Secretagogue-induced digestive enzyme activation and cell injury in rat pancreatic acini

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We have utilized the secretagogue-induced model of acute pancreatitis to explore these issues. When given to rats in doses that exceed those required to elicit a maximal rate of digestive enzyme secretion from the pancreas, the CCK-analog caerulein rapidly induces an acute, edematous form of pancreatitis (16). We have shown that evidence of trypsinogen activation within the pancreas can be detected within 15–30 min of the start of caerulein infusion and that trypsinogen activation occurs before evidence of acinar cell injury can be detected (11). More recently, we have found that a supramaximally stimulating concentration of caerulein can also cause trypsinogen activation within acini incubated in vitro with the secretagogue (23). Similar to the in vivo model of pancreatitis, in vitro caerulein-induced trypsinogen activation is rapid (i.e., 15–30 min), dependent on the presence of supramaximally stimulating concentrations of caerulein, and mediated via low-affinity CCK-A receptors. Because of the similarities between this in vitro system and the in vivo model of pancreatitis, we reasoned that the in vitro system might be exploited for studies designed to elucidate the mechanisms responsible for intrapancreatic digestive enzyme activation and acinar cell injury in pancreatitis.

In the currently reported studies, we have utilized the in vitro system to examine the role of Ca\(^{2+}\) in the process of intra-acinar cell trypsinogen activation and the relationship between trypsinogen activation and acinar cell injury. We report that caerulein-induced intra-acinar cell activation of trypsinogen is calcium-dependent, which requires Ca\(^{2+}\) in the suspending medium and a sustained rise in intracellular Ca\(^{2+}\) levels. We have found that intracellular trypsinogen activation precedes cell injury and that inhibition of either trypsinogen activation or trypsin activity can prevent cell injury. These findings indicate that Ca\(^{2+}\)-dependent intra-acinar cell activation of trypsinogen is an early and critical event that leads to cell injury in pancreatitis.

**MATERIALS AND METHODS**

Wistar rats of either sex weighing 100–125 g were obtained from Charles River Laboratories. Caerulein was purchased from Research Plus (Bayonne, NJ), and collagenase was from Worthington Biochemicals (Freehold, NJ). Propidium iodide (PI), 4-(2-aminoethyl)benzenesulfonyl fluoride (Pefabloc), and secretin were from Sigma Chemical (St. Louis, MO). The trypsin substrate Boc-Glu-Ala-Arg-4-methylcoumaryl-7-amide (MCA) was procured from Peptides International (Louisville, KY). 1,2-Bis(2-aminoethoxy)ethane-N,N',N''-tetra-acetic acid (BAPTA)-AM and fura 2-AM, carbamylcholine, L-364,718, benzamidine, thapsigargin, and ionomycin were purchased from Research Plus (Bayonne, NJ). 1,2-Bis(2-aminoethoxy)ethane-N,N',N''-tetra-acetic acid (BAPTA)-AM and fura 2-AM, carbamylcholine, L-364,718, benzamidine, thapsigargin, and ionomycin were purchased from Research Plus (Bayonne, NJ).
RESULTS

Secretagogue-induced trypsinogen activation. We have previously shown that incubation of freshly prepared rat pancreatic acini at 37°C for 30 min with caerulein results in trypsinogen activation and that activation occurs only when caerulein concentrations in excess of those causing a maximal rate of digestive enzyme secretion (i.e., 10–100 µM) are used (23). This relationship between caerulein concentration and in vitro trypsinogen activation is shown in Fig. 1. Little or no activation is observed when acini are incubated without caerulein. Half-maximal caerulein-induced trypsinogen activation is observed in the presence of 1–10 nM caerulein, and complete inhibition of caerulein-induced activation is found when the CCK-A receptor antagonist L-364,718 (0.1 µM) is included in the incubation mixture. Trypsinogen activation is also noted when acini are incubated with the cholinergic agonist carbachol (data not shown). Activation is observed when concentrations of carbachol in excess of 0.1 µM are used; maximal activation is seen with 100 µM carbachol, and activation is prevented by inclusion of the muscarinic cholinergic antagonist atropine (100 µM) in the incubation mixture. In contrast to caerulein and carbachol, which both induce in vitro trypsinogen activation, no activation is observed when acini are incubated with secretin (0.1–1.0 µM) (Table 1) or CCK-J MV-180 (0.1 mM; Ref. 23).

Role of extracellular Ca\(^{2+}\) in secretagogue-induced trypsinogen activation. Digestive enzyme secretion by acini in response to stimulation with caerulein or carbachol, but not secretin, is known to depend on the presence of Ca\(^{2+}\) in the suspending medium (4, 28). To evaluate the role of extracellular Ca\(^{2+}\) in secretagogue-induced trypsinogen activation, acini were obtained from Molecular Probes. All other reagents were of the highest purity commercially available.

Preparation of pancreatic acini. Dispersed rat pancreatic acini were prepared by collagenase digestion and gentle shearing as described previously (22, 27). The acini were suspended in HEPES-Ringer buffer (pH 7.4), which contained 115 mM NaCl, 5 mM glucose, and 0.1% BSA. The buffer was saturated with O\(_2\) by bubbling. Viability of acini at the start of each experiment was >95% as assessed by trypan blue exclusion. After incubation of the acini with various agents and under various conditions, the suspending buffer was harvested and the acini were washed twice with HEPES-Ringer buffer. They were then homogenized in cold (4°C) buffer using a motorized glass-Teflon homogenizer. The resulting homogenate was centrifuged (50 g, 5 min), and the supernatant was taken for assay.

Quantitation of cell injury. Cell injury was monitored using two independent but complementary methods: lactate dehydrogenase (LDH) leakage from cells (5) and PI intercalation into nuclear DNA (9). LDH leakage was evaluated by measuring LDH activity in the suspending buffer during incubation of acini and quantitating that activity as a percentage of the total cellular LDH activity measured after homogenization of the acini. PI intercalation was evaluated by incubating acini with 50 µg/ml of PI for 20 min before quantitating fluorescence (excitation at 536 nm, emission at 617 nm).

Measurement of intracellular Ca\(^{2+}\) concentration. Acini were loaded with fura 2 by incubation for 30 min at 23°C with 5 µM fura 2-AM. Acini were then washed and resuspended in fresh buffer, and intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) was evaluated using a SPEX fluorometer as previously described (6, 12).

Assays. Trypsin activity was measured fluorometrically using Boc-Glu-Ala-Arg-MCA as the substrate according to the method of Kawabata et al. (14). For that measurement, a 200-µl aliquot of the acinar cell homogenate was added to a cuvette containing assay buffer (50 mM Tris, 150 mM NaCl, 1 mM CaCl\(_2\), and 0.1% BSA, pH 8.0). The reaction was initiated by addition of substrate, and the fluorescence emitted at 440 nm in response to excitation at 380 nm was monitored. Trypsin activity in the samples was calculated using a standard curve generated by assaying purified trypsin obtained from Worthington and expressed as microgram equivalents of trypsin activity per milligram DNA. To permit pooling of results from several independent experiments, trypsin activity was generally expressed as the percentage of the maximal noted in each experiment with 0.1 µM caerulein. In general, trypsin activity in homogenized acini exposed to caerulein (0.1 µM) for 30 min at 37°C ranged from 0.8 to 1.5 µg trypsin equivalents/mg pancreatic DNA. LDH activity was quantitated as described by Amador et al. (2). In preliminary experiments, Pefabloc (2 mM) was found to completely inhibit trypsin activity but not to reduce LDH activity (data not shown). DNA was measured fluorometrically using Hoechst dye 33258 and calf thymus DNA as the standard as previously described (15).

Data presentation. The results reported here represent means ± SE values for multiple determinations from at least three separate preparations of acini. The significance of the changes was evaluated using Student's t-test when the data consisted of two groups only or by ANOVA when comparing three or more groups. If ANOVA indicated significant differences, the data were analyzed using Tukey's method as a post hoc test for the difference between the groups. P < 0.05 was considered to be significant.

Fig. 1. Caerulein-induced in vitro trypsinogen activation in pancreatic acini. Freshly prepared acini were incubated for 30 min with varying concentrations of caerulein either alone (○) or with the CCK-A receptor antagonist L-364,718. (●). Trypsin activity was measured as described in the text and expressed as a percentage of the value obtained in the presence of 0.1 µM caerulein. Results represent means and vertical bars the SE values obtained from 3 or more independent preparations and incubations. *P < 0.05 compared with basal values obtained when acini were incubated in buffer alone.
suspended in buffer containing varying concentrations of Ca\textsuperscript{2+} with or without caerulein (0.1 µM), and trypsin activity was measured. As shown in Fig. 2, no caerulein-induced trypsinogen activation is detected when acini are incubated in nominally Ca\textsuperscript{2+}-free buffer, maximal activity is noted in 1 mM Ca\textsuperscript{2+}-containing buffer, and half-maximal activation is observed in 0.3 mM Ca\textsuperscript{2+}-containing buffer. Addition of Ca\textsuperscript{2+} to the incubation mixture without caerulein does not result in trypsinogen activation.

The time-dependent effects of either withdrawing or adding Ca\textsuperscript{2+} (1 mM) to the incubation mixture are shown in Fig. 3. Maximal caerulein-induced trypsinogen activation is noted when 1 mM Ca\textsuperscript{2+} is present during the full 30-min incubation. Withdrawal of Ca\textsuperscript{2+} at various times causes a time-dependent decrease in the overall extent of caerulein-induced trypsinogen activation, whereas readdition of Ca\textsuperscript{2+} results in a time-dependent restoration of caerulein-induced trypsinogen activation. The effect of either withdrawal or readdition of Ca\textsuperscript{2+} is a continuous function of the time of Ca\textsuperscript{2+} exposure, i.e., it is relatively slow and approximately half-maximal activation is noted when acini are exposed to extracellular Ca\textsuperscript{2+} during only the final (Fig. 3A) or initial (Fig. 3B) 15 min of the total 30-min incubation with caerulein.

Role of intracellular Ca\textsuperscript{2+}. Digestive enzyme secretion in response to either caerulein or carbamylcholine but not secretin is known to be associated with a rise in [Ca\textsuperscript{2+}] (4, 28). To evaluate the role of a rise in [Ca\textsuperscript{2+}] in secretagogue-induced trypsinogen activation, acini were suspended in buffer containing varying concentrations of Ca\textsuperscript{2+} with or without caerulein (0.1 µM), and trypsin activity was measured. As shown in Fig. 2, no caerulein-induced trypsinogen activation is detected when acini are incubated in nominally Ca\textsuperscript{2+}-free buffer, maximal activity is noted in 1 mM Ca\textsuperscript{2+}-containing buffer, and half-maximal activation is observed in 0.3 mM Ca\textsuperscript{2+}-containing buffer. Addition of Ca\textsuperscript{2+} to the incubation mixture without caerulein does not result in trypsinogen activation.

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Table 1. Effects of caerulein, secretin, ionomycin, and thapsigargin on trypsinogen activation

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration, µM</th>
<th>Trypsin Activity, % of maximal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
<td>19 ± 5</td>
</tr>
<tr>
<td>Caerulein</td>
<td>0.1</td>
<td>100</td>
</tr>
<tr>
<td>Secretin</td>
<td>0.1</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>Secretin</td>
<td>1.0</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>1.0</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>1.0</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>10</td>
<td>14 ± 5</td>
</tr>
</tbody>
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Values are means ± SE obtained from 3 or more independent preparations and incubations. Acini were incubated for 30 min in 1 mM Ca\textsuperscript{2+} buffer containing the indicated concentrations of each agent. Trypsin activity was measured as described in the text and expressed as a percentage of that noted with 0.1 µM caerulein.

Fig. 2. Effect of extracellular Ca\textsuperscript{2+} on caerulein-induced trypsinogen activation. Acini were incubated for 30 min in nominally Ca\textsuperscript{2+}-free buffer or in buffer containing the indicated concentrations of Ca\textsuperscript{2+}. C, Results for acini incubated with caerulein (0.1 µM). •, Results for acini incubated without caerulein. Trypsin activity was measured as described in the text and expressed as described in Fig. 1 legend. Results represent means and vertical bars the SE values obtained from 3 or more independent preparations and incubations. *P < 0.05 compared with values obtained in nominally Ca\textsuperscript{2+}-free buffer.

Fig. 3. Effect of addition or withdrawal of Ca\textsuperscript{2+} on caerulein-induced trypsinogen activation. Pancreatic acini were incubated in the presence (open bars) or absence (closed bars) of caerulein (0.1 µM) for 30 min. Trypsin activity was measured as described in the text and expressed as described in Fig. 1 legend. A: Ca\textsuperscript{2+} (1.0 mM final concentration) was added at the start of the incubation (i.e., time 0) or after 5, 10, 15, and 30 min of incubation. B: Ca\textsuperscript{2+} was withdrawn from the medium at the start of the incubation (i.e., time 0) or after 5, 10, 15, and 30 min of incubation. Results represent means and vertical bars the SE values for 3 or more independent preparations and incubations. *P < 0.05 when samples incubated with caerulein were compared with those incubated without caerulein. #Values for samples incubated with caerulein that are different (P < 0.05) from values for samples incubated with caerulein at the immediately preceding time.

Digestive enzyme secretion in response to either caerulein or carbamylcholine but not secretin is known to be associated with a rise in [Ca\textsuperscript{2+}] (4, 28). To evaluate the role of a rise in [Ca\textsuperscript{2+}] in secretagogue-induced trypsinogen activation, acini were
preloaded with the Ca\(^{2+}\)-chelating agent BAPTA and then exposed to caerulein (0.1 µM). Preliminary experiments (not shown) using fura-2-loaded and BAPTA-loaded acini, confirmed the observation previously reported (7, 24) that, under these conditions, addition of caerulein did not result in a measurable rise in [Ca\(^{2+}\)]. As shown in Fig. 4, preloading acini with BAPTA prevents caerulein-induced trypsinogen activation.

Effects of ionomycin and thapsigargin. To determine whether a rise in [Ca\(^{2+}\)] is, by itself, sufficient to induce trypsinogen activation in the absence of secretagogue stimulation, acini were incubated with either the Ca\(^{2+}\) ionophore ionomycin or the Ca\(^{2+}\)-ATPase inhibitor thapsigargin. Preliminary experiments using fura-2-loaded acini (not shown) confirmed the observation previously reported (18, 19, 25) that, under these conditions, sustained [Ca\(^{2+}\)] levels in excess of the sustained rises observed using supramaximally stimulating concentrations of caerulein were achieved. As shown in Table 1, neither ionomycin nor thapsigargin addition to the incubation medium under these conditions results in trypsinogen activation.

Caerulein-induced cell injury. Two separate but complementary methods, LDH leakage and PI incorporation, were used to quantitate cell injury during in vitro exposure of acini to a supramaximally stimulating concentration of caerulein. A rise in either or both LDH leakage from cells and the extent of PI intercalation into DNA indicate cellular membrane injury has occurred (8). As shown in Fig. 5A, LDH leakage is not increased when acini are incubated with caerulein in 1 mM Ca\(^{2+}\) buffer for 15 min. Both parameters of cell injury are modestly increased when acini are exposed to caerulein in 1 mM Ca\(^{2+}\) buffer for 30 min. The extent of these changes increases with time over the next 1 h, but no evidence of cell injury is noted when the CCK-A receptor antagonist L-364,718 is included, along with caerulein in the incubation medium. Cell injury is also markedly reduced when acini are exposed to caerulein in nominally Ca\(^{2+}\)-free buffer.

Effect of protease inhibition on cell injury. We have employed the cell-permeant and potent protease inhibitor Pefabloc (3) to evaluate the effects of protease inhibition on caerulein-induced acinar cell injury. As shown in Fig. 6B, addition of Pefabloc (2 mM) to the incubation mixture before caerulein addition prevents...
caerulein-induced acinar cell injury. Under these conditions, no caerulein-induced increase in trypsin activity is detected (Fig. 6A). When Pefabloc is added to the incubation mixture 30 min after incubation with caerulein, i.e., after maximal caerulein-induced trypsinogen activation has occurred (Fig. 3) but when caerulein-induced acinar cell injury is minimal (Fig. 5), the otherwise time-dependent rise in caerulein-induced LDH leakage is halted (Fig. 7). Similar results were obtained when the protease inhibitor benzamidine (10 mM) was used (data not shown).

**DISCUSSION**

Activated digestive enzymes, present within the pancreas, are generally believed to be responsible for the initiation and/or progression of cell injury in acute pancreatitis. The recent observation (26) that some forms of hereditary pancreatitis are the result of a genetic mutation that renders trypsin resistant to inactivation has strengthened this belief. We have previously reported that intra-acinar cell activation of trypsinogen is an early event that precedes overt pancreatitis in the secretagogue-induced model of pancreatitis (11), and, more recently, we have shown that trypsinogen activation is mediated by the lysosomal enzyme cathepsin B in an in vitro version of the secretagogue-induced pancreatitis model (23).

The currently reported studies have used this in vitro system, in which freshly prepared but otherwise normal pancreatic acini are exposed, in vitro, to supramaximally stimulating concentrations of secretagogues, to examine the mechanisms responsible for secretagogue-induced trypsinogen activation as well as the relationship between enzyme activation and cell injury in pancreatitis. Secretagogue-induced trypsinogen activation in the in vitro system shares many of the features that characterize intrapancreatic trypsinogen activation during secretagogue-induced pancreatitis in vivo (13). In both cases, trypsinogen activation 1) occurs rapidly (i.e., <30 min), 2) requires supramaximally stimulating concentrations of caerulein, 3) is mediated by low-affinity CCK-A receptors, 4) is associated with subcellular redistribution of lysosomal enzymes, and 5) occurs within cytoplasmic vacuoles, which contain digestive enzyme zymogens as well as lysosomal hydrolases. Thus this in vitro system would seem to be an ideal tool with which to explore issues basic to the in vivo process of pancreatitis while avoiding the complexity and difficulty in controlling conditions inherent to whole animal studies.

In addition to caerulein, which acts via CCK-A receptors, we now report that carbamylcholine, acting
via muscarinic cholinergic receptors, can also induce in vitro trypsinogen activation. On the other hand, secretin stimulation of acini does not lead to trypsinogen activation (Table 1). Similar responses occur in vivo as well, i.e., supramaximally stimulating doses of agents acting via CCK-A and muscarinic cholinergic receptors induce pancreatitis (1, 10, 11), but high doses of secretin do not induce the disease (Saluja and Steer, unpublished observations). Rat pancreatic acinar cell CCK-A and muscarinic cholinergic receptors mediate secretion via the phospholipase A-inositol trisphosphate-Ca$^{2+}$ messenger cascade, whereas secretin stimulation of secretion is mediated via the adenylate cyclase-CAMP system (4, 28). Thus our observation that caerulein and carbamylcholine, but not secretin, can cause trypsinogen activation suggested to us that in vitro secretagogue-induced trypsinogen activation might be a Ca$^{2+}$-dependent phenomenon. To evaluate the role of Ca$^{2+}$ in secretagogue-induced trypsinogen activation, three series of experiments were performed.

In the first series of experiments, the dependence of intracellular trypsinogen activation on the presence of extracellular Ca$^{2+}$ was examined. As shown in Fig. 2, little or no secretagogue-induced trypsinogen activation can be detected when acini are suspended in nominally Ca$^{2+}$-free buffer for the entire 30-min period of incubation. The process of caerulein-induced trypsinogen activation can be either halted or restored by removal or reintroduction, respectively, of Ca$^{2+}$ (Fig. 3). These observations indicate that caerulein-induced trypsinogen activation is dependent on the continued presence of extracellular Ca$^{2+}$ and that extracellular Ca$^{2+}$ plays a relatively slow but continuous, as opposed to either an early or a late, role in facilitating trypsinogen activation.

Secretagogue-induced temporal changes in [Ca$^{2+}$]$_i$ are known to be dependent on the presence or absence of Ca$^{2+}$ in the suspending medium (4, 28). Exposure of acini to a supramaximally stimulating concentration of caerulein in Ca$^{2+}$-containing buffer results in an early and large increase in [Ca$^{2+}$]$_i$, reflecting release of Ca$^{2+}$ from intracellular stores, followed by a sustained but lower level elevation of [Ca$^{2+}$]$_i$, which is believed to reflect influx of Ca$^{2+}$ from the extracellular space. In Ca$^{2+}$-free medium, the initial large rise in [Ca$^{2+}$]$_i$ is still seen, but, thereafter, [Ca$^{2+}$]$_i$ levels decay to the basal value (20, 21). Our observation that secretagogue-induced trypsinogen activation either is markedly reduced or does not occur in Ca$^{2+}$-free medium indicates, therefore, that the initial but transient large rise in [Ca$^{2+}$]$_i$, caused by release of Ca$^{2+}$ from internal stores, is not, by itself, sufficient to permit trypsinogen activation. Our findings could suggest, however, that the sustained but lower level rise in [Ca$^{2+}$]$_i$, mediated by influx of Ca$^{2+}$ from the extracellular space plays a critical role in secretagogue-induced trypsinogen activation (see below).

Our second series of experiments was designed to determine whether caerulein-induced trypsinogen activation is dependent on the presence of extracellular Ca$^{2+}$ alone, a rise in [Ca$^{2+}$]$_i$ alone, or on both phenomena. To evaluate this issue, acini were preloaded with the Ca$^{2+}$-chelating agent BAPTA and then suspended in Ca$^{2+}$-containing buffer. We and many others have previously noted that preloading cells with BAPTA prevents the rise in [Ca$^{2+}$], which is otherwise seen when acini are exposed to a secretagogue such as caerulein (7, 24). As shown in Fig. 4, preloading acini with BAPTA prevents caerulein-induced trypsinogen activation even in the presence of Ca$^{2+}$-containing buffer. This observation indicates that secretagogue-induced trypsinogen activation depends on a secretagogue-induced rise in [Ca$^{2+}$], and that the presence of extracellular Ca$^{2+}$ is not, by itself, sufficient to permit trypsinogen activation.

Our third and final series of experiments performed to characterize the role of Ca$^{2+}$ in secretagogue-induced trypsinogen activation was designed to determine whether a sustained rise in [Ca$^{2+}$]$_i$ is, by itself, sufficient to permit enzyme activation. We have taken advantage of the known ability of calcium ionophores and of Ca$^{2+}$-ATPase inhibitors, when added to acini in Ca$^{2+}$-containing medium, to cause a sustained rise in [Ca$^{2+}$]$_i$ (18, 19, 25). We have chosen conditions in which the sustained rise in [Ca$^{2+}$]$_i$ achieved with the ionophore ionomycin or the Ca$^{2+}$-ATPase inhibitor thapsigargin equals or exceeds the sustained elevation in [Ca$^{2+}$]$_i$ achieved when acini were exposed to a supramaximally stimulating concentration of caerulein in Ca$^{2+}$-containing buffer. As shown in Table 1, neither ionomycin nor thapsigargin addition, under these conditions, results in intracellular trypsinogen activation. These results indicate that a sustained rise in [Ca$^{2+}$]$_i$, if achieved by a nonsecretagogue method, is not by itself sufficient to cause intra-acinar cell trypsinogen activation.

Taken together, these results indicate that caerulein-induced trypsinogen activation depends on a sustained rise in [Ca$^{2+}$]$_i$, which, presumably, reflects influx of Ca$^{2+}$ from the extracellular medium. Neither the marked but transient initial rise in [Ca$^{2+}$]$_i$ induced by caerulein in Ca$^{2+}$-free buffer nor the lesser but sustained rise in [Ca$^{2+}$]$_i$ induced by ionomycin or thapsigargin is, by itself, sufficient to cause enzyme activation. Rather, it would appear that the sustained rise in [Ca$^{2+}$]$_i$ must, itself, be associated with exposure to a supramaximally stimulating concentration of a Ca$^{2+}$-associated secretagogue.

Two potential explanations for these findings should be considered. Perhaps the simplest explanation is that the supramaximally stimulating concentrations of caerulein and carbamylcholine, but not ionomycin or thapsigargin, cause sustained elevations of [Ca$^{2+}$]$_i$ to occur at the subcellular site, which is actually critical to trypsinogen activation. The second, and perhaps more intriguing, explanation for our observations is that, in addition to causing a sustained rise in [Ca$^{2+}$]$_i$, the supramaximally stimulating concentrations of secretagogues bring about one or more additional changes that permit trypsinogen activation to occur. One of the features shared by caerulein and carbamylcholine but not secretin is the fact that, at supramaximally stimu-
lating concentrations, both secretagogues inhibit digestive enzyme secretion from acinar cells (28). It is, therefore, tempting to speculate that inhibition of secretion combined with a sustained rise in \([\text{Ca}^{2+}]i\), is the critical event that leads to intra-acinar cell trypsinogen activation and to postulate that inhibition of secretion contributes to this process by promoting cathepsin B-catalyzed trypsinogen activation.

The relationship between intra-acinar cell digestive enzyme activation and acinar cell injury in pancreatitis is of potentially great importance, but this relationship remains poorly understood. We now report that secretagogue-induced intra-acinar cell activation of trypsinogen is associated with biochemical evidence of cell injury. Similar observations have been recently reported in abstract form by others (17). In our studies (Fig. 5), cell injury was quantitated by measuring LDH leakage from acinar cells or intercalation of PI into acinar cell DNA. With the use of both techniques, cell injury was first detected 30 min after exposure to a supramaximally stimulating concentration of caerulein, and the extent of cell injury was noted to increase, in a time-dependent manner, thereafter. Cell injury was either prevented or markedly reduced when CCK-A receptors were blocked with L-364,718 or when \(\text{Ca}^{2+}\) was not added to the incubation medium. These observations should be compared with those reported previously (23) and earlier in this paper (Figs. 1–3), which indicate that caerulein-induced in vitro trypsinogen activation can be detected within 5 min of supramaximal caerulein stimulation (23), that it is maximal within 30 min of supramaximal secretagogue stimulation (23), that it can be prevented by inclusion of the CCK-A receptor antagonist L-364,718, and that it is dependent on the presence of \(\text{Ca}^{2+}\) in the suspending medium. Together, these observations indicate that caerulein-induced trypsinogen activation and caerulein-induced cell injury are closely linked phenomena and that trypsinogen activation occurs prior, rather than subsequent, to cell injury.

We have used the protease inhibitors Pefabloc and benzamidine to further examine the relationship between trypsinogen activation and cell injury. Similar results were obtained with both agents. Because of its greater potency and cell permeance, Pefabloc may be better suited for these studies, since these qualities permit use of lower concentrations of the inhibitor. No trypsin activity could be detected within the acinar cell homogenate when the protease inhibitors were added before caerulein even when measurements were performed as late as 30 min after caerulein addition. Under these conditions, supramaximal stimulation with caerulein did not result in cell injury as measured by LDH leakage from acinar cells (Fig. 6). These observations are compatible with the conclusion that caerulein-induced acinar cell injury depends on proteolytic activity, which can be inhibited by either Pefabloc or benzamidine.

To further examine this issue, acini were preincubated with caerulein in \(\text{Ca}^{2+}\)-containing buffer for 30 min to permit full trypsinogen activation and the initiation of cell injury. At that time, Pefabloc was added. As expected, trypsin activity in the acinar cell homogenate could not be detected after addition of Pefabloc. Of greater importance, however, was the finding that inhibition of trypsin activity by Pefabloc prevented the otherwise expected increase in the extent of cell injury (Fig. 7).

These observations indicate that cell injury is a manifestation of the continued presence of proteolytic activity (which is inhibited by Pefabloc) within acinar cells. Taken together, our results lead us to conclude that caerulein-induced acinar cell injury in this in vitro model of secretagogue-induced pancreatitis is a two-step process that is initiated by \(\text{Ca}^{2+}\)-dependent trypsinogen activation and completed by protease-mediated cell injury.

We thank H. Wattanga and E. Donovan for technical assistance. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-31396. Address for reprint requests and other correspondence: M. L. Steer, Dept. of Surgery, Beth Israel Deaconess Medical Center, 330 Brookline Ave., Boston, MA 02215. (E-mail: msteer@bidmc.harvard.edu.)

Received 21 September 1998; accepted in final form 9 December 1998.

REFERENCES


25. Toescu, E. C., and O. H. Petersen. The thapsigargin-evoked increase in [Ca\textsuperscript{2+}] involves an InsP\textsubscript{3}-dependent Ca\textsuperscript{2+} release process in pancreatic acinar cells. Pflügers Arch. 427: 325–331, 1994.

