Zymogen proteolysis within the pancreatic acinar cell is associated with cellular injury

T. Grady, M. Mah'moud, T. Otani, S. Rhee, M. M. Lerch, and F. S. Gorelick

MATERIALS AND METHODS

Materials

Rats were obtained from the Charles River Breeding Laboratories (Wilmington, MA) and housed at the Veterans Affairs Connecticut Healthcare System, West Haven, CT. Caerulein, CCK-8, and bombesin were purchased from Research Plus (Bayonne, NJ). The o-phenylmethyl ester of CCK (OPE) was generously provided by L. J. Miller, Rochester, MN (7). An inability to obtain caerulein during the study resulted in the use of CCK-8 for some studies. However, the two agents were found to have an equivalent effect in all in vitro assays.

The anesthetics used were Rompun (xylazine hydrochloride; Mobay, Shawnee, KS), Ketaset (ketamine hydrochloride; Aveco, Fort Dodge, IA), and Metofane (methoxyflurane, Aveco). Heparin was purchased from Elkins-Sinn, Cherry Hill, NJ. Trypan blue stain was purchased from Gibco BRL, Gaithersburg, MD. Dithiothreitol, b-naphthylamine, 3-(N-morpholino)propanesulfonic acid, HEPES, hexadecyltrimethylammonium bromide, tetramethylbenzidine (TMB), catalase, N,N-dimethylformamide, HPLC-grade water for the electrophoresis procedures, and the LDH determination assay kit LDH-500 were all purchased from Sigma Chemical, St. Louis, MO. 125I-labeled goat anti-rabbit IgG was purchased from DuPont-New England Nuclear, Billerica, MA.

Materials used for protein electrophoresis and Western blot analysis were purchased from Bio-Rad Laboratories, Hercules, CA. Immobilon-P membranes were purchased from Millipore, Bedford, MA. Nylon meshes (400, 200, and 20 µm) were purchased from Small Parts, Miami, FL. The Phadebas
Amylase assay kit was purchased from Pharmacia Diagnostics, Atlanta, GA. The lipase turbidimetric assay kit was obtained from Boehringer Mannheim Diagnostics, Indianapolis, IN.

**In Vivo Experimental Design**

Hyperstimulation with caerulein and bombesin. Male Wistar rats (125–150 g) were fed laboratory chow and water ad libitum. Fasted rats were given a constant intravenous infusion for 3 h of 0.9% NaCl as a control, caerulein (5 µg·kg⁻¹·h⁻¹), or bombesin (500 µg·kg⁻¹·h⁻¹) dissolved in 0.9% NaCl. This caerulein dose has been shown to induce pancreatitis in rats (9). The dose of bombesin was chosen because our in vitro studies revealed that bombesin is ~100-fold less potent than caerulein in stimulating amylase release from acinar cells.

Markers of pancreatitis. Serum amylase and lipase activity were determined as described (4, 24). The water content of the pancreas was quantified by comparing the wet weight to the same sample after desiccation and expressed as a percentage. Pancreatic myeloperoxidase (MPO) activity was determined using a spectrophotometric assay that detects its oxidation of TMB as described (8) and was expressed as units per gram wet tissue weight.

**In Vitro Experimental Design**

Preparation of isolated pancreatic acini. Isolated pancreatic acini were prepared from fasted male Wistar rats (40–80 g) by enzymatic digestion as described (9). Acini were suspended in Tris-Ringer buffer (pH 7.4) containing (in mM) 40 trisoma base, 95 NaCl, 4.7 KCl, 0.6 MgCl₂, 1.3 CaCl₂, 0.5% bovine serum, and 0.01% soybean trypsin inhibitor and solubilized from gravity-sedimented acini by aspiration and washed 3 times with 100 µl of medium. To prevent interference with substances added to the incubation medium, the enzyme was precipitated from the fractions with ice-cold methanol (80%) and resuspended in 25 mM HEPES, pH 7.5, and 100 mM NaCl. LDH release was expressed as the activity in the medium as a percent of the total. Preliminary studies demonstrated that longer equilibration periods reduced markers of cell injury in control and physiologically stimulated cells without changing their secretory responsiveness. Accordingly, assays of cell injury were performed on acini after a 3-h equilibration period unless otherwise noted.

Detection of TAP. Affinity-purified antibodies to the five amino acids (Asp-Asp-Asp-Asp-Lys) adjacent to the activation site in trypsinogen were prepared as described (5). For immunofluorescence studies, the treated pancreatic acini were fixed with a buffer (pH 7.2) containing (in mM) 50 HEPES, 100 NaCl, 1 EGTA, 5 benzamidine, and 10 µg/ml aprotinin. Trypsinogen permeabilized in 0.05% saponin in buffer without fixative and saturated with oxygen at 37°C. The dispersed cells then underwent a 3-h equilibration period unless otherwise stated. Preparations that demonstrated at least 95% viability using trypan blue exclusion were used for experimentation.

Detection of zymogenic conversion (PCA1 to CA1) dissolved in 24-well plates. After 30 min of treatment, the acini were solubilized in sample buffer (13) with 10% SDS-polyacrylamide (10%) gels at a constant voltage (150 V) and processed for quantitative immunoblot analysis as described (14). The relative level of conversion of PCA1 to CA1 was expressed as the ratio of stimulated over basal conversion (14). In a few studies, the percent of PCA1 converted to CA1 was calculated by quantifying PCA1 and CA1 and expressed as a percent conversion (CA1/CA1 + PCA1).

Markers of cell injury. Trypan blue retention in isolated acini was quantitated with the use of a spectrophotometric assay and expressed as an A₀.₀₂₅₀ ratio (12). LDH was measured in the medium and isolated acini that had been solubilized in 0.1% Triton X-100. To prevent interference with substances added to the incubation medium, the enzyme was precipitated from the fractions with ice-cold methanol (80%) and resuspended in 25 mM HEPES, pH 7.5, and 100 mM NaCl. LDH release was expressed as the activity in the medium as a percent of the total. Preliminary studies demonstrated that longer equilibration periods reduced markers of cell injury in control and physiologically stimulated cells without changing their secretory responsiveness. Accordingly, assays of cell injury were performed on acini after a 3-h equilibration period unless otherwise noted.

**RESULTS**

In Vivo Studies

Caerulein, but not bombesin, hyperstimulation generates pancreatitis in vivo. As reported, caerulein hyperstimulation (5 µg·kg⁻¹·h⁻¹) for 3 h resulted in edematous pancreatitis indicated by hyperamylasemia, hyperlipasemia, and increased tissue water content and MPO activity (Table 1). Bombesin hyperstimulation (500 µg·kg⁻¹·h⁻¹) of rats resulted in none of the parameters indicative of acute pancreatitis (Table 1), with the exception of a small increase in tissue water. These studies confirm that caerulein hyperstimulation causes pancreatitis and that high doses of bombesin do not (21). Because zymogen activation within the pancreatic acinar cell has been linked to generation of pancreatitis, the effects of CCK or its analog caerulein and bombesin on zymogen processing in isolated acini were examined.

Detection of zymogen proteolysis (conversion) in vitro. Dispersed acini were incubated in a 24-well plate with 250 µl of cells distributed into each well. After 30 min of treatment, the acini were solubilized in sample buffer (13) with 10% β-mercaptoethanol and boiled for 10 min and the solubilized proteins (~60 µg/lane) were subjected to electrophoresis on SDS-polyacrylamide (10%) gels at a constant voltage (150 V) and processed for quantitative immunoblot analysis as described (14). The relative level of conversion of PCA1 to CA1 was expressed as the ratio of stimulated over basal conversion (14). In a few studies, the percent of PCA1 converted to CA1 was calculated by quantifying PCA1 and CA1 and expressed as a percent conversion (CA1/CA1 + PCA1).
Table 1. In vivo induction of pancreatitis by caerulein and not bombesin

<table>
<thead>
<tr>
<th></th>
<th>Serum Amylase, U/ml</th>
<th>Serum Lipase, U/ml</th>
<th>Tissue MPO, U/g</th>
<th>Tissue Water, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.72 ± 0.31 (21)</td>
<td>0 (21)</td>
<td>9.23 ± 0.98 (7)</td>
<td>74.36 ± 0.62 (21)</td>
</tr>
<tr>
<td>Caerulein</td>
<td>22.82 ± 2.5 (16)*</td>
<td>132 ± 13 (16)*</td>
<td>36.7 ± 0.85 (7)*</td>
<td>89.22 ± 0.77 (16)*</td>
</tr>
<tr>
<td>Bombesin</td>
<td>3.76 ± 0.47 (13)†</td>
<td>2 ± 1 (13)†</td>
<td>14.7 ± 1.22 (7)†</td>
<td>76.96 ± 0.66 (13)†</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses indicate no. of rats. Rats were treated with saline (control), caerulein (5 µg·kg⁻¹·h⁻¹), or bombesin (500 µg·kg⁻¹·h⁻¹) for 3 h by intravenous infusion through an in-dwelling catheter. MPO, myeloperoxidase. *P < 0.05 compared with control. †P < 0.05 compared with corresponding caerulein value.

In Vitro Studies

Secretagogue stimulation is associated with the generation of TAP in isolated pancreatic acini. In the absence of stimulation (Fig. 1A) or after physiological stimulation by CCK (10⁻¹⁰ M) (Fig. 1B), little or no TAP immunoreactivity was detected within pancreatic acinar cells. However, after hyperstimulation using CCK (Fig. 1C), caerulein (Fig. 1D), or bombesin (Fig. 1E), TAP immunoreactivity appeared in irregular vesicular structures that were located in a juxtanuclear distribution in acinar cells. These structures were similar in size, morphology, and distribution to those generated after in vivo caerulein hyperstimulation (20). Although the effects of caerulein on TAP generation were expected, the appearance of TAP after bombesin stimulation was surprising in light of the inability of bombesin to generate pancreatitis. To confirm that both caerulein and bombesin stimulated zymogen proteolysis, the processing of PCA₁ to CA₁ was examined in isolated acini.

PCA₁ processing to CA₁ is stimulated by caerulein and bombesin. The dose-related effects of caerulein and bombesin on amylase secretion and proteolytic zymogen processing were examined in isolated acini. The maximal stimulatory concentration of caerulein for amylase secretion was 10⁻¹⁰ M; higher concentrations led to a progressive decrease in enzyme discharge (Fig. 2A). Bombesin stimulated secretion in a monophasic manner, with maximal secretion observed at 10⁻⁶ M. The processing of PCA₁ to CA₁ was also concentration dependent, increasing between 10⁻¹¹ and 10⁻⁷ M caerulein (Fig. 2A). This result is indistinguishable from that previously found for CCK-8 (14). Similar to caerulein and CCK (14), bombesin stimulated the proteolytic conversion of PCA₁ to CA₁; a concentration of 10⁻⁷ M resulted in maximal processing. In unstimulated acini, 1–2% of PCA₁ was processed to CA₁; in caerulein-hyperstimulated acini, 4–8% of PCA₁ was converted to the active form. The levels of CA₁ generated by bombesin were ~60–70% of that generated by caerulein. The addition of OPE (10⁻¹⁰–10⁻⁶ M for 30 min), a selective agonist of the high-affinity CCK receptor that stimulates the monophasic release of amylase (7), did not stimulate the processing of PCA₁ to CA₁ (not shown).

The release of CA₁ from the cell would be predicted to follow the same pattern as amylase secretion if the processing of zymogens to active forms takes place in a secretory compartment. Accordingly, the release of CA₁ into the incubation medium was examined. In unstimulated acini, little of the mature enzyme CA₁ was found in the medium (Fig. 3). Although caerulein (10⁻⁷ M) hyperstimulation induced maximum levels of CA₁ (Fig. 2B), virtually none of the active form was detected in the medium (Fig. 3). In contrast, CA₁ appeared in the medium after bombesin stimulation (Fig. 3). Thus CA₁ generated by caerulein hyperstimulation is retained within the acinar cell while much of that generated by bombesin is released into the medium. The conversion induced by caerulein (10⁻⁷ M) and bombesin (10⁻⁷ M) was blocked by 10 mM benzamidine, but not by the addition of soybean trypsin inhibitor (1 mg/ml), a high molecular-weight trypsin inhibitor that would not be expected to enter the cell. This observation demon-
strates that PCA$_1$ is likely processed to CA$_1$ in a secretory compartment. Next, we sought evidence that caerulein might also stimulate CA$_1$ processing in a secretory compartment.

The secretion of CA$_1$ into the medium was measured as a function of increasing concentrations of caerulein (Fig. 4) in parallel to its effect on amylase secretion (Fig. 2A). The amount of CA$_1$ released into the medium decreased when the concentration of caerulein increased above $10^{-10}$ M. These findings demonstrate that CA$_1$ generated by low concentrations of stimulation is released from the acinar cell and that higher concentrations of caerulein ($>10^{-10}$ M) cause both reduced amylase secretion and the retention of CA$_1$ within the acinar cell.

High concentrations of CCK generate cell injury. To determine whether zymogen activation and the retention of active enzymes are associated with acinar cell injury, pancreatic acini were exposed to CCK for up to 2 h. Unstimulated controls or those maximally stimulated ($10^{-10}$ M) by CCK exhibited the same levels of LDH release and trypan blue retention (not shown). However, supraphysiological ($10^{-7}$ M) concentrations of CCK released more LDH and retained more trypan blue, and the effects of CCK hyperstimulation on both markers of cell injury were time dependent; LDH release and trypan blue retention were elevated after 30 min of treatment and continued to increase during the next 90 min (Fig. 5). To determine whether serine proteases contributed to cell injury, the effects of the inhibitor benzamidine were examined.

Pretreatment (15 min) with benzamidine (10 mM) has been found to block CCK-stimulated PCA$_1$ processing to CA$_1$ in isolated acini without altering the secre-
tory response to CCK (14). Benzamidine reduced both LDH release and trypan blue retention (Fig. 6). The fact that neither LDH release nor the degree of trypan blue retention returned to baseline levels after benzamidine suggests that some injury may be present. The protective effects of benzamidine were time dependent; after 30–60 min of hyperstimulation, it was less effective in reducing injury. Addition of the soybean protease inhibitor (1 mg/ml), a high-molecular-weight trypsin inhibitor that would not be expected to enter the cell, to the incubation medium did not prevent cellular injury (not shown). These findings suggest that activation of intracellular serine proteases soon after the initiation of CCK hyperstimulation contributes to acinar cell injury. To test the hypothesis that enzymes might not cause cell damage if they are secreted, the effects of bombesin on acinar cell injury were examined.

Acini were exposed to concentrations of bombesin that induced nearly maximum conversion of PCA1 to CA1 (Fig. 2B). In contrast to CCK, bombesin did not increase either LDH release or trypan blue retention (Fig. 7). To provide additional evidence that CCK but not bombesin induced injury, the effects of treatments on the acinar ultrastructure were examined.

The electron microscopy appearance of control acini was similar to that of those exposed to CCK (10^{-10} M) or bombesin (10^{-5} M). However, acini hyperstimulated with CCK (10^{-7} M) developed protrusions of the basal membrane. The membrane “blebs” are a characteristic response of isolated acini to secretagogue hyperstimulation (1, 3) and were virtually eliminated by pretreatment with benzamidine (data not shown). Mitochondrial swelling and a loss of membrane definition are characteristic of cell injury. Mitochondria from control acini were elongated and had well-defined cristae (Fig. 8A). In contrast, mitochondria from the hyperstimulated condition were rounded and swollen and demonstrated poorly defined cristae (Fig. 8B). The mitochondria from benzamidine pretreatment (Fig. 8C) with or exposure to bombesin (Fig. 8D) were not swollen and had well-defined cristae. These morphological changes were confirmed by using the ratio of the short to long axis of the mitochondria as a marker for swelling (Table 2).
DISCUSSION

The existence of a relationship between enzyme activation and acinar cell injury was previously unknown. To examine these potential associations, the effects of secretagogues on zymogen processing and cell damage were examined. First, we confirmed that in vivo caerulein hyperstimulation causes pancreatitis, but high doses of bombesin do not. To correlate zymogen processing with the generation of pancreatitis, antibodies to TAP were used as a marker for trypsino-gen conversion to trypsin in isolated pancreatic acini. The TAP antigen was not detected in control conditions or after physiological stimulation by CCK or caerulein. Similar to in vivo studies (20), hyperstimulation by CCK (10^{-7} M) or its analog, caerulein (10^{-7} M), resulted in the generation of TAP within the acinar cell. Surprisingly, bombesin also stimulated the intracellular generation of TAP. The morphological appearance of the TAP-positive compartment was indistinguishable among the secretagogues or from that generated by caerulein hyperstimulation in vivo (20) and did not overlap with zymogen granules. Parallel studies in isolated acini demonstrated that caerulein and bombesin also stimulated the processing of the zymogen PCA_1 to the active form, CA_1. However, the trafficking of CA_1 differed among secretagogues. Whereas most CA_1 was retained in the acinar cell after caerulein (10^{-7} M) or CCK hyperstimulation (10^{-7} M) (14), CA_1 was secreted from the cell after bombesin treatment. These findings suggest that stimulation of zymogen processing may be a generalized response of the acinar cell to stimulation and that this processing takes place within the acinar cell.

A link between zymogen processing, secretion of active enzyme forms, and cell injury was next examined. With the use of the biochemical criteria of LDH release and trypan blue retention and the morphological criteria of mitochondrial swelling and vacuole formation, caerulein hyperstimulation of acini was found to cause injury, whereas bombesin did not. The caerulein-induced injury could be reduced by benzamidine, a serine protease inhibitor that blocks the conversion of PCA_1 to CA_1 (14). Whether the protective effect of benzamidine is a result of blocking zymogen activation or inhibiting serine proteases that become activated during hyperstimulation is unclear. Notably, a concentration of benzamidine that has been found to block CCK-induced processing of PCA_1 to CA_1 did not reduce injury to background levels. This suggests that serine proteases play a role in acinar cell injury but that additional mechanisms, such as activated proteases that are not inhibited by benzamidine or other factors, may contribute to cell damage.

Although both caerulein and bombesin stimulate the generation of TAP in the acinar cell and PCA_1 process-

| Table 2. Mitochondrial morphology in pancreatic acini is affected by secretagogues |
|---------------------------------|----------|----------|----------|----------|
|                                  | Control  | CCK      | Benz + CCK| Bombesin |
| Short axis/long axis             | 0.41 ± 0.038 | 0.71 ± 0.040 | 0.42 ± 0.037 | 0.43 ± 0.041 |

Each value represents mean ± SE (per 20 mitochondria) from 2 or 3 rats for each condition. All treatments were for 120 min. Control, unstimulated; CCK, 10^{-7} M CCK hyperstimulation; Benz + CCK, 10^{-7} M benzamidine with 10^{-7} M CCK; Bombesin, 10^{-5} M bombesin hyperstimulation. Statistical differences (P < 0.01, Student's t-test): control vs. CCK, CCK vs. Benz + CCK, CCK vs. bombesin. No statistical difference: control vs. Benz + CCK, control vs. bombesin.
ing, there are differences in the response to the two agents. In the case of caerulein, maximum zymogen conversion occurs at supramaximal concentrations, whereas bombesin stimulates maximum conversion at the same concentration that causes maximum amylase secretion. An agonist of the high-affinity CCK receptor, OPE, did not significantly stimulate the processing of PCA1. Notably, the OPE peptide generates a similar monophasic pattern of amylase secretion as generated by bombesin and stimulates maximal amylase secretion at ~10^{-7} M (7). To summarize, maximal secretory concentrations of OPE that stimulate amylase release do not stimulate processing. Maximal secretory concentrations of bombesin stimulate both maximal amylase release and processing. Finally, much higher concentrations of CCK or caerulein than required to maximally stimulate amylase secretion are required to stimulate processing. These findings suggest that different intracellular signals may regulate secretion and zymogen processing.

The observation that much of CA1 is released from the acinar cell after bombesin stimulation or low concentrations of caerulein has two important implications. First, because acinar cells are not injured under these conditions, this finding would suggest that CA1 is being retained after bombesin stimulation or low concentrations of caerulein. Notably, the OPE peptide generates a similar monophasic pattern of amylase secretion as generated by bombesin and stimulates maximal amylase secretion at ~10^{-7} M (7). To summarize, maximal secretory concentrations of OPE that stimulate amylase release do not stimulate processing. Maximal secretory concentrations of bombesin stimulate both maximal amylase release and processing. Finally, much higher concentrations of CCK or caerulein than required to maximally stimulate amylase secretion are required to stimulate processing. These findings suggest that different intracellular signals may regulate secretion and zymogen processing.

In summary, the present study provides direct evidence that zymogen processing occurs within the pancreatic acinar cell. The fact that the processed form of PCA1, CA1, is released from the acinar cell after stimulation by bombesin or low concentrations of caerulein suggests that the processing takes place within a secretory compartment. The fact that caerulein or CCK and bombesin stimulate PCA1 processing but have different effects on the retention of CA1 in the acinar cell suggests that different signaling mechanisms may regulate processing and secretion. Finally, zymogen processing is associated with the generation of acinar cell injury. However, injury is observed only when zymogens are both activated and retained in the acinar cell.

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Address for reprint requests: F. S. Gorelick, Research Bldg. 27, VA Connecticut Healthcare, West Haven, CT 06516.

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