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Trypsin activity is not involved in premature, intrapancreatic trypsinogen activation

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Halangk, Walter, Burkhard Krüger, Manuel Ruthenbürger, Jörg Stürzebecher, Elke Albrecht, Hans Lippert, and Markus M. Lerch. Trypsin activity is not involved in premature, intrapancreatic trypsinogen activation. *Am J Physiol Gastrointest Liver Physiol* 282: G367–G374, 2002. First published September 21, 2001; 10.1152/ajpgi.00315.2001.—A premature and intracellular activation of digestive zymogens is thought to be responsible for the onset of pancreatitis. Because trypsin has a critical role in initiating the activation cascade of digestive enzymes in the gut, it has been assumed that trypsin also initiates intracellular zymogen activation in the pancreas. We have tested this hypothesis in isolated acini and lobules from rat pancreas. Intracellular trypsinogen activation was induced by supramaximal secretagogue stimulation and measured using either specific trypsin substrates or immunoreactivity of the trypsinogen activation peptide (TAP). To prevent a trypsin-induced trypsinogen activation, we used the cell-permeant, highly specific, and reversible inhibitor *N*α-(2-naphthylsulfonyl)-3-amidinophenylalanine-carboxymethylpiperazide (S124), and to prevent cathepsin-induced trypsinogen activation, we used the cysteine protease inhibitor E-64d. Incubation of acini or lobules in the presence of S124 completely prevented the generation of trypsin activity in response to supramaximal caerulein but had no effect whatsoever on the generation of TAP. Conversely, when trypsin activity was recovered at the end of the experiment by either washout of S124 from acini or extensive dilution of lobule homogenates, it was up to 400% higher than after caerulein alone and corresponded, in molar terms, to the generation of TAP. Both trypsin activity and TAP release were inhibited in parallel by E-64d. We conclude that caerulein-induced trypsinogen activation in the pancreas is caused by an E-64d-inhibitable mechanism such as cathepsin-induced trypsinogen activation, and neither involves nor requires intracellular trypsin activity. Specific trypsin inhibition, on the other hand, prevents 80% of trypsin inactivation or autodegradation in the pancreas.

autoactivation; cathepsin B; acute pancreatitis

ACUTE PANCREATITIS HAS LONG been thought to represent an autodigestion of the pancreas by its own digestive proteases (1). Recent evidence suggests that a premature activation of digestive proteases does indeed occur within pancreatic acinar cells (6, 10, 13) and has been closely associated with the proteolytic damage to the organ (3, 23). What have remained controversial are the triggering events that initiate the premature and intrapancreatic activation of these proteolytic enzymes that are physiologically synthetized, stored, and secreted from the pancreas as inactive precursor zymogens.

In a series of elegant studies from the first half of the last century, Kunitz and Northrop (11) have firmly established that highly purified trypsin cannot only activate other serine protease precursors, such as chymotrypsinogen, but can also convert itself from an inactive (trypsinogen) to an active (trypsin) protease (19). On the basis of these fundamental observations, others have postulated an activation cascade in which trypsinogen is initially activated by the brush border protease enterokinase (enteropeptidase) in the gut and then proceeds to activate all other digestive proteases including itself (20). Whereas this protease cascade with trypsin as the crucial trigger enzyme has been shown to be operative in the small intestine, the conditions within the pancreas are different in several respects. Because enterokinase as an initial activator of trypsinogen is absent in the pancreas, other mechanisms must account for the conversion from trypsinogen to trypsin before a proteolytic cascade, which would be analogous to the one in the gut, could be expected to occur. Therefore, several hypotheses have been proposed to explain trypsinogen activation within the pancreas in the absence of enterokinase. The two most prominent theories that have addressed this issue appear to be mutually exclusive. The first predicts that cathepsin B, a lysosomal enzyme that has been found to colocalize with digestive zymogens in the pancreas during early pancreatitis, accounts for the initial activation of trypsinogen (25, 26, 29). We and

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others have already confirmed that a significant proportion of intrapancreatic trypsinogen activation depends on the presence of cathepsin B in the pancreas (4, 24). The second hypothesis predicts that trypsinogen autoactivates (31) and, therefore, a trypsin-induced trypsinogen activation represents the triggering event for acute pancreatitis. In the study presented here, we have tested the latter hypothesis.

With the use of a cell-permeant, highly specific, and reversible trypsin inhibitor, we were able to completely inhibit trypsin activity in isolated rat pancreatic acini or lobules. Because of the reversibility of the inhibitor, however, we were able to determine not only to what extent trypsin had been activated, by measuring trypsinogen activation peptide (TAP), but also how much free intracellular trypsin activity had been generated by supramaximal secretagogue stimulation at the end of the experiment. Our data indicate that the conversion of trypsinogen to trypsin in response to supramaximal caerulein remains completely unaffected by the presence of a specific trypsin inhibitor and thus by the presence or absence of free trypsin activity within the acinar cells. Autoactivation of trypsinogen is therefore not an initiating factor for the intrapancreatic proteolytic cascade. Intracellular trypsin, on the other hand, was responsible for the rapid inactivation of ~80% of the prematurely generated free trypsin activity in the pancreas.

MATERIALS AND METHODS

Chemicals and reagents. The reversible cell-permeant and highly specific trypsin inhibitor *N*α-(2-naphthylsulfonyl)-3-amidinophenylalanine-carboxymethylpiperazide (S124), which belongs to the class of benzamidine-type serine protease inhibitors but has a much higher affinity to trypsin than benzamidine, was kindly provided by Dr. P. Wikström (Basel, Switzerland). The protease substrates (CBZ-Ile-Pro-Arg)$_2$-rhodamine 110, BOC-Gln-Ala-Arg-AMC, and Z-Arg-Arg-AMC were purchased from Molecular Probes (Eugene, OR) and Bachem (Heidelberg, Germany), respectively. Caerulein was obtained from Pharmacia & Upjohn (Erlangen, Germany). E-64d, bovine trypsin (T-8003; Sigma), cathepsin B, and soybean trypsin inhibitor were obtained from Sigma (Deisenhofen, Germany). The TAP enzyme immunoassay kit was purchased from Biotrin International (Sinsheim-Reihen, Germany). The protein assay was obtained from BioRad (Munich, Germany)

Preparation and incubation of pancreatic acini and lobules. For all experiments involving animals, the permissions of the respective animal care and use committees were obtained. Male Wistar rats (HsdCpb:WU; 150–180 g) were purchased from Harlan-Winckelmann (Borchen, Germany), kept in nalgene shoebox cages at a 12:12-h light-dark cycle, and left for a minimum of 5 days to adjust to laboratory conditions. Acini or lobules from rat pancreas were prepared after an overnight fast of the animals with access to water ad libitum. Acini were prepared by collagenase (Serva, Heidelberg, Germany), as previously reported (16, 32), and incubated in a medium containing (in mM) 24.5 HEPES, 96 NaCl, 6 KCl, 1 $MgCl_2$, 2.5 Na_2HPO_4, 0.5 $CaCl_2$, 11.5 glucose, 5 Na pyruvate, 5 Na glutamate, and 5 Na fumarate, with minimum essential medium (1% vol/vol) and bovine serum albumin, fraction V (1% wt/vol), at pH 7.4.

Lobules were prepared by needle dissection according to Lerch et al. (15) in an ice-cold medium consisting of (in mM) 140 NaCl, 2.8 KCl, 1.3 $CaCl_2$, 0.6 $MgCl_2$, 0.8 Na_2HPO_4, 10 glucose, 2 Na glutamine, and 10 mM HEPES with essential amino acids (pH 7.4) gassed with oxygen. Incubations of lobule preparations (10–12 mg protein/ml) were performed at 37°C. After the incubation, aliquots of the lobule incubations were transferred into four volumes of iced medium containing 5 mM MOPS, 1 mM $MgSO_4$, and 250 mM sucrose at pH 6.5, homogenized using an Ultra-Turrax, sonicated, and centrifuged for 5 min at 16,000 *g*. Sample preparation for TAP assay was performed by supplementing the lobule homogenate with 1 mM EDTA and Triton X-100 (0.1% vol/vol) and boiling the solution for 10 min. Supernatants were stored at −80°C.

Trypsin activity and TAP immunoreactivity measurements. Trypsin activity was determined in supernatants of lobule homogenates with the fluorogenic substrate BOC-Gln-Ala-Arg-AMC (64 μM) at 37°C. AMC fluorescence was recorded at 360 nm (excitation)/450 nm (emission) wavelength using a Perkin-Elmer LS50 B fluorometer. Trypsin activity was calculated as soybean trypsin inhibitor (SBTI)-sensitive fluorescence increase in relation to bovine trypsin as standard and expressed as picomoles of trypsin.

Trypsin activity in intact living acini was quantitated as previously reported in detail (9). In brief, after an equilibration of 30 min, the cholecystokinin analog caerulein was added at either supramaximal (10 nM) or maximal (0.1 nM) concentrations for up to 60 min and at 37°C. Acini were then washed and resuspended in medium without secretagogue but in the presence of the cell-permeant synthetic trypsin substrate (CBZ-Ile-Pro-Arg)$_2$-rhodamine 110 (10 μM). To quantitate substrate cleavage, acini, together with the substrate, were transferred to 96-well microtiter plates and the $\Delta F/\Delta t$ ratio was determined by cytofluorometry (Ex 485 nm, Em 530 nm; CytoFluor 2350, Millipore, Bedford, MA) over 60 min. For localization experiments, a high-resolution digital fluorescence imaging system (Nikon-Improvision, Coventry, UK; Ex 485 nm, Em 530 nm) was used, and acini were viewed continuously under the microscope in an optical incubation chamber. TAP was determined in supernatants of boiled lobule homogenates using an enzyme immunoassay kit from Biotrin International (7). Protein content was determined according to Bradford using the BioRad reagent.

Statistical analysis of data. Data are given as mean values (±SD) for at least four separate experiments in each group. Treatment and control values at the different time intervals were tested for statistically significant differences by means of Student's *t*-test with Bonferroni correction for multiple comparisons wherever appropriate. A P value of <0.05 was considered to be statistically significant.

RESULTS

One of the difficulties in investigating the role of trypsin in its own activation or degradation has been the absence of appropriate research tools. Although trypsin autoactivation as a triggering event for acute pancreatitis appeared an attractive hypothesis, it could not be tested. The available trypsin inhibitors used in the in vitro studies of the past were either nonspecific, would not readily cross the acinar cell membrane, or inhibited the enzyme in a nonreversible manner. Moreover, trypsin activity was the only available parameter to determine trypsinogen activation, and inhibition of trypsin would therefore prevent any

assessment of autoactivation. A number of recent developments has greatly aided the design of studies addressing trypsinogen activation. The first is the generation of specific antibodies directed against the NH_2-terminal activation (penta)-peptide of trypsinogen (TAP) and an according assay based on immunoreactivity (7). The antibody detects neither trypsinogen nor trypsin but only TAP after cleavage from trypsinogen. The advantage of this approach is that trypsinogen activation can be determined without the need to measure catalytic trypsin activity and even in the presence of trypsin inhibitors. The second is the development of cell-permeant and highly specific trypsin inhibitors that block enzymatic activity completely but reversibly in the nanomolar range.

Characterization of trypsin inhibition by S124. One of these novel high-affinity inhibitors is S124, a benzamidine type inhibitor that does not cross-react with other pancreatic serine proteases (5, 27). When we tested the sensitivity and specificity of S124 in purified trypsin preparations in vitro, we found a concentration-dependent inhibition of the catalytic activity beginning at 10 nM and complete inhibition at 1 μM (Fig. 1*A*). The in vitro activity of purified cathepsin B, on the other hand, remained completely unaffected by the presence or absence of S124 over the entire range of concentrations.

Whereas this in vitro observation indicates a high inhibitor specificity, it provided no evidence for membrane permeability. To prove this, we used isolated pancreatic acini and exposed them to supramaximal concentrations of the secretagogue caerulein (10 nM). This resulted in intracellular cleavage of the trypsin-specific substrate $(CBZ\text{-}Ile\text{-}Pro\text{-}Arg)_2$-rhodamine 110 and a rapid increase in rhodamine 110 fluorescence in living acini as previously reported (9, 10). Addition of 10 μM S124 either before or even 30 min after exposure to supramaximal caerulein completely prevented the increase in fluorescence and therefore blocked trypsin activity in living acinar cells (Fig. 1*B*). To determine the ideal concentration at which S124 inhibited trypsin activity in isolated acini, we used a range of S124 concentrations in parallel experiments. Concentrations of 10 μM or greater inhibited caerulein-induced trypsin activity completely and even below control levels (Fig. 1*C*). The latter indicates that even under control conditions, a basal amount of free trypsin activity, which amounts to between 10 and 20% of the trypsin activity generated by supramaximal concentrations of caerulein and to <0.1% of the total cellular trypsinogen content (9, 10), is constantly as well as physiologically generated in living pancreatic acinar cells and can be inhibited with S124. These results demonstrate further that S124 is readily cell-permeant and specifically inhibits trypsin in vitro and in vivo. Although enzyme kinetic data have shown a competitive type of inhibition for S124 (27), the reversibility of S124 action in intact cells had to be demonstrated.

Reversibility of trypsin inhibition by S124. To this end, we stimulated isolated pancreatic acini for 30 min with supramaximal concentrations of caerulein and subsequently studied intracellular trypsin activity

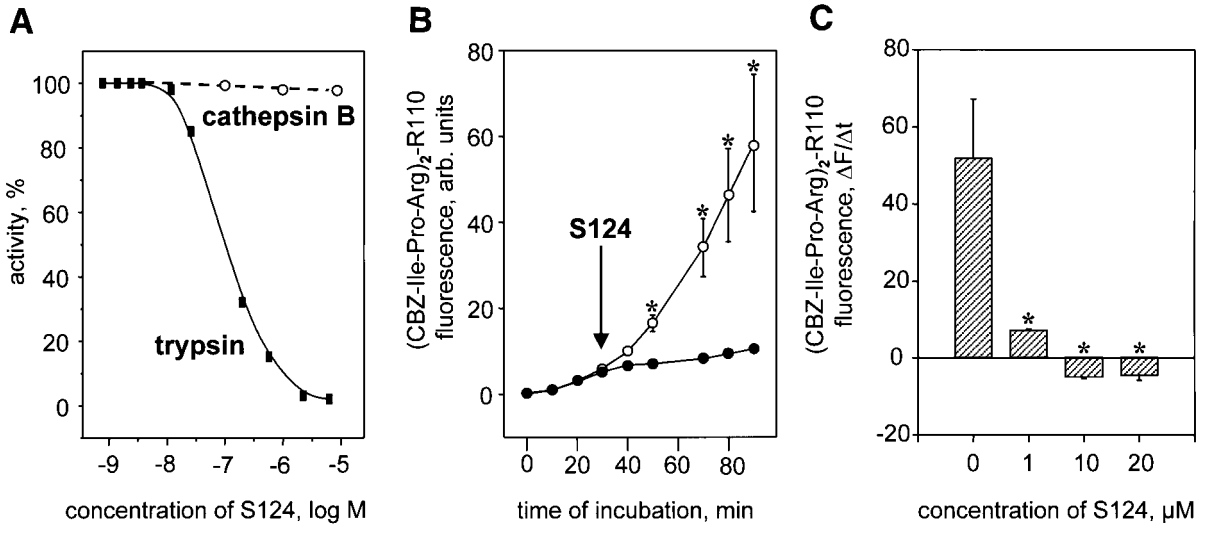

Fig. 1. Characterization of trypsin inhibition by *N*α-(2-naphthylsulfonyl)-3-amidinophenylalanine-carboxymethylpiperazide (S124). *A*: in vitro effects of different concentrations of S124 on the activity of purified trypsin (■) and of purified cathepsin B (○) are shown as inhibitor titration curves that are representative of at least 3 experiments with each enzyme. *B*: living pancreatic acini were incubated with supramaximal concentrations of caerulein (10 nM), and the generation of free intracellular trypsin activity was quantitated as cleavage of the cell-permeant trypsin substrate $(CBZ\text{-}Ile\text{-}Pro\text{-}Arg)_2$-rhodamine 110 by fluorometry. When, after 30 min, S124 (10 μM) was added to the buffer, this resulted in an almost immediate arrest of the increasing fluorescence curve (●), whereas the trypsin activity in caerulein-stimulated acini without S124 continued to rise (○). *C*: cumulative trypsin activity generated in isolated acini in response to caerulein (10 nM) over 20 min is shown together with the effect of different concentrations of S124 in the medium. The relative fluorescence (ΔF/Δt) of control acini (no caerulein or S124) was taken as baseline. Note that S124 can somewhat decrease trypsin activity below the levels found in controls. *Significant differences from respective control values.

over 60 min by fluorometry of the living cell using the cell-permeant substrate (CBZ-Ile-Pro-Arg)$_2$-rhodamine 110. When S124 (20 μM) was present during caerulein stimulation and during subsequent activity measurements, no substrate cleavage could be detected, and fluorescence levels corresponded to control acini (Fig. 2). Supramaximal caerulein alone led to a rapid increase of fluoresence followed by a subsequent decline, the latter indicating a loss of trypsin activity rather than substrate depletion as previously shown (9). When S124 was present during caerulein stimulation only and not during the subsequent incubation for cytofluorometry, an even greater fluorescence increase than with caerulein alone was found, and no subsequent decline in fluorescence occurred over 60 min (Fig. 2). This already indicates that S124 can rapidly dissociate from trypsin and that a single wash of acini before the transfer to cytofluorometry is sufficient to recover trypsin activity. When we studied these events by direct digital imaging of living acini, no activity was detected at the start of the experiment in acini after exposure to either caerulein alone (10 nM) or to caerulein + S124 (10 μM, Fig. 3, *A* and *D*, respectively).

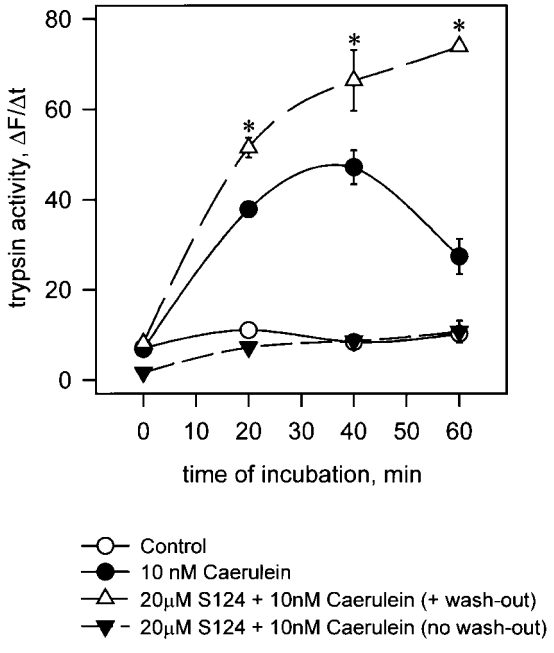

Fig. 2. Effect of reversible trypsin inhibition in pancreatic acini. Intact living acini were incubated for 30 min with supramaximal caerulein concentrations and the trypsin inhibitor S124. They were then transferred to 96-well plates for fluorometry of intracellular cleavage of the trypsin substrate (CBZ-Ile-Pro-Arg)$_2$-rhodamine 110 over 60 min. In control acini without prior caerulein incubation, only small amounts of trypsin activity were generated, and these remained constant over the entire experiment. The same result was obtained when acini were stimulated with supramaximal caerulein, but S124 was present during stimulation as well as during fluorometry. Caerulein alone induced a rapid increase of fluorescence followed by a continuous decline after 40 min. When caerulein stimulation for 30 min was done in the presence of S124 but subsequent fluorometry without S124, trypsin activity showed a more rapid and higher increase than after caerulein alone and did not decline over 60 min. *Significant differences between the caerulein and the caerulein + S124 washout group.

Thirty minutes after caerulein stimulation alone, a maximum of rhodamine 110 fluorescence had developed in the apical compartment of the acinar cells (Fig. 3*B*), whereas no fluorescence could be detected in acini that had been exposed to caerulein + S124 simultaneously and in whose buffer S124 was still present (Fig. 3*E*). When caerulein-stimulated acini were followed for up to 120 min, fluorescence progressively and completely disappeared (Fig. 3*C*). When the S124-containing buffer was continuously replaced by S124-free buffer (washout of S124), bright apical fluorescence rapidly developed, was still detectable after 120 min of incubation, and was more prominent than the fluorescence generated in response to caerulein alone (Fig. 3*F*). This indicates that trypsin inhibition by S124 is completely reversible and that the trypsin activity that is generated in response to supramaximal caerulein stimulation can be recovered by washout of S124 from living acinar cells. It also suggests that the actual trypsin activity that is detected as cleavage of a fluorescent trypsin substrate at any given point in time is the result of two independent processes, the activation of trypsinogen and the degradation of active trypsin, and that S124 can affect both of these events.

Role of trypsin in trypsinogen activation. To study the role of trypsin and cathepsin B in converting trypsinogen to active trypsin in pancreatic acinar cells, we used an isolated lobule assay because of the easier comparison between trypsin activity measurements and TAP generation data and because the preparation of lobules does not involve proteolytic digestion of pancreatic tissue. In analogy to the experiments with acini, pancreatic lobules were stimulated with supramaximal concentrations of caerulein for 60 min and S124 (10 μM) was either present or absent during the entire duration of the assay. TAP generation, as determined by immunoreactivity assay, was minimal in control lobules and in lobules incubated with S124 alone (Fig. 4*A*). Surprisingly, the generation of TAP that followed supramaximal caerulein stimulation was completely unaffected by trypsin inhibition with S124. This indicates that the conversion from trypsinogen to trypsin and thus the intracellular activation of trypsinogen is totally independent of the presence or absence of available free trypsin activity in acinar cells. It further proves that the mechanism responsible for caerulein-induced trypsinogen activation is independent of S124-inhibitable serine proteases and therefore rules out an autoactivation of trypsin. When lobules exposed to supramaximal caerulein concentrations for up to 60 min were homogenized at different time points during the experiment and the homogenates were diluted 10^3-fold to wash out S124, the substrate-specific trypsin activity could readily be recovered and quantitated. Lobules that had been supramaximally stimulated in the presence of S124 were found to contain 400% of the trypsin activity that had been generated after caerulein exposure alone (Fig. 4*B*). When the extent of TAP generation and free trypsin activity were compared and calculated in molar terms, it was found that TAP immunoreactivity corresponded to trypsin

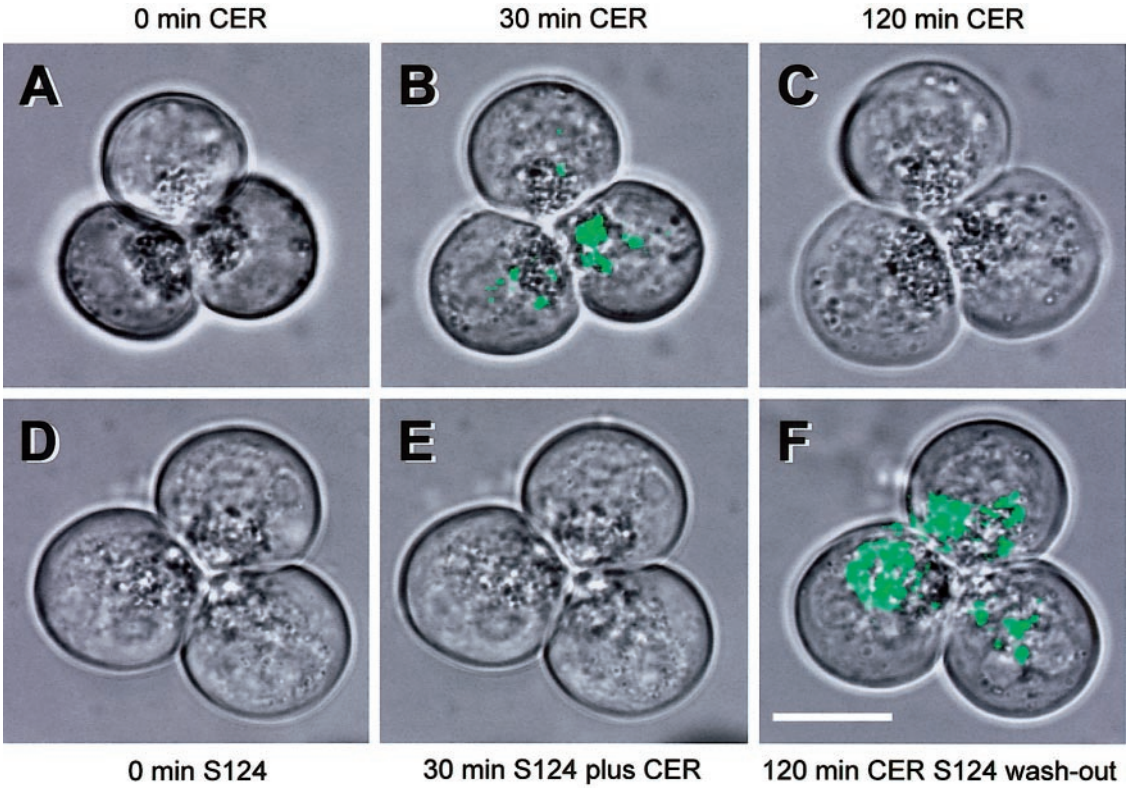

Fig. 3. Digital imaging of trypsin activity in pancreatic acini. Acini were stimulated with supramaximal caerulein (CER) concentrations as indicated in Fig. 2. Instead of fluorometry, they were continuously observed for up to 120 min under a high-resolution fluorescence microscope and digital phase contrast. Acini shown are representative of 4 or more samples per experiment. The acinus in *A* was exposed to supramaximal CER alone and photographed at the start of the experiment (*A*) and after 30 (*B*) and 120 min (*C*). Note the bright fluorescence in the apical portion of the acinus in *B* that had completely disappeared after 120 min (*C*). The acinus in *D* was stimulated with CER (10 nM) in the presence of S124. By 30 min (*E*), no fluorescence was detectable. When the incubation buffer was continuously replaced by medium without S124, a bright apical fluorescence rapidly developed and was still very prominent after 120 min (*F*). Bar indicates 20 μm.

activity in the lobules that were incubated in the presence of S124. The trypsin activity detected in lobules that were treated with caerulein alone amounted to only ~20% of TAP. This indicates not only that trypsin inhibition with S124 has no effect on trypsinogen activation but that it prevents the degradation of 80% of the trypsin activity that is generated in response to supramaximal caerulein stimulation. Trypsin activity within acinar cells is therefore not involved in trypsinogen activation or autoactivation but represents an extremely potent factor for the elimination of free intracellular trypsin activity (Figs. 2–4).

Because we have previously shown that intracellular trypsinogen activation is highly dependent on the presence of cathepsin B in the pancreas, we compared the effect of trypsin inhibition with that of cysteine protease inhibition on the generation of trypsin activity in pancreatic lobules. With the use of E-64d as a cell-permeant cysteine protease inhibitor, which has been shown not to interact with trypsin activity (24), together with S124 to inhibit trypsin activity and degradation, we found that the cysteine protease inhibitor significantly reduced the generation of free trypsin activity and confirmed that S124 induced a dramatic increase in intracellular trypsin activity (Fig. 5). In experiments in which we measured the release of TAP in response to supramaximal caerulein stimulation, we found that, again, inhibition of cysteine proteases significantly reduced TAP generation and trypsinogen activation in parallel, whereas the inhibition of intracellular trypsin activity with S124 had no effect on TAP release (Fig. 6). These results must be regarded as further evidence that cysteine proteases such as cathepsin B play a critical role in the activation of trypsinogen and that free trypsin activity is *1*) largely responsible for its own degradation and *2*) is not involved in trypsinogen activation.

DISCUSSION

In a series of elegant experiments that go back to the beginning of the last century, it was firmly established that trypsin, once activated by the brush-border enzyme enterokinase, can activate all other digestive proteases of the exocrine pancreas, including its own precursor zymogen trypsinogen (11, 19). Trypsin was therefore regarded as the "key activator" of the proteolytic cascade of pancreatic zymogens, and its premature and intracellular activation was thought to represent a critical factor for the development of acinar cell

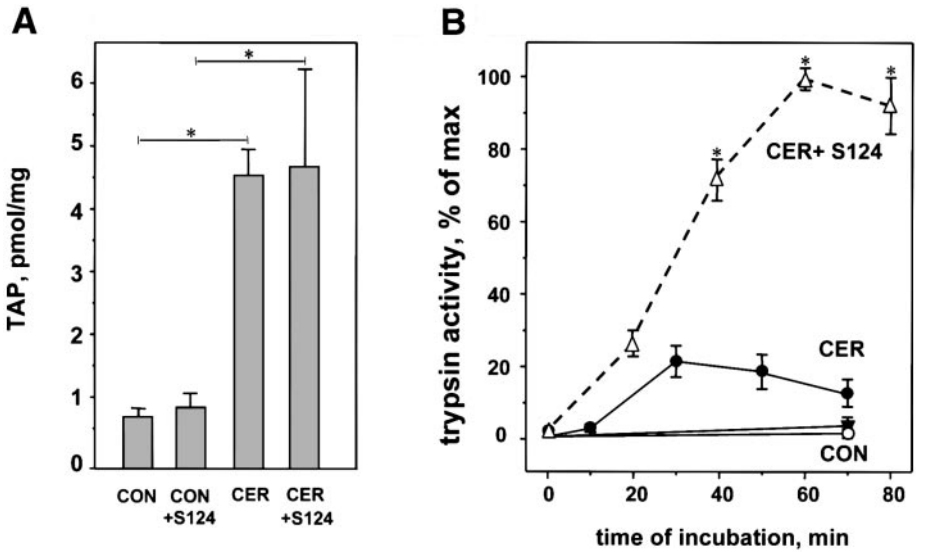

Fig. 4. Effect of S124 on trypsinogen activation peptide (TAP) generation and trypsin activity in pancreatic lobules. Pancreatic lobules were prepared, stimulated with supramaximal CER concentrations (100 nM), and homogenized as described in MATERIALS AND METHODS. After 60 min of incubation with CER and in either the presence or absence of S124 (10 μM), TAP immunoreactivity was measured in the lobule homogenates (*A*). Supramaximal CER induced a significant increase in TAP immunoreactivity regardless of whether or not trypsin activity was inhibited by S124. To quantitate trypsin activity directly at different time intervals of incubation with CER and S124, the homogenates were diluted 10^3-fold before activity measurements with the fluorogenic substrate (*B*). The intracellular trypsin activity recovered when trypsin that had been inhibited with S124 during CER stimulation (△) was 5 times higher than after CER incubation alone (●). Activities shown are expressed as %maximal trypsin activity, which corresponds to 5.38 ± 0.52 pmol trypsin/mg protein at 60 min. Controls (CON) refer to incubations in the absence of CER (▼) and with S124 alone (○). Data represent means ± SD of 4 or more experiments; *significant differences between the CER group or the CER + S124 group and their respective controls in *A* and between the CER group and the CER + S124 washout group in *B*. The small lag period of 10 min between the time courses of the latter groups was a technical necessity to allow for immediate homogenization on ice.

injury (3, 23) and the onset of pancreatitis. This conclusion, however, was entirely based on experiments using either pancreatic juice or pancreatic homogenates or purified pancreatic enzyme preparations (21). Our current concepts with which we try to explain the premature and intrapancreatic activation of digestive proteases, and hence pancreatic autodigestion during pancreatitis, are therefore derived entirely from in vitro observations and may not reflect the complex and compartimentalized conditions within the pancreatic acinar cell. Only recent developments in biotechnical methods have allowed the intracellular events involved in premature zymogen activation in living pancreatic acinar cells to be addressed directly (14).

Support for a crucial role of trypsin in the onset of pancreatitis came from genetic studies that have linked the phenotype of hereditary pancreatitis to point mutations in the cationic trypsinogen gene (31). In view of the role of trypsin in activating all other digestive proteases in the gut, it was proposed that hereditary pancreatitis could be caused by either a higher rate of trypsin autoactivation, an extended period of intracellular trypsin activity, or a greater resistance to degradation of the mutated trypsin (30). All of these possibilities would require that trypsin is, indeed, the trigger of an activation cascade in the pancreas. Studies that have addressed the functional role of disease-relevant trypsin mutations had to rely, as of today, on in vitro experiments in which the activation kinetics of recombinant mutant trypsin were compared with the wild-type enzyme (22, 28).

By using a technique that allows for studying trypsinogen activation in living pancreatic acini, and thus under physiological intracellular in vivo conditions, and by employing a novel, highly specific, and cell-permeant inhibitor for trypsin, we were able to completely block the intracellular trypsin activity that is generated within pancreatic acinar cells in response to supramaximal caerulein concentrations. In vivo administration of supramaximal concentrations of the cholecystokinin analog caerulein is known to result in an experimental variety of acute pancreatitis (12, 17), and in vitro exposure to supramaximal caerulein has been shown to be associated with premature and intracellular activation of digestive proteases (6, 9, 10, 13) and subsequent proteolytic damage (3, 23). When we inhibited trypsin activity in acinar cells, the rate at which trypsinogen was converted to trypsin (as indicated by the release of TAP) was found to be completely unaffected. Moreover, when we recovered the trypsin activity at the end of the experiment, which was possible because trypsin inhibition by S124 is a reversible process, it was up to 400% higher than the activity recovered after supramaximal caerulein stimulation alone. When, on the other hand, we measured trypsinogen activation in response to caerulein in the presence of the cysteine protease inhibitor E-64d, both trypsin

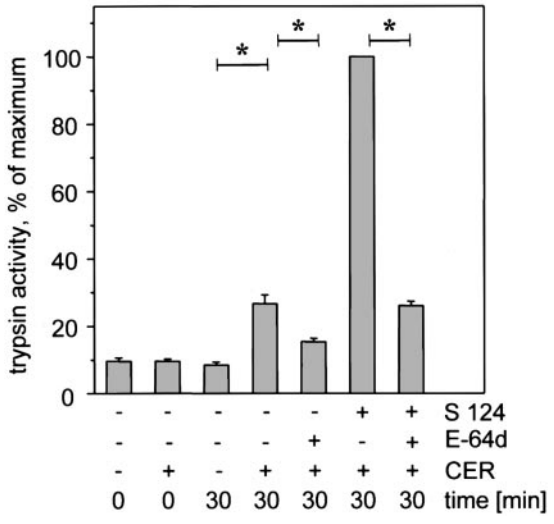

Fig. 5. Effect of combined cysteine and serine protease inhibition on trypsinogen activation. Pancreatic lobules were preincubated for 15 min with S124 (20 μM), with E-64d (2 mM), or with both inhibitors and subsequently stimulated with supramaximal secretagogue while the respective inhibitors remained in the medium for the remaining 30 min of the experiment. Trypsin activity was measured as before after lobule homogenization and 10^3-fold dilution of the homogenate. Note that only E-64d significantly inhibited trypsinogen activation regardless of whether S124 was present or not. The mean trypsin activities shown are representative for 6 experiments; *significant differences.

activity and TAP release were found to be significantly reduced.

These observations have a number of important implications. *1*) Whether or not trypsin activity is inhibited within the pancreatic acinar cell is completely immaterial for the rate at which the conversion from the zymogen trypsinogen to the active protease trypsin occurs. This effectively rules out that a trypsin-induced trypsinogen activation or an autoactivation of trypsinogen plays any significant role as the triggering event for premature protease activation within the pancreas. Although the zymogen trypsinogen itself has some autocatalytic property that is independent of a prior cleavage of the activation peptide (8), this autocatalytic potential is much too small to account for the trypsin activity and TAP release that are generated in response to supramaximal caerulein. *2*) The premature intracellular trypsin activity as well as the generation of TAP are both highly dependent on the activity of lysosomal cysteine proteases such as cathepsin B. This observation is in line with earlier reports using either lysosomal enzyme inhibitors (24) or knockout strains of mice in which the cathepsin B gene was deleted (4). Although our present experiments cannot exclude that nonlysosomal cysteine proteases (such as calpain) or lysosomal hydrolases other than cathepsin B (e.g., cathepsin L, K, and H) account for some of the E-64d effect reported here and elsewhere, our studies using cathepsin B-deleted animals have firmly established the role of this particular cysteine protease in triggering trypsinogen activation (4). *3*) The predominant role of prematurely activated trypsin appears to be the elimination of prematurely activated trypsin. Whereas trypsin activity appears to play no role in triggering intracellular trypsinogen activation, the trypsin-induced inactivation of trypsin, most probably by means of autodegradation, appears to be highly effective. Because TAP and recovered free trypsin activity were found to be equimolar only in the presence of S124, >80% of the trypsin activity that was generated within 1 h of supramaximal caerulein stimulation must have been inactivated by an S124-sensitive protease. Although we cannot completely rule out the possibility that another, as yet unidentified, serine protease of the exocrine pancreas is responsible for this rapid degradation of activated trypsin, the high specificity and affinity of S124 for trypsin makes this assumption unlikely.

As far as human pancreatitis is concerned, the most critical conclusion of our study, that trypsin is not involved in trypsinogen activation but accounts for 80% of trypsin degradation, must be interpreted with caution. Whereas this interpretation appears unequivocal as far as secretagogue-induced trypsinogen activation in the pancreas of rodents is concerned, it remains unclear whether frequent human causes of pancreatitis such as alcohol or gallstones are associated with an equivalent intracellular proteolytic cascade. The human pancreas, although also susceptible to secretagogue-induced pancreatitis, may contain a different composition of cysteine as well as serine proteases, and human cationic trypsin is known to have a higher capacity for autoactivation at a pH <6 than trypsin from, e.g., the bovine pancreas (2). What remains unequivocal is the fact that trypsinogen activation, as

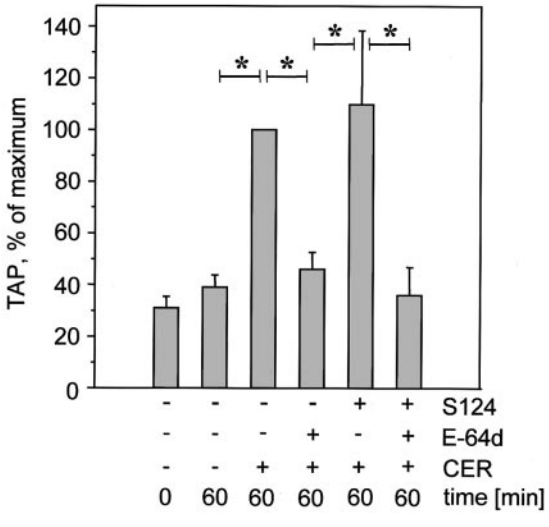

Fig. 6. Effect of combined cysteine and serine protease inhibition on TAP generation. Pancreatic lobules were preincubated for 15 min with S124 (20 μM), with E-64d (2 mM), or with both inhibitors and subsequently stimulated with supramaximal concentrations of secretagogue, whereas the respective inhibitors remained in the medium for the remaining experiment. The generation of TAP was measured in lobule homogenates as described under MATERIALS AND METHODS. Note that E-64d significantly inhibited TAP generation, whereas trypsin inhibition with S124 did not. The means are representative for 6 experiments; *significant differences.

determined by the release of TAP into the serum and urine of affected patients, is a frequent characteristic of clinical pancreatitis and occurs independent of the etiology of pancreatitis, and its extent reflects disease severity (18). Whether it represents a triggering event or a protective defense mechanism in pancreatitis remains to be shown.

For the pathophysiology of hereditary pancreatitis, the results from our study suggest the possibility of a previously unrecognized mechanism: that disease-causing trypsinogen mutations change the enzyme's conformation in a manner that, although completely immaterial to the events that lead to activation, could interfere with the autodegradation of free intracellular trypsin. If that were the case, any physiological or pathological stimulus associated with premature intracellular protease activation could result, analogous to our experiments, in a 5- to 10-fold increase in uninhibited free trypsin activity within acinar cells.

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REFERENCES

1. **Chiari H.** über die Selbstverdauung des menschlichen pankreas. *Zeitschrift für Heilkunde* 17: 69–96, 1896.
2. **Figarella C, Amouric M, and Guy-Crotte O.** Proteolysis of human trypsinogens. I. Pathogenetic implications in chronic pancreatitis. *Biochem Biophys Res Commun* 118: 154–161, 1984.
3. **Grady T, Otani T, Mah'moud M, Rhee S, Lerch MM, and Gorelick FS.** Zymogen proteolysis within the pancreatic acinar cell is associated with cellular injury. *Am J Physiol Gastrointest Liver Physiol* 275: G1010–G1017, 1998.
4. **Halangk W, Lerch MM, Brandt-Nedelev B, Roth W, Ruthenbuerger M, Reinheckel T, Domschke W, Lippert H, Peters C, and Deussing J.** Role of cathepsin B in intracellular trypsinogen activation and the onset of acute pancreatitis. *J Clin Invest* 106: 773–781, 2000.
5. **Halangk W, Stürzebecher J, Matthias R, Schulz HU, and Lippert H.** Trypsinogen activation in rat pancreatic acinar cells hyperstimulated by caerulein. *Biochim Biophys Acta* 1362: 243–251, 1997.
6. **Hofbauer B, Saluja AK, Lerch MM, Bhagat L, Bhatia M, Lee HS, Frossard JL, Adler G, and Steer ML.** Intra-acinar cell activation of trypsinogen during caerulein-induced pancreatitis in rats. *Am J Physiol Gastrointest Liver Physiol* 275: G352–G362, 1998.
7. **Hurley PR, Cook A, Jehanli A, Austen BM, and Hermon-Taylor J.** Development of radioimmunoassays for free tetra-aspartyl-L-lysine trypsinogen activation peptides (TAP). *J Immunol Methods* 111: 195–203, 1988.
8. **Kassel B and Kay J.** Zymogens of proteolytic enzymes. *Science* 180: 1022–1027, 1973.
9. **Krüger B, Albrecht E, and Lerch MM.** The role of intracellular calcium signaling in premature protease activation and the onset of pancreatitis. *Am J Pathol* 157: 43–50 2000.
10. **Krüger B, Lerch MM, and Tessenow W.** Direct detection of premature protease activation in living pancreatic acinar cells. *Lab Invest* 78: 763–764, 1998.
11. **Kunitz M and Northrop JH.** Crystalline chymotrypsin and chymotrypsinogen. I. Isolation, crystallization, and general properties of a new proteolytic enzyme and its precursor. *J Gen Physiol* 18: 433–453, 1935.
12. **Lampel M and Kern HF.** Acute interstitial pancreatitis in the rat induced by excessive doses of a pancreatic secretagogue. *Virchows Arch* 373: 97–117, 1977.
13. **Leach SD, Modlin IM, Scheele GA, and Gorelick FS.** Intracellular activation of digestive zymogens in rat pancreatic acini. Stimulation by high doses of cholecystokinin. *J Clin Invest* 87: 362–366, 1991.
14. **Lerch MM and Gorelick FS.** Trypsinogen activation in acute pancreatitis. *Med Clin North Am* 84: 549–563, 2000.
15. **Lerch MM, Hoppe-Seyler P, and Matern S.** In vitro perfusion and incubation of rat pancreatic lobules in a kinetic system. *Biomed Biochim Acta* 50: 1115–1125, 1991.
16. **Lerch MM, Saluja AK, Dawra R, Saluja M, and Steer ML.** The effect of chloroquine administration on two experimental models of acute pancreatitis. *Gastroenterology* 104: 1768–1779, 1993.
17. **Lerch MM, Weidenbach H, Gress TM, and Adler G.** Effect of kinin inhibition in experimental acute pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 269: G490–G499, 1995.
18. **Neoptolemos JP, Kemppainen EA, Mayer JM, Fitzpatrick JM, Raraty MG, Slavin J, Beger HG, Hietaranta AJ, and Puolakkainen PA.** Early prediction of severity in acute pancreatitis by urinary trypsinogen activation peptide: a multicentre study. *Lancet* 355: 1955–1960, 2000.
19. **Northrop JH and Kunitz M.** Isolation of protein crystals possessing tryptic activity. *Science* 73: 262–263, 1931.
20. **Rinderknecht H.** Activation of pancreatic zymogens, normal activation, premature intrapancreatic activation, protective mechanisms against inappropriate activation. *Dig Dis Sci* 31: 324–321, 1986.
21. **Rinderknecht H.** Pancreatic secretory enzymes. In: *The Pancreas: Biology, Pathobiology, and Disease*, edited by Go VLW, DiMagno EP, Gardner JD, Lebenthal E, Reber HA, and Scheele GA. New York: Raven, p. 219–251, 1993.
22. **Sahin-Toth M and Toth M.** Gain-of-function mutations associated with hereditary pancreatitis enhance autoactivation of human cationic trypsinogen. *Biochem Biophys Res Commun* 278: 286–289, 2000.
23. **Saluja AK, Bhagat L, Lee HS, Bhatia M, Frossard JL, and Steer ML.** Secretagogue-induced digestive enzyme activation and cell injury in rat pancreatic acini. *Am J Physiol Gastrointest Liver Physiol* 276: G835–G842, 1999.
24. **Saluja AK, Donovan EA, Yamanaka K, Yamaguchi Y, Hofbauer B, and Steer ML.** Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. *Gastroenterology* 113: 304–310, 1997.
25. **Saluja A, Sadamitsu H, Saluja M, Powers M, Meldolesi J, and Steer M.** Subcellular redistribution of lysosomal enzymes during caerulein-induced pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 253: G508–G516, 1987.
26. **Steer ML and Meldolesi J.** The cell biology of experimental pancreatitis. *N Engl J Med* 316: 144–150, 1987.
27. **Stürzebecher J, Prasa D, Hauptmann J, Vieweg H, and Wikstroem P.** Synthesis and structure-activity relationships of potent thrombin inhibitors: piperazides of 3-amidinophenylalanine. *J Med Chem* 40: 3091–3099, 1997.
28. **Teich N, Ockenga J, Hoffmeister A, Manns M, Moessner J, and Keim V.** Chronic pancreatitis associated with an activation peptide mutation that facilitates trypsin activation. *Gastroenterology* 119: 461–465, 2000.
29. **Watanabe O, Baccino FM, Steer ML, and Meldolesi J.** Supramaximal caerulein stimulation and ultrastructure of rat pancreatic acinar cell: early morphological changes during development of experimental pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 246: G457–G467, 1984.
30. **Whitcomb DC.** New insights into hereditary pancreatitis. *Curr Gastroenterol Rep* 1: 154–160, 1999.
31. **Whitcomb DC, Gorry MC, Preston RA, Furey W, Sossenheimer MJ, Ulrich CD, Martin SP, Gates LK, Amann ST, Toskes PP, Liddle R, McGrath K, Uomo G, Post JC, and Ehrlich GD.** Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. *Nat Genet* 14: 141–145, 1996.
32. **Williams JA, Korc M, and Dormer RL.** Action of secretagogues on a new preparation of functionally intact, isolated pancreatic acini. *Am J Physiol Endocrinol Metab Gastrointest Physiol* 235: E517–E524, 1978.