Apoptosis and necrosis are critical parameters of pancreatitis, the mechanisms of which remain unknown. Many characteristics of pancreatitis can be studied in vitro in pancreatic acini treated with high doses of cholecystokinin (CCK). We show here that CCK stimulates apoptosis and death signaling pathways in rat pancreatic acinar cells, including caspase activation, cytochrome c release, and mitochondrial depolarization. The mitochondrial dysfunction is mediated by upstream caspases (possibly caspase-8) and, in turn, leads to activation of caspase-3. CCK causes mitochondrial alterations through both permeability transition pore-dependent (cytochrome c release) and permeability transition pore-independent (mitochondrial depolarization) mechanisms. Caspase activation and mitochondrial alterations also occur in untreated pancreatic acinar cells; however, the underlying mechanisms are different. In particular, caspases protect untreated acinar cells from mitochondrial damage. We found that caspases not only mediate apoptosis but also regulate other parameters of CCK-induced acinar cell injury that are characteristic of pancreatitis; in particular, caspases negatively regulate necrosis and trypsin activation in acinar cells. The results suggest that the observed signaling pathways regulate parenchymal cell injury and death in CCK-induced pancreatitis. Protection against necrosis and trypsin activation by caspases can explain why the severity of pancreatitis in experimental models correlates inversely with the extent of apoptosis.

Cholecystokinin (CCK) is a major physiological regulator of digestive enzyme secretion by pancreatic acinar cells (1–3). However, supraphysiological doses of CCK that cause inhibition of secretion are injurious to pancreas, causing pancreatitis (2–4). Pancreatitis induced in rats and mice by high doses of CCK or its analogue, cerulein, is a widely used experimental model that mimics many features of the human disease (4–6). CCK is also involved in other models of pancreatitis; for example, it synergizes with ethanol to cause pancreatitis (7).

Inflammation and acinar cell death are hallmarks of both human and experimental pancreatitis (6, 8–10). Although significant progress has been achieved over the past decade in understanding the inflammatory response (9–15), mechanisms of acinar cell death in pancreatitis remain largely unexplored. We and others (11–13, 16–18) have demonstrated that both apoptosis and necrosis occur in various models of pancreatitis and, in particular, in cerulein-induced pancreatitis. Of note, in these models an inverse correlation was observed between the extent of apoptosis on the one hand and necrosis and the severity of the disease on the other hand.

Many characteristics of pancreatitis can be studied in vitro in isolated pancreatic acini stimulated with supraphysiological doses of CCK, such as up-regulation of pro-inflammatory cytokines and adhesion molecules, and the pathological, intra-acinar cell conversion of trypsinogen to active trypsin (13–15, 19). The latter is considered an important event in the development of pancreatitis because trypsin can cause cell injury and activation of other potentially harmful digestive enzymes in the acinar cell (4, 6, 20). It remains unknown whether CCK (or cerulein) can directly induce apoptosis in pancreatic acinar cells and, if so, what signaling pathways are involved.

A central event in apoptosis is activation of specific cysteine proteases, the caspases (21). Two major pathways of caspase activation have been identified (21–23). One is triggered through “death domain” receptors, such as the tumor necrosis factor family of receptors, and involves the formation of a “death-inducing signaling complex” leading to activation of initiator caspases, i.e. caspase-8 (21–23). Initiator caspases then directly activate effector caspases such as caspase-3 (21–23).

A second major pathway for apoptosis involves mitochondrial alterations, in particular, the release of cytochrome c (21–24). Cytochrome c release into the cytosol is necessary for activation of caspase-9, which, in turn, activates the effector caspases. Cytochrome c release may be mediated by opening of the mitochondrial permeability transition pore (PTP), although the issue is much debated (24–27). Another characteristic of mitochondrial dysfunction is loss of the mitochondrial transmembrane potential, ΔΨm (24, 25).

Mitochondrial dysfunction can be mediated by upstream caspases. For example, caspase-8 can activate the mitochondrial pathway by cleaving Bid, a pro-apoptotic member of the Bcl-2 protein family, which then causes cytochrome c release from mitochondria (28). This “amplification” pattern of apopto-
sis is thought to be used by cells that do not have high enough level of death-inducing signaling complex formation and activation of caspase-8 (22). However, caspase-3 also can proteolytically activate caspase-8 so that stimuli that directly cause mitochondrial dysfunction may subsequently activate caspase-8 downstream of cytochrome c release (23).

Little is known about the mechanisms of apoptosis induced by receptors unrelated to tumor necrosis factor family, in particular, by G protein-coupled receptors. One exception is somatostatin, whose receptor is coupled to pertussis toxin-sensitive G$_{i/o}$ protein (29). Somatostatin was recently shown to stimulate apoptosis in breast cancer cells by recruiting a nonmembrane tyrosine phosphatase, resulting in activation of caspase-8 independent of mitochondria (30). It is not clear whether the apoptotic pathway activated by somatostatin is unique or is utilized by other G protein-coupled receptors as well. The CCK-A receptor in rodent pancreatic acinar cells is coupled to a pertussis toxin-insensitive G$_{i/o}$ protein that activates phospholipase C (1–3).

We report here that CCK stimulates death signaling pathways in rat pancreatic acinar cells, including caspase activation, cytochrome c release, and mitochondrial depolarization, leading to apoptosis. The mitochondrial dysfunction is mediated by upstream caspase(s). CCK causes mitochondrial alterations through both PTP-dependent and PTP-independent mechanisms. In addition to apoptosis, caspases also regulate other processes in the pancreatic acinar cell that play key roles in pancreatitis; in particular, caspases negatively regulate necrosis and intra-acinar cell activation of trypsin. Caspase-mediated protection against necrosis and trypsin activation can explain the inverse correlation between the extent of apoptosis on the one hand and necrosis and the severity of the disease on the other hand observed in experimental models of pancreatitis. These signaling mechanisms may play an important role in acinar cell injury and death in pancreatitis.

**EXPERIMENTAL PROCEDURES**

**Isolation of Pancreatic Acini**—Dispersed rat pancreatic acini were prepared using a previously published collagenase digestion method (12, 13, 15, 31). Isolated acini were washed and resuspended in 199 medium supplemented with penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 0.5% bovine serum albumin. The cells were plated at a concentration of 5 × 10$^6$/ml in 25-cm tissue-culture flasks and incubated at 37 °C in a 5% CO$_2$ humidified atmosphere.

**DNA Extraction and Gel Electrophoresis**—DNA extraction and gel electrophoresis were performed as described previously (12). Briefly, isolated pancreatic acini were collected by centrifugation, lysed in a buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM EDTA, 300 μg/ml proteinase K, and 1% (w/v) SDS, and incubated at 48 °C until the mixture became clear. DNA was purified by phenol/chloroform extraction (1:1, v/v), precipitated overnight with 0.3 M sodium acetate at –20 °C, and collected by centrifugation at 15,000 × g for 15 min at 4 °C. The pellet was resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and treated with RNase (200 μg/ml) for 2 h at room temperature, followed by overnight incubation with proteinase K (200 μg/ml) at 48 °C. Finally, the mixture was re-extracted with phenol/chloroform and chloroform, precipitated with ethanol, and resuspended in TE buffer. The DNA fragments were electrophoretically separated on 1.8% agarose gel containing 0.5 μg/ml ethidium bromide in 0.5 × TBE buffer (1 TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA). Quantification of DNA Fragmentation—Quantification of DNA fragmentation was performed as described below (12). Briefly, isolated pancreatic acini were collected by centrifugation and suspended in TE lysis buffer containing 10 mM Tris (pH 7.5), 10 mM EDTA, and 0.2% (w/v) Triton X-100. High and low molecular weight DNA molecules were separated by centrifugal elution of the samples for 15 h at 15,000 × g. Supernatants containing fragmented DNA and pellets containing high molecular weight DNA were each incubated overnight at 4 °C in 1 ml of TE lysis buffer and 0.25 ml of 50% (v/v) trichloroacetic acid. DNA in precipitates from both supernatants and pellets was hydrolyzed by heating at 70 °C for 20–25 min in 1 ml of 5 M HClO$_4$ and quantified by the diphenylamine method of Burton (32).

**Quantification of Apoptosis**—The cells were suspended in phosphate-buffered saline, plated on polylysine-coated glass coverslips, fixed for 10 min with methanol at –20 °C, and stained with 5 μg/ml Hoechst 33258 as described previously (12). The slides were examined by fluorescence microscopy. The cells with nuclei containing condensed and/or fragmented chromatin were defined as apoptotic cells.

**Measurement of LDH Release**—Acinar cell necrosis was determined by the release of LDH into incubation medium (12). LDH activity was measured spectrophotometrically as the production of NAD from pyruvic acid and NADH. The values for LDH release are presented as the percentages of total cellular LDH determined by permeabilizing cells with Triton X-100.

**Western Blot Analysis**—The proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Non-specific binding was blocked by 1 h of incubation of the membranes in 5% (w/v) nonfat dry milk in TTBS (0.05% (v/v) Tween 20 in Tris-buffered saline), washed three times with TTBS, and finally incubated for 1 h with a peroxidase-labeled secondary antibody in the antibody buffer. The blots were developed for visualization using enhanced chemiluminescence detection kit (Pierce). The band intensities in the immunoblots were quantified by densitometry using Scion Image software (Scion Corporation).

**Measurement of Caspase Activation with Fluorimetric Assay**—The acini were collected, washed with ice-cold phosphate-buffered saline, and resuspended in lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, and 0.5 mM EDTA. The cells were pelleted for 30 min on a rotator at 4 °C and centrifuged for 15 min at 15,000 × g, and the supernatants were collected. The proteolytic reactions were carried out at 37 °C in a buffer containing 25 mM HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 1 mM dithiothreitol, and 800 μg/ml of cytosolic protein, and 20 μM specific fluorogenic substrate. For caspase-3, the substrate was Ac-Asp-Glu-Val-Asp-AMC (Worthington); for caspase-8, the substrate was Ac-Ile-Glu-Thr-Asp-AMC (AnaSpec). Cleavage of the caspase substrate relieves AMC, which emits a fluorescent signal with excitation at 380 nm and emission at 440 nm. The fluorescence was calibrated using a standard curve for AMC. The data are expressed as pmol AMC/mg protein/min.

**Mitochondrial and Cytosolic Fractions**—Mitochondrial and cytosolic fractions were obtained as described previously (33). The cells were washed twice with ice-cold phosphate-buffered saline (pH 7.2) and resuspended in an extraction buffer containing 250 mM sucrose, 20 mM HEPES-KOH (pH 7.0), 10 mM KCl, 1 mM EDTA, 2 mM ethylenediaminetetraacetic acid (EDTA), and protease inhibitor mixture (5 μg/ml each of pepstatin, leupeptin, chymostatin, antipain, and aprotinin). The cells were incubated for 30 min on ice and then lysed in a glass Dounce homogenizer (80 strokes with a pestle). The nuclei were removed by centrifugation at 1000 × g for 10 min at 4 °C. The supernatant was then centrifuged for 1 h at 100,000 × g, and both the pellet (mitochondrial fraction) and supernatant (cytosolic fraction) were collected separately and used for Western blotting.

**Mitochondrial Membrane Potential**—Mitochondrial membrane potential (ΔΨ$_{m}$) was determined as described previously (34) by measuring the retention of the dye 3,3'-dihexyloxacarbocyanine (DiOC$_6$) (3) (Molecular Probes). The cells were loaded with 1 μM DiOC$_6$ (3) during the last 30 min of treatment. The cells were then collected by centrifugation, the supernatant was removed, and the pellet was washed twice with ice-cold phosphate-buffered saline. The pellet was then lysed by the addition of 1 ml of H$_2$O. The amount of DiOC$_6$(3) retained by cells was determined using a Shimadzu RF-1501 spectrofluorimeter with excitation at 488 nm and emission at 500 nm. The amount of DiOC$_6$(3) retained by cells pretreated for 30 min with 10 μM CCCP (a protonophore) was considered to correspond to a ΔΨ$_{m}$ value of 0.

**Trypsin Activity**—Trypsin activity was measured in cell homogenates by a fluorimetric assay as described previously (13, 35). Briefly, pancreatic acini were homogenized using a glass Teflon homogenizer in an ice-cold buffer containing 5 mM MES (pH 6.5), 1 mM MgSO$_4$, and 250 mM sucrose. The homogenates were mixed in an assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl$_2$, 0.1 mg/ml bovine serum albumin) with a specific substrate, Boc-Glu-Arg-Ala-Arg-AMC (Pepid International, Inc.). The reaction was started by the addition of the trypsin product. The product emits fluorescence at 440 nm with excitation at 380 nm. Trypsin was determined using a standard curve for purified trypsin (Worthington).
incubated for 30 min at 37 °C with 3 μM Fura-2-AM in a buffer containing 20 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, 10 mM sodium pyruvate, 10 mM ascorbic acid, 0.1% bovine serum albumin, and 0.01% soybean trypsin inhibitor. The cells were then washed twice by centrifugation and resuspended in the extracellular medium and the total cellular amylase determined by permeabilizing cells with 0.1% SDS in 10 mM phosphate buffer (pH 7.8). 

**Amylase Secretion**—Amylase secretion was measured spectrophotometrically using Phadebrom amylase kit from Pharmacia Diagnostics as described previously (37). The values for amylase secretion are expressed as ratios between the amount of amylase released into the same buffer. 

**Statistical Analysis of Data**—Statistical analysis of the data was done using unpaired Student’s t test. The values of p < 0.05 were considered statistically significant.

**Antibodies and Reagents**—Antibodies against caspases-3, -8, and -9 were from Santa Cruz Biotechnology; those against cytochrome c were from Pharmingen. CCK-8 was from Peninsula Laboratories. Caspase inhibitors zVAD-fmk and zIETD-fmk were from Enzyme Systems Products and Calbiochem, respectively; aristolochic acid and cyclosporin A were from Biomol Research Laboratories; Fura-2-AM was from Molecular Probes; and Bio-Rad protein assay was from Bio-Rad Laboratories. Other reagents were from Sigma.

**RESULTS**

**CCK Stimulates Activity of Caspases-3, -8, and -9 in Rat Pancreatic Acinar Cells**—Western blot analysis showed that CCK-8 induced activation of both the effector caspase-3 and initiator caspases-8 and -9 in isolated rat pancreatic acinar cells (Fig. 1). Caspase-3 activation is recognized by a time-dependent decrease of the 45-kDa isoform with concomitant increase in the 44-kDa product. Activation of caspase-8 was recognized by a time-dependent decrease of the 54-kDa isoform with concomitant increase in the 50-kDa product (Fig. 1B). Western blot analysis for caspase-8 in pancreatic acinar cells detected a doublet at ~50 and 54 kDa (Fig. 1B) possibly corresponding to caspases-8a and -8b (39). Stimulation with CCK-8 decreased the intensities of both p54 and p50 bands at all times studied, compared with untreated cells. The changes in the p54 isoform were usually more pronounced than in p50 (Fig. 1B). CCK-induced cleavage of p54 and p50 was associated with the accumulation of a ~45-kDa product. In untreated cells, processing of caspase-8 was recognized by a time-dependent decrease of the 54-kDa isoform with concomitant increase in the p45 product (Fig. 1B).

**Cytochrome c Release and Mitochondrial Depolarization; the Former but Not the Latter, Is Prevented by PTP Inhibitors**—CCK-8 dose-dependently increased the cytochrome c level in the cytosol, with a concomitant decrease in the mitochondrial cytochrome c content (Fig. 3A). CCK-induced cytochrome c release was evident at the hormone concentrations ≥0.1 nM.

Cytosolic cytochrome c was detected in the cytosolic fractions from normal pancreatic tissue (not shown). We observed a decrease in the cytosolic cytochrome c almost at freshly isolated acinar cells, which increased with incubation of untreated cells (Fig. 3B). Compared with untreated cells, CCK-8 stimulated cytochrome c release into the cytosol at each time point tested, which was already manifest after 30 min of incubation with the hormone (Fig. 3B).

Cytochrome c release from mitochondria is often associated with dissipation of the mitochondrial membrane potential (ΔΨm). We measured changes in ΔΨm by using the fluorescent dye DiOC6(3) that accumulates in the negatively charged mitochondrial matrix according to ΔΨm (34). As shown in Fig. 3C, CCK-8 caused a pronounced mitochondrial depolarization in pancreatic acinar cells at concentrations ≥0.1 nM, that is, in the same concentration range in which it induced cytochrome c release. We also observed a slight decrease in ΔΨm with incubation of untreated acinar cells (Fig. 3D). At each time point, CCK-8
further stimulated mitochondrial depolarization, its effect being evident after 30 min of incubation (Fig. 3D).

One mechanism for cytochrome c release from mitochondria is via opening of PTP (24–27). To determine whether cytochrome c release in untreated and CCK-treated pancreatic acinar cells was mediated by PTP, we applied the PTP inhibitor cyclosporin A (CsA; Refs. 25–27 and 47) or the combination of CsA and aristolochic acid (ArA). Aristolochic acid, an inhibitor of phospholipase A2, can enhance or prolong the inhibitory effect of CsA on PTP opening, and in some cases its combination with CsA is necessary to block cytochrome c release (34, 48). CsA alone and, to a greater extent, the combination of CsA plus ArA prevented CCK-induced cytochrome c release (Fig. 3D). In untreated acinar cells the PTP inhibitors did not inhibit but, in contrast, potentiated cytochrome c release (Fig. 3B). These data suggest that PTP opening contributes to CCK-induced cytochrome c release but that it does not mediate the “basal” cytochrome c release in untreated pancreatic acinar cells.

The effect of the PTP inhibitors on caspase-3 processing (Fig. 3E) correlated with that on cytochrome c release; CsA + ArA blocked CCK-induced cleavage of caspase-3 but potentiated...
CCK Induces Apoptosis in Pancreatic Acinar Cells

Caspases mediate CCK-induced cleavage of PKCδ. Pancreatic acini were incubated for 3 h without and with 100 nM CCK-8 in the absence or presence of 100 μM zVAD-fmk. Whole cell lysates were subjected to Western blot analysis using an antibody against PKCδ. The blots were reprobed for α-tubulin to confirm equal protein loading. A longer exposure was used to better visualize the 42-kDa cleaved PKCδ product.

caspase-3 processing in untreated acinar cells (Fig. 3E). With the fluorogenic assay, we measured that CsA + ArA inhibited CCK-induced DEVDase activity by over 80% (data not shown).

Both CsA and the combination of CsA plus ArA decreased ΔΨm in untreated acinar cells (Fig. 3F). Moreover, in the presence of the PTP inhibitors, CCK-8 further depolarized the mitochondria (Fig. 3F). These results indicate that PTP opening does not mediate the CCK-induced mitochondrial depolarization, although it contributes to CCK-induced cytochrome c release and caspase-3 activation.

zVAD-fmk and zIETD-fmk Inhibit Cytochrome c Release and Mitochondrial Depolarization in CCK-treated but Not in Untreated Pancreatic Acinar Cells—To determine whether the cytochrome c release and mitochondrial depolarization in acinar cells are mediated by upstream caspase activation, we measured the effect of caspase inhibitors on these parameters. As seen from a representative immunoblot (Fig. 4A) and the quantification data (Fig. 4B), CCK-induced cytochrome c release was inhibited by zVAD-fmk and (to even a greater extent) the caspase-8 inhibitor zIETD-fmk. In contrast, both inhibitors did not inhibit but only potentiated cytochrome c release in untreated acinar cells (Fig. 4, A and B). These results suggest that upstream caspase(s), possibly caspase-8, mediate the CCK-induced cytochrome c release in pancreatic acinar cells. By contrast, in untreated cells cytochrome c release is not mediated by upstream caspases.

Both caspase inhibitors prevented CCK-induced decrease in ΔΨm (Fig. 4C), suggesting its regulation by upstream caspase(s), possibly caspase-8. By contrast, in untreated acinar cells the caspase inhibitors caused mitochondrial depolarization (Fig. 4C) similar in extent to that induced by 100 nM CCK.

CCK Stimulates Apoptosis in Pancreatic Acinar Cells—In pancreatic acini, we observed internucleosomal DNA fragmentation, a hallmark of apoptosis, which was stimulated by CCK-8. In particular, after 3 h of incubation, DNA laddering was prominent in acinar cells incubated with CCK-8 but not in untreated cells (Fig. 5A). After 6 h of incubation, internucleosomal DNA fragmentation was observed in both untreated and CCK-treated cells, but it was much more pronounced under the action of CCK-8 (Fig. 5A). DNA fragmentation was not detected in freshly isolated acini and in acini incubated for 1 h with or without CCK-8 (not shown). 100 μM zVAD-fmk prevented both basal and CCK-stimulated DNA laddering (Fig. 5A).

CCK-8 also increased the percentage of fragmented DNA in acinar cells (Fig. 5B). The hormone-induced DNA fragmentation increased dose-dependently and was manifest at CCK-8 concentrations >0.1 nM.

With Hoechst staining, we detected apoptotic nuclear morphology in both control and CCK-treated cells after 6 h of incubation (Fig. 5C). CCK-8 increased about 3-fold the number of apoptotic cells with condensed or fragmented chromatin, whereas zVAD-fmk completely prevented the appearance of cells with apoptotic morphology (Fig. 5D).

Caspases Regulate CCK-induced Amylase Secretion—Fig. 6A shows a typical biphasic dose-response curve for CCK-8-induced secretion of amylase from rat pancreatic acinar cells, with stimulation up to ~0.1 nM CCK-8 and subsequent reduction of amylase release at supramaximal concentrations of the agonist. This blockade of pancreatic exocrine secretion is a critical event in pancreatitis (4, 6, 50). The maximal level of secretion (with 0.1 nM CCK-8) was 26% of the total cellular amylase; the background amylase release from untreated acinar cells was less than 4%. Preincubation with zVAD-fmk decreased the down slope of the curve (in the range of CCK concentrations above 0.1 nM). For example, 100 nM CCK-8 induced 78% versus 56% of maximal amylase release in the presence and absence of zVAD-fmk, respectively (Fig. 6). Thus zVAD-fmk partially reversed the blockade of amylase secretion observed with supramaximal CCK-8 concentrations, indicating the involvement of caspases. We recently showed (49) that the caspase-8 inhibitor, zIETD-fmk, similarly reversed the blockade of amylase secretion in acinar cells stimulated with supramaximal (>0.1 nM) concentrations of CCK-8.

Blockade of Caspases Potentiates CCK-induced Trypsin Activation and Necrosis in Pancreatic Acinar Cells—Intrapancreatic trypsin activation and acinar cell necrosis are considered key features of pancreatitis (4, 6, 11, 16, 20). These events also occur in vitro in isolated pancreatic acinar cells treated with supramaximal doses of CCK-8 (19). To determine whether caspases regulate CCK-induced trypsin activation, we measured the effect of zVAD-fmk on trypsin activity in both untreated and CCK-treated pancreatic acinar cells. Trypsin activity was measured at 1 h because CCK-induced trypsin activation in isolated rat acinar cells is transient and by 3 h falls back almost to the basal level (19). Fig. 7A shows that zVAD-fmk markedly potentiated the CCK-induced trypsin activation. The basal trypsin activity was not affected by caspase inhibition.

The results indicate that trypsin activation by CCK-8 is not mediated by preceding caspase activation. To the contrary, active caspases negatively regulate CCK-induced trypsin activation in acinar cells and thus may protect from damage caused by intra-acinar cell trypsin activation.

To assess necrosis in pancreatic acinar cells, we measured LDH release into the extracellular medium. Necrotic cells have damaged plasma membranes and, hence, release LDH. High dose CCK-8 stimulated acinar cell necrosis (Fig. 7B). zVAD-fmk potentiated CCK-induced LDH release, indicating that caspase activation protects acinar cells from CCK-induced necrosis. In contrast, the level of basal LDH release was unaffected by zVAD-fmk (Fig. 7B).

DISCUSSION

Apoptosis and necrosis are key characteristics of both human and experimental pancreatitis (6, 8, 11–13, 16–20); however, the mechanisms underlying these processes remain unknown. This study shows that CCK-8, a major agonist of pancreatic acinar cells, activates multiple death signaling pathways in rat pancreatic acinar cells, namely, caspase activation, cytochrome c release, and mitochondrial depolarization. This activation occurs with supraphysiological concentrations of CCK-8 (>0.1 nM) at which it induces pancreatitis.

CCK-8 stimulated the effector and the initiator caspases-3,-8, and -9; caspase activation and mitochondrial alterations were already evident after 30 min of stimulation with CCK. CCK-induced cytochrome c release was blocked in the presence of CsA and ArA, inhibitors of mitochondrial PTP. The PTP inhibitors also prevented CCK-induced activation of caspase-3,
indicating that it is mitochondria-dependent. On the other hand, CsA and ArA did not prevent the CCK-induced mitochondrial depolarization in acinar cells, suggesting a mechanism distinct from PTP opening. The role of PTP and the relations between cytochrome c release and mitochondrial depolarization are a matter of much debate (24–27). Our data indicate that CCK causes mitochondrial dysfunction in pancreatic acinar cells through both PTP-dependent and -independent pathways and that the mechanisms of CCK-induced cytochrome c release and mitochondrial depolarization are different.

CCK-induced cytochrome c release and mitochondrial depolarization were inhibited by both the broad spectrum caspase inhibitor zVAD-fmk and the caspase-8 inhibitor zIETD-fmk. This suggests that the CCK-induced mitochondrial alterations...
are mediated by upstream caspase(s), possibly caspase-8 (23, 25–28). Further studies are required to determine the mechanism of CCK-induced activation of caspase-8 (or another upstream caspase), in particular, whether the CCK-A receptor triggers death-inducing signaling complex formation and, if so, what adapter proteins are involved.

Interestingly, the mitochondrial alterations were more sensitive to the action of CCK than caspase activation. For example, the extent of both cytochrome c release and mitochondrial depolarization were similar with 1 and 100 nM CCK-8 (Fig. 3), whereas the effect on caspase-3 processing and DEVDase activity was much more pronounced at 100 nM than at 1 nM CCK-8 (Fig. 1). This suggests that in CCK-treated cells caspases are additionally regulated at the post-mitochondrial level.

Untreated pancreatic acinar cells also displayed time-dependent activation of caspases-3, -8, and -9, and the mitochondrial alterations. However, the mechanisms of death signaling in untreated acinar cells are different from those induced by CCK-8. First, cytochrome c release in untreated acinar cells was not inhibited by PTP inhibitors. In fact, CsA and the combination of CsA plus ArA stimulated cytochrome c release and mitochondrial depolarization in untreated acinar cells.

Second, cytochrome c release in untreated cells was not blocked by caspase inhibitors. This indicates that in untreated acinar cells, the mitochondrial alterations are not triggered by upstream caspases. Such a pathway is typical for stress-induced apoptosis, in particular, for apoptosis triggered by growth factor withdrawal (23, 24). Moreover, both zVAD-fmk and zIETD-fmk stimulated cytochrome c release and mitochondrial depolarization in untreated acinar cells. The mechanisms by which PTP and caspase inhibitors induce mitochondrial dysfunction in acinar cells remain to be determined. One possibility is an increased generation of reactive oxygen species, which was reported for both CsA (51, 52) and zVAD-fmk (53, 54). In turn, reactive oxygen species are known to cause mitochondrial dysfunction (24, 25, 55).

Thus our data show that CCK does not simply enhance the signals mediating death of pancreatic acinar cells, but it also triggers mechanisms not operating in untreated cells. As a result, the role of some of these signals changes; for example, caspases mediate cytochrome c release and mitochondrial depolarization in CCK-treated cells, whereas they protect untreated acinar cells from mitochondrial damage.

CCK-8 increased the number of cells with apoptotic nuclear morphology and stimulated internucleosomal DNA fragmentation. Both effects were prevented by zVAD-fmk. These results demonstrate a novel biological activity of CCK: stimulation of
apoptosis in pancreatic acinar cells. In rat pancreatic acinar cells CCK-8 acts through the Gq-coupled CCK-A receptor (1–3).

Hence, our data describe cell death signaling pathways triggered via a G protein-coupled receptor; these pathways remain poorly characterized.

The results also demonstrate that untreated acinar cells die through apoptosis, extending our previous observations (12). Apoptosis in untreated acinar cells is probably caused by a lack of survival factors as well as detachment of cells from extracellular matrix that occurs with their isolation from tissue (56). Without stimulation with growth factors, hormones, etc., pancreatic acinar cells usually die within 8–10 h after isolation.

As stated above, the mechanisms of apoptosis in pancreatitis have not been studied. Our data suggest that apoptosis in CCK-induced (or cerulein-induced) pancreatitis is mediated by caspase activation and mitochondrial dysfunction in acinar cells. Depolarization and structural changes were reported previously in mitochondria isolated from pancreas of rats with cerulein pancreatitis (57, 58).

Our results also show that, in addition to apoptosis, caspases regulate other key parameters of pancreatitis: intra-acinar cell activation of trypsin, necrosis, and inhibition of amylase secretion. In particular, we found that caspase inhibition with zVAD-fmk potentiated CCK-induced trypsin activation in acinar cells, indicating that caspases protect the cells from this pathological process. Although a subject of intensive studies, the mechanism and regulation of intra-acinar cell trypsin activation are not well understood (4, 59).

Caspase inhibition with zVAD-fmk potentiated CCK-induced necrosis of acinar cells, measured by LDH release. The

**Fig. 5.** CCK induces apoptosis in pancreatic acinar cells that is mediated by caspases. A, CCK stimulates internucleosomal DNA cleavage. Pancreatic acini were incubated for indicated times without and with 100 nM CCK-8 in the absence or presence of 100 μM zVAD-fmk. DNA was isolated and analyzed as described under “Experimental Procedures.” B, zVAD-fmk inhibits CCK-induced DNA fragmentation. The percentage of DNA fragmentation was measured in acini incubated for 6 h without and with indicated concentrations of CCK-8. DNA fragmentation was also measured in the presence of 100 μM zVAD-fmk (open triangle). To quantify DNA fragmentation, low and high molecular weight DNA were separated by centrifugation, and the amount of DNA in the supernatant and pellet was determined with diphenylamine method. The values are the means ± S.E. (n = 4). C, the morphology of apoptosis in pancreatic acini incubated for 6 h with 100 nM CCK-8 was evaluated by staining with Hoechst 33258. Apoptotic nuclei are indicated by arrows. D, zVAD-fmk inhibits CCK-induced apoptosis. Pancreatic acini were incubated for 6 h with 100 nM CCK-8 in the absence or presence of 100 μM zVAD-fmk. The percentage of cells with apoptotic nuclei was measured with Hoechst 33258 staining. For each condition, at least 1,000 cells were counted in three different acinar preparations. The values are the means ± S.E. (n = 4). *, p < 0.05 compared with untreated cells.
mechanism(s) of such a protective effect of caspases against 
CCK-induced necrosis remain to be determined. One mecha-
nism could be caspase-mediated cleavage and deactivation of a 
pro-necrotic molecule, poly(ADP-ribose) polymerase, a major 
substrate of caspase-3 (60). We found that poly(ADP-ribose) 
polymerase activity was markedly stimulated both in cerulein 
pancreatitis and in vitro, in CCK-treated rat pancreatic acinar 
cells (data not shown).

The caspase-mediated apoptosis and protection against ne-
crosis and intra-acinar trypsin activation could explain the 
inverse correlation between the extent of apoptosis on the one 
hand, and necrosis and the severity of the disease, on the other

**FIG. 6. Caspases regulate CCK-induced amylase secretion from pancreatic acinar cells.** Pancreatic acini were preincubated for 30 min without and with 100 μM zVAD-fmk and then incubated for additional 30 min with different concentrations of CCK-8. Basal and CCK-induced amylase release was measured as described under “Experimental Procedures.” A, dose dependences of CCK-induced amylase release in the absence and presence of zVAD-fmk. The values are the means ± S.E. from two to four independent experiments. B, effect of zVAD-fmk on amylase secretion induced by 0.1 and 100 nM CCK-8. The maximal amylase release (i.e. induced by 0.1 nM CCK-8) was considered to be 100%. Open bars, CCK-8 alone; closed bars, CCK-8 in the presence of zVAD-fmk. The values are the means ± S.E. (n = 4). *, p < 0.05 compared with CCK alone.

**FIG. 7. Caspases regulate trypsin activation and necrosis in pancreatic acinar cells.** A, effect of zVAD-fmk on CCK-induced trypsin activation. Pancreatic acini were incubated for 1 h without and with 100 nM CCK-8 in the absence or presence of 100 μM zVAD-fmk. Trypsin activity was measured in whole cell lysates using a specific fluorogenic substrate, as described under “Experimental Procedures.” The values are the means ± S.E. (n = 5). *, p < 0.05 compared with untreated cells. #, p < 0.05 compared with cells treated with CCK alone. B, effect of zVAD-fmk on CCK-induced LDH release. Pancreatic acini were incubated for 6 h without and with 100 nM CCK-8, in the absence or presence of 100 μM zVAD-fmk. The percentage of total cellular LDH released into the incubation medium was measured spectrophotometrically. The values are the means ± S.E. (n = 5). *, p < 0.05 compared with untreated cells. #, p < 0.05 compared with cells treated with CCK alone.
hand, that we and others observed in different experimental models of pancreatitis (11–13, 16–18).

Using zVAD-fmk, we also found that caspases mediate processing of PKCδ, which in other cell types was shown to result in PKCδ activation (44–46). PKCδ is a major PKC isofrom activated by CCK (3, 43), and PKC activation is believed to mediate inhibition of amylase secretion with supraphysiological doses of CCK (61–63). Based on the results obtained in this study and in Ref. 49, it is tempting to speculate that caspases may regulate amylase secretion with supraphysiological doses of CCK.

In conclusion, this report shows that high dose CCK, through its G1 protein-coupled receptor, stimulates death signaling mechanisms in pancreatic acinar cells, including caspase activation, cytochrome c release, and mitochondrial depolarization, leading to apoptosis. Caspases not only mediate apoptosis, but they also negatively regulate necrosis and trypsin activation, key parameters of pancreatitis. The results suggest that these signaling mechanisms may play an important role in parenchymal cell injury and death in CCK-induced pancreatitis.

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Anna S. Gukovskaya, Ilya Gukovsky, Yoon Jung, Michelle Mouria and Stephen J. Pandol

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