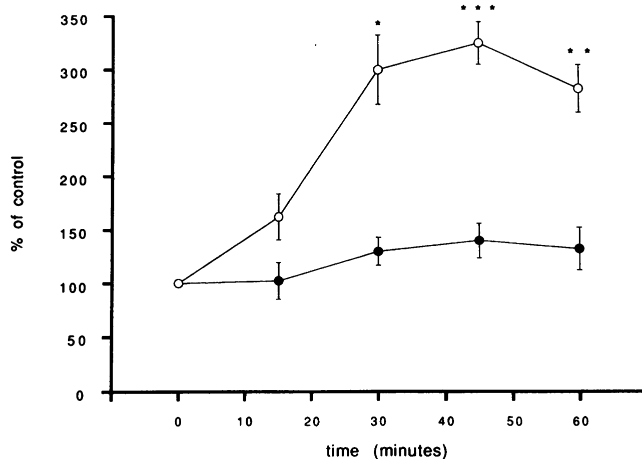

Figure 4. Quantification of the time course of CCK (100 nM) stimulated conversion of procarboxypeptidase A1 to carboxypeptidase A1. Following detection with an ^{125}I-labeled secondary antibody, the fraction converted was determined as described in Methods, and expressed as percentage of unstimulated control (●, controls; ○, CCK-treated). Values depicted represent mean±SEM (n = 6). *P < 0.1; **P < 0.001; and ***P < 0.0001 vs. corresponding control value by unpaired t test.

of [^{3}H]leucine into TCA-precipitable protein, had no effect on the CCK-stimulated generation of carboxypeptidase A1.

These findings suggested that high dose CCK stimulated the conversion of presynthesized procarboxypeptidase A1 to carboxypeptidase A1 within the acinar cell. To detect the conversion of other pancreatic zymogens to their active forms, immunoblotting was performed using antibodies to procarboxypeptidase B and chymotrypsinogen 2. Similar to procarboxypeptidase A1, ~10% of procarboxypeptidase B immunoreactivity was converted to a 35-kD form consistent with active carboxypeptidase B following treatment with 100 nM CCK. In the case of chymotrypsinogen 2, electrophoresis in the presence of β-mercaptoethanol revealed several lower molecular weight bands following CCK treatment, a finding consistent with the

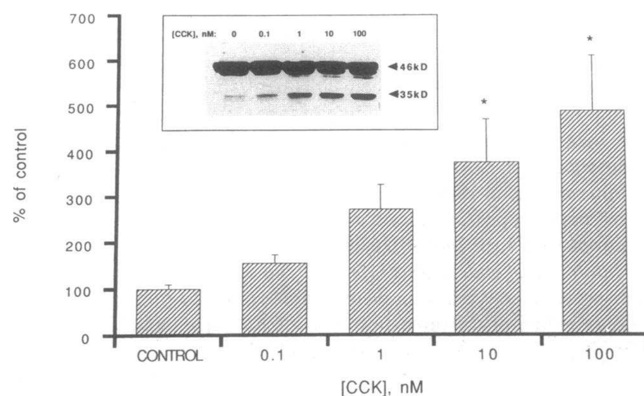

Figure 5. CCK stimulates the conversion of procarboxypeptidase A1 to carboxypeptidase A1 in a dose-dependent manner. After exposure to increasing concentrations of CCK for 30 min, treated acini were processed for immunoreactivity. Values represent mean±SEM (n = 6). *P < 0.05 vs. control by analysis of variance. (*Inset*) Representative autoradiograph depicting procarboxypeptidase A1 (46 kD) and carboxypeptidase A1 (35 kD) immunoreactivity under corresponding treatment conditions.

three-chain structure of chymotrypsin (20). Although clearly discernible, these bands were faint in comparison to the precursor and represented < 2% of total immunoreactivity.

Effects of subcellular pH manipulation. To test the effects of dissipating intracellular pH gradients on the CCK-stimulated conversion of procarboxypeptidase A1 to its active form, acini were exposed to either 40 μM chloroquine or 10 μM monensin for 30 min before CCK stimulation. These pretreatments completely prevented CCK-induced conversion to carboxypeptidase A1 (Fig. 6). For both agents, these doses effectively neutralized subcellular pH gradients as assessed by acridine orange cytofluorescence (not shown).

Effects of endogenous protease inhibition. The differential effects of pretreatment with the cysteine protease inhibitor E64 and the serine protease inhibitor benzamidine are also demonstrated in Fig. 6. In this system, E64 (100 μM) was effective in inhibiting 99% of cellular cathepsin B activity, as assayed at pH 6.5, the optimum pH for cathepsin B-mediated hydrolysis of *N*-CBZ-arg-arg-4-methoxy-B-naphthylamide (23). This inhibition of cathepsin B had no effect on the CCK-stimulated conversion of procarboxypeptidase to the 35-kD form. In contrast, pretreatment with benzamidine (10 mM) completely inhibited these CCK-induced changes.

Discussion

This investigation emphasizes the ability of high dose CCK to stimulate activation of digestive zymogens within populations of isolated pancreatic acini. Using an immunologic approach, untreated pancreatic acini were found to contain small amounts of a 35-kD species having the same net charge and electrophoretic mobility as carboxypeptidase A1. Treatment of acini with high dose CCK dramatically increased the conversion of procarboxypeptidase A1 to carboxypeptidase A1 in this system. Similarly, this treatment resulted in changes in the electrophoretic mobility of immunoreactive procarboxypeptidase B and chymotrypsinogen 2, consistent with activation of these zymogens. These changes were only detectable in acinar cell pellets and not in the incubation medium, suggesting an intracellular site of conversion. The conversion of procarboxypeptidase A1 to carboxypeptidase A1 did not require ongoing protein synthesis, suggesting activation of a presynthesized pool. Finally, this conversion was found to depend on the presence of an acidic subcellular compartment, and to require serine, but apparently not cysteine protease activity.

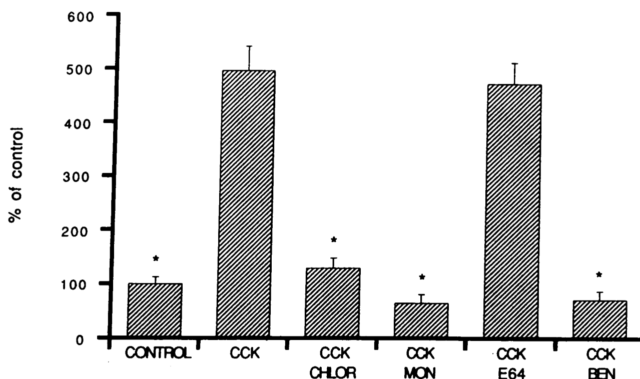

Figure 6. Subcellular pH manipulation and specific protease inhibitors affect the CCK-stimulated conversion of procarboxypeptidase A1 to carboxypeptidase A1. Isolated acini were pretreated with agents for 30 min, then exposed to CCK (100 nM) for 30 min. Agents included chloroquine sulfate (*chlor;* 40 μM); monensin (*mon:* 10 μM), E64 (100 μM), and benzamidine (*ben;* 10 mM). Values represent mean±SEM ($n = 6$). $^*P < 0.01$ vs. CCK alone by analysis of variance.

Under the short-term treatment conditions used in this study, no changes in the catalytic activities of trypsin (25) or carboxypeptidase A (26) could be detected in homogenates of pancreatic acini (data not shown). There are several possible explanations for these results. First, the amount of the zymogen activated may be too small for detection by enzymatic assay. Second, the active enzymes may be exposed to a proteolytically active compartment in which they are degraded during homogenization. Finally, the presence of endogenous inhibitory factors, including pancreatic secretory trypsin inhibitor (27, 28), may prohibit direct measurements of enzymatic activities. However, the CCK-stimulated changes in the electrophoretic mobility of several pancreatic zymogens demonstrated in this study strongly suggest that this treatment results in the conversion of a subset of pancreatic zymogens to their activated enzyme forms.

The ability of CCK to stimulate zymogen activation in this system was prevented by pretreatment with either chloroquine or monensin. Although these agents vary in their mechanisms of action, their major biologic effect under the conditions used in this study is to neutralize acidic subcellular compartments. Thus, a low pH environment is likely required for the intracellular conversion of procarboxypeptidase A1 to its active form. The participation of an acidic subcellular compartment in various forms of experimental pancreatitis has been previously proposed (10). This concept was based upon the observation that cytoplasmic vacuoles that develop during various forms of in vivo pancreatitis accumulate acridine orange, suggesting the presence of an acidic subcellular compartment having the potential to support acid-hydrolase activities. The identity of the acidic compartments observed in vivo and their relationship to those required for the activation of zymogens within isolated acini is unclear.

The mechanism by which an acidic compartment participates in CCK-stimulated zymogen activation is also unknown. Two distinct proteolytic pathways are likely candidates for mediating the pH-dependent activation of digestive zymogens. The first pathway would involve activation of trypsinogen by the lysosomal enzyme cathepsin B, a cysteine protease. This reaction occurs only within a narrow pH range (2.5–4.0), with an optimum pH of 3.6 (6). The second pathway would be initiated by the autoactivation of trypsinogen, a serine protease. This event occurs optimally at pH 5.0 (6). The respective pH optima of these pathways would appear to favor the autoactivation of trypsinogen as the mechanism responsible for intracellular zymogen activation. In a preliminary report, we have estimated the pH within a population of CCK-induced acinar cell vacuoles to be ~ 5.2 (29). The presence of an acinar cell compartment with a pH near the optimum for cathepsin B-mediated trypsinogen activation has yet to be demonstrated (30).

This study provides further information on which of these mechanisms may mediate CCK-stimulated zymogen activation. First, the activation of digestive zymogens in this system occurs within 15 min. This event appears to temporarily precede the redistribution of pancreatic zymogens and lysosomal hydrolases into a common subcellular fraction that is reported to require at least 1 h of treatment (31, and personal communi-

cation, A. Saluja). Second, the cysteine protease inhibitor E64, an agent previously shown to inhibit lysosomal hydrolase activities in intact hepatocytes (32), had no effect on CCK-stimulated zymogen activation. This agent was ineffective even at doses that inhibited 99% of acinar cathepsin B activity. In contrast, benzamidine, a serine protease inhibitor, totally blocked the effects of CCK in this system. Although these findings do not exclude other proteolytic pathways, they support the hypothesis that pH-dependent trypsinogen autoactivation is the mechanism responsible for initiating zymogen activation in this system.

The specific acidic compartment that participates in CCK-stimulated zymogen activation remains unknown. Several possibilities exist. First, alterations in zymogen traffic may result in proenzyme entry into a separate acidic organelle such as the late endosome/lysosome compartment (8). Alternatively, zymogen activation may occur within a component of the classical secretory pathway that undergoes enhanced or prolonged acidification in response to CCK. An examination of subcellular pH changes within the acinar cell after short-term CCK treatment may provide some information regarding this possibility

In summary, this study provides evidence that exposure of isolated pancreatic acini to high dose CCK is associated with the intracellular activation of digestive zymogens. This system may provide a convenient model for the investigation of initiating events in experimental pancreatitis.

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