Chapter

21

Molecular Basis of Diseases of the Exocrine Pancreas

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ACUTE PANCREATITIS

Acute pancreatitis presents clinically as a sudden inflammatory disorder of the pancreas, and is caused by premature intracellular activation of pancreatic proteases leading to (i) self-destruction of acinar cells and (ii) autodigestion of the organ. Necrotic cell debris resulting from this process produces a systemic inflammatory reaction, which may lead to multiorgan failure in due course. The incidence of acute pancreatitis differs regionally from 20 to 120 cases per 100,000 population. Acute pancreatitis varies considerably in severity and can be categorized into two forms of the disease. The majority of cases (85%) present with a mild form of disease, classified as edematous pancreatitis, with absent or only transient extrapancreatic organ failure. In the remaining minority of cases (15%), pancreatitis follows a severe course accompanied by sustained multiorgan failure. This latter form of pancreatitis is commonly referred to as severe or necrotizing pancreatitis. Severe necrotizing pancreatitis is associated with high mortality (10%–20%) and may lead to long-term complications such as the formation of pancreatic pseudocysts or impairment of exocrine and endocrine function of the pancreatic gland.

In many patients (approximately 50%) the underlying cause of acute pancreatitis is the migration of a gallstone resulting in obstruction of the pancreatic duct at the papilla of Vater. Development of acute pancreatitis is frequently triggered by alcohol abuse. In 25%–40% of acute pancreatitis patients, increased or excess alcohol consumption is regarded as the cause of the disease. Removal of the underlying disease-causing agent results in complete regeneration of the pancreas and preserved exocrine and endocrine function in the majority of cases. Recurrent attacks of the disease can result from chronic alcohol abuse, repeated gallstone passage, genetic predispositions, sphincter dysfunction, metabolic disorders, or pancreatic duct strictures. All of these factors can contribute to the development of chronic pancreatitis as well. In the remaining cases (10%–20%), no apparent clinical cause or etiology of the disease can be identified. These cases are referred to as idiopathic pancreatitis. It became clear during the last decade that previously unknown genetic factors play a major role in these cases. The initial cellular mechanism causing acute pancreatitis is probably independent of the underlying etiology of the disease [1].

Early Events in Acute Pancreatitis and the Role of Protease Activation

Acute pancreatitis is an inflammatory disorder whose pathogenesis is not well understood. The pancreas is known as the enzyme factory of the human organism, producing and secreting large amounts of potentially hazardous digestive enzymes, many of which are synthesized as pro-enzymes known as zymogens. Under physiological conditions the pancreatic digestive enzymes are secreted in response to hormonal stimulation [2]. Activation of the pro-enzymes (or zymogens) requires hydrolytic cleavage of their activation peptide by protease enzymes. After entering the small intestine, the pancreatic zymogen trypsinogen is first activated to trypsin by the intestinal protease enterokinase (or enteropeptidase). Trypsin then proteolytically processes other pancreatic enzymes to their active forms. Under physiological conditions pancreatic proteases remain inactive during synthesis, intracellular transport, secretion from acinar cells, and transit through the pancreatic duct. They are activated only upon reaching the lumen and brush-border of the small intestine (Figure 21.1).

More than a century ago, Hans Chiari proposed that the underlying pathophysiological mechanism for the development of pancreatitis was autodigestion of the exocrine pancreatic tissue by proteolytic enzymes [3]. Today, this theory is well accepted. Nevertheless, this theory suggests that disease results

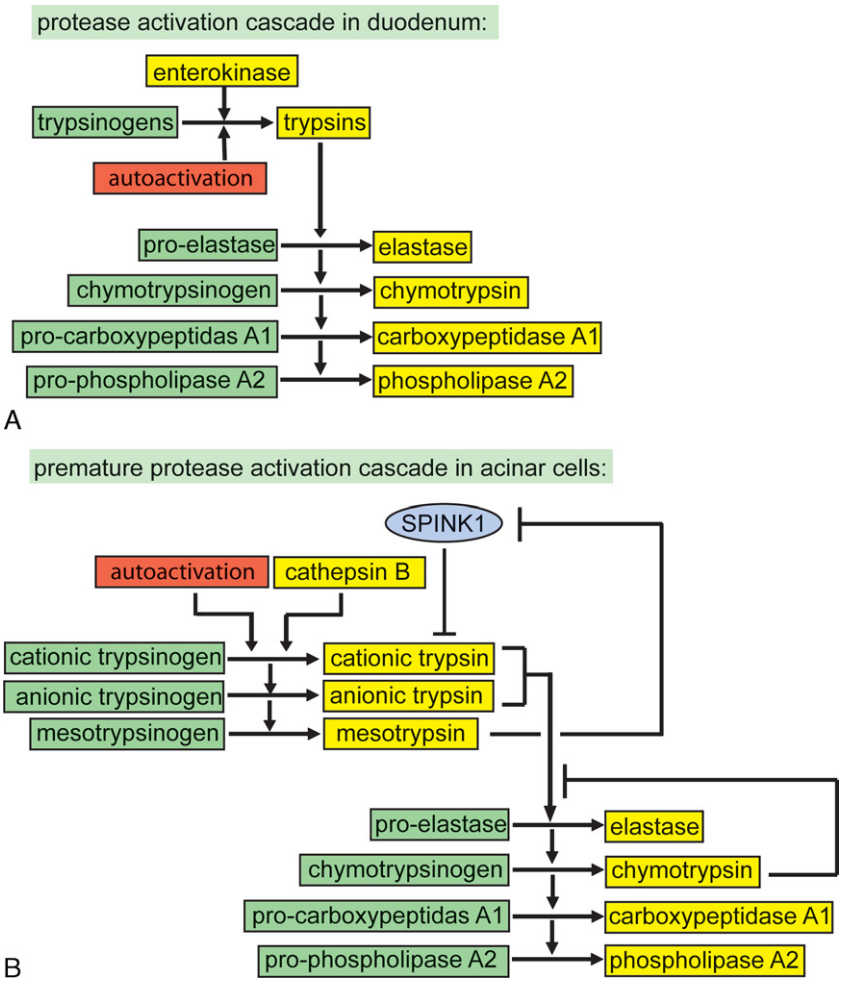

Figure 21.1 Activation of pancreatic proteases under normal conditions and in disease. (A) The protease activation cascade in the duodenum. Enterokinase activates trypsinogen by proteolytic cleavage of the trypsin activation peptide (TAP), and autoactivation contributes to this process. Trypsin then activates other digestive pro-enzymes in a cascade-like fashion. **(B)** Within acinar cells, premature activation of trypsinogen by cathepsin B is involved in the setting of the proteolytic cascade. Intracellular trypsin may also activate other digestive pro-enzymes, in spite of presence of the trypsin inhibitor SPINK-1 and the trypsin-degrading enzyme chymotrypsin C.

from the premature intracellular activation of zymogens and that this occurs in the absence of enterokinase. Furthermore, this theory suggests that protease inactivation takes place despite several physiological defense mechanisms, including the synthesis of endogenous protease inhibitors and the storage of proteases in a membrane-confined compartment of zymogen granules.

Much of our current knowledge regarding the onset of pancreatitis was not gained from studies involving the human pancreas or patients with pancreatitis, but from animal or isolated cell models [4]. There are several reasons why these models have been used: (i) the pancreas is a rather inaccessible organ because of its anatomical location in the retroperitoneal space (unlike in the colon or stomach, biopsies of human pancreas are difficult to obtain for ethical and medical reasons), and (ii) patients who are admitted to the hospital with acute pancreatitis have usually progressed beyond the initial stages of the disease where the triggering early events could have been studied. Particularly, the autodigestive process that characterizes this disease has remained a significant impediment for investigations that address initiating pathophysiological events. Therefore, the issue of premature protease activation has mostly been studied in animal models of the disease and before randomized placebo-controlled trials to evaluate antiproteolytic therapeutic concepts in humans could be performed. Experimental models are now irreplaceable tools in studying etiological factors, pathophysiology, new diagnostic tools, and treatment options for acute pancreatitis [5].

Data from various animal models suggest that after the initial insult a variety of pathophysiological factors determine disease onset. These include (i) a block in secretion, (ii) the co-localization of zymogens with lysosomal enzymes, (iii) the activation of trypsinogen and other zymogens, and (iv) acinar cell injury. *In vitro* and *in vivo* studies have demonstrated the importance of premature zymogen activation in the pathogenesis

of pancreatitis since the inception of the hypothesis by Chiari [6]. The activation of trypsinogen and other pancreatic zymogens can be demonstrated in pancreatic homogenates from experimental animals and this zymogen activation appears to be an early event. Trypsin activity is detected as early as 10 minutes after supramaximal stimulation with the cholecystokinin-analogue cerulein in rats and increases over time. The activation of trypsinogen requires the hydrolytic cleavage of a 7–10 amino acids propeptide called trypsin activation peptide (TAP) on the N-terminus of trypsinogen. Measuring an increase of immunoactive TAP after cerulein-induced pancreatitis in rats showed trypsinogen activation in the secretory compartment of acinar cells [7]. Furthermore, TAP was detected in serum and urine of patients with pancreatitis [8], and the amount of TAP appears to correlate with the severity of the disease [9]. Not only the activity of trypsin increases in the early phase in acute experimental pancreatitis, but also elastase activity [10]. In addition to the activation peptide of trypsinogen (TAP), the activation peptide of carboxypeptidase A1 (PCA1) can also be identified in serum at an early stage of pancreatitis [11]. Premature activation of these pro-enzymes leads to the development of necrosis and to autodigestion of pancreatic tissues [12–20]. Recent studies defining the localization of early activation of pro-enzymes suggest that these processes, which lead to pancreatitis and pancreatic tissue necrosis, originate in acinar cells (Figure 21.1) [6,21,22].

Additional evidence for the association between premature protease activation and severity of pancreatitis resulted from experiments with serine-protease inhibitors, where cell injury could be significantly reduced compared to controls [23,24]. On the other hand, protease inhibitors have a protective effect for the prevention of acute pancreatitis only [25]. Therapeutic application of protease inhibitors after the initial disease phase does not result in a significant beneficial effect on survival or severity of the disease [26].

In conclusion, premature intracellular activation of zymogens to active proteases in the secretory compartment of acinar cells results in acinar necrosis and contributes to the onset of pancreatitis. As a direct result of cellular injury, the acinar cells release chemokines and cytokines which initiate the later events in pancreatitis, including recruitment of inflammatory cells into the tissue. Trypsin seems to be the key enzyme in the process of activating other digestive pro-enzymes prematurely, and one of the crucial questions in understanding the pathophysiology of acute pancreatitis is to identify the mechanism which prematurely activates trypsinogen inside acinar cells. However, it must be noted that the term trypsin, as defined by the cleavage of specific synthetic or protein substrates, comprises a group of enzymes whose individual role in the initial activation cascade may differ considerably.

The Mechanism of Zymogen Activation

One hypothesis for the initiation of the premature activation of trypsinogen suggests that during the early stage of acute pancreatitis, pancreatic digestive zymogens become co-localized with lysosomal hydrolases. Recent data show that the lysosomal cysteine proteinase cathepsin B may play an important role for the activation of trypsinogen. Many years ago *in vitro* data demonstrated activation of trypsinogen by cathepsin B [27–29]. Most lysosomal hydrolases are synthesized as inactive pro-enzymes, but in contrast to digestive zymogens, they are activated by post-translational processing in the cell. During protein sorting in the Golgi system, lysosomal hydrolases are sorted into prelysosomes, whereas zymogens are packaged into condensing vacuoles. The sorting of lysosomal hydrolases depends on a mannose-6-phosphate-dependent pathway [30], which leads to a separation of lysosomal hydrolases from other secretory proteins and to the formation of prelysosomal vacuoles. However, this sorting is incomplete. Under physiological conditions, a significant fraction of hydrolases enter the secretory pathway [31–34]. It has been suggested that these mis-sorted hydrolases play a role in the regulation of zymogen secretion [35]. In acute pancreatitis the separation of digestive zymogens and lysosomal hydrolases is impaired. This leads to further co-localization of lysosomal hydrolases and zymogens within cytoplasmic vacuoles of acinar cells [36]. This co-localization has also been shown in electron microscopy [37], as well as in subcellular fractions isolated by density gradient centrifugation [38]. The redistribution of cathepsin B from the lysosome-enriched fraction was noted within 15 minutes of the start of pancreatitis induction, and trypsinogen activation was observed in parallel [39–41]. There are two main theories trying to explain the co-localization of cysteine and serine proteases: (i) fusion of lysosomes and zymogen granules [42] or (ii) incorrect sorting of zymogens and hydrolases in the process of vacuole maturation [37]. Wortmannin, a phosphoinositide-3-kinase inhibitor, prevents the intracellular mis-sorting of hydrolases and zymogens, and subsequently prevents trypsinogen activation to trypsin during acute pancreatitis [43].

Further experiments focused on cathepsin B as the main enzyme driving the intracellular activation of trypsinogen. Cathepsin B is the most abundant lysosomal hydrolase in acinar cells. Pretreatment of rat pancreatic acini with E64d, a cell-permeable cathepsin B inhibitor, leads to complete inhibition of cathepsin B and completely abrogates trypsinogen activation [44,45]. Final evidence that cathepsin B is involved in activation of trypsinogen during cerulein-induced experimental pancreatitis comes from experiments in cathepsin B knockout mice. In these animals, after induction of experimental pancreatitis, trypsin activity was reduced to less than 20% compared to wild-type animals and the severity of the disease was markedly ameliorated [46]. These data showed unequivocally the importance of cathepsin B for the pathogenesis of acute pancreatitis (Figure 21.1) [47].

The cathepsin B theory implies one further critical point—that trypsinogen is expressed and stored in the presence of different potent intrapancreatic trypsin inhibitors. To activate trypsinogen, cathepsin B

needs to override these defensive mechanisms to initiate the premature intracellular activation cascade [18]. Recently, it has become clear that cathepsin B cannot only activate cationic and anionic trypsinogen, but also mesotrypsinogen [48]. Mesotrypsin, the third trypsin isoform expressed in the human pancreas, is resistant against trypsin inhibitors like SPINK-1 or soybean trypsin inhibitor (SBTI) [49]. Moreover, mesotrypsin is able to degrade trypsin inhibitors. Under physiological conditions, mesotrypsin is activated in the duodenum by enterokinase, where it degrades exogenous trypsin inhibitors to ensure normal tryptic digestion. Mesotrypsin rapidly inactivates trypsin inhibitors like SPINK-1 by proteolytic cleavage *in vitro* [48,50]. Therefore, activation of trypsins by cathepsins might not only trigger a proteolytical cascade, but also involve the removal of trypsin inhibitors such as SPINK-1 via the activation of mesotrypsin.

The role of cathepsin B in chronic pancreatitis was recently addressed by an Indian group from Hyderabad. In 140 patients suffering from tropical pancreatitis, they found a significant difference between patients and controls for the C76G polymorphism in the *CTSB* gene [51]. Unfortunately, these data could not be confirmed in a Caucasian cohort [52]. Thus, the role of CTSB in human pancreatitis remains inconclusive. Taken together, these experimental observations represent compelling evidence that cathepsin B can contribute to premature, intracellular zymogen activation and the initiation of acute pancreatitis not only through co-localization with trypsinogen, but also through activation of mesotrypsin, rendering endogenous pancreatic protease inhibitors inactive.

The Degradation of Active Trypsin

During the early phase of pancreatitis, trypsinogen and other zymogens are rapidly activated, while later in the disease course their activity declines to physiological levels, suggesting degradation of the active enzymes. This phenomenon has been termed autolysis or autodegradation. Since this process self-limits autoactivation of trypsinogen, it is regarded as a safety mechanism to counteract premature zymogen activation.

One theory to possibly explain how uncontrolled trypsinogen activation can be antagonized is based on the existence of a serine protease that is capable of trypsin degradation. In 1988 Heinrich Rinderknecht discovered an enzyme which rapidly degrades active cationic and anionic trypsin and named this protease enzyme Y [53]. Recent *in vitro* data suggest that the autodegradation of trypsin is a very slow process and that most of trypsin degradation is not mediated by trypsin itself but by another enzyme. Chymotrypsin C has the capability to proteolytically cleave cationic trypsin at Leu81–Glu82 in the Ca^{2+} binding loop. This leads to rapid autodegradation and catalytic inactivation of trypsin by additional cleavage at the Arg122–Val123 [54]. Thus, chymotrypsin C has the capability to induce trypsin-mediated trypsin autodegradation during pancreatitis and is most likely identical with the enzyme Y that Rinderknecht proposed in 1988. However, chymotrypsin C has also the ability to induce trypsin-mediated trypsinogen autoactivation by proteolytic cleavage at the Phe18–Asp19 position of cationic trypsinogen [55]. The balance between autoactivation and autodegradation of cationic trypsin mediated by chymotrypsin C is regulated via the Ca^{2+} concentration. In the presence of 1 mM Ca^{2+}, degradation of trypsin is blocked and autoactivation of trypsinogen is induced. Under physiological conditions in the duodenum, high Ca^{2+} concentrations facilitate the activation of trypsinogen to promote digestion. In the absence of high Ca^{2+} concentrations, chymotrypsin C degrades active trypsin and protects against premature activation of trypsin (Figure 21.2).

Calcium Signaling

The second messenger calcium plays an important role in multiple different intracellular processes such as metabolism, cellular secretion, cell differentiation, and cell growth. Under physiological conditions, pancreatic acinar cells maintain a Ca^{2+} gradient across the plasma membrane with low intracellular concentration and high extracellular concentration of calcium. In response to hormonal stimulation, Ca^{2+} is released from intracellular stores to regulate signal-secretion coupling. In pancreatic acinar cells, acetylcholine (ACh) and cholecystokinin (CCK) regulate the secretion of digestive enzymes via the generation of repetitive local cytosolic Ca^{2+} signals [56]. In response to secretagogue stimulation with ACh or CCK, Ca^{2+} is initially released from intracellular stores near the apical pole of acinar cells [57]. This induces the fusion of zymogen granules with the apical plasma membrane [58], and activation of Ca^{2+} dependent Cl^- channels in the apical membrane [59]. The pattern of intracellular calcium signal in response to secretagogues stimulation is dependent on the neurotransmitter or hormone concentration. ACh at physiological concentrations elicits repetitive Ca^{2+} spikes and oscillation of Ca^{2+} concentrations, but these oscillations are restricted to the secretory pole of the cell. High concentrations of cholecystokinin lead to short-lasting spikes followed by longer Ca^{2+} transients that spread to the entire cell. Each oscillation is associated with a burst of exocytotic activity and the release of zymogen into the duct lumen [60]. In contrast, supramaximal stimulation of acinar cells induces a completely different pattern of Ca^{2+} signals. Instead of oscillatory activity observed with physiological doses of cholecystokinin, there is a much larger rise followed by a sustained elevation associated to a block of enzyme secretion and premature intracellular protease activation [56,61,62]. Ca^{2+} is released from the endoplasmatic reticulum (ER) in response to stimulation. The ER is located in the basolateral part of the acinar cell with extensions in the apical part enriched with zymogen granules. While the entire ER contains Ca^{2+}, release of Ca^{2+} in response to cholecystokinin or ACh occurs only at the apical pole due to the higher density of Ca^{2+} release channels at the apical pole of

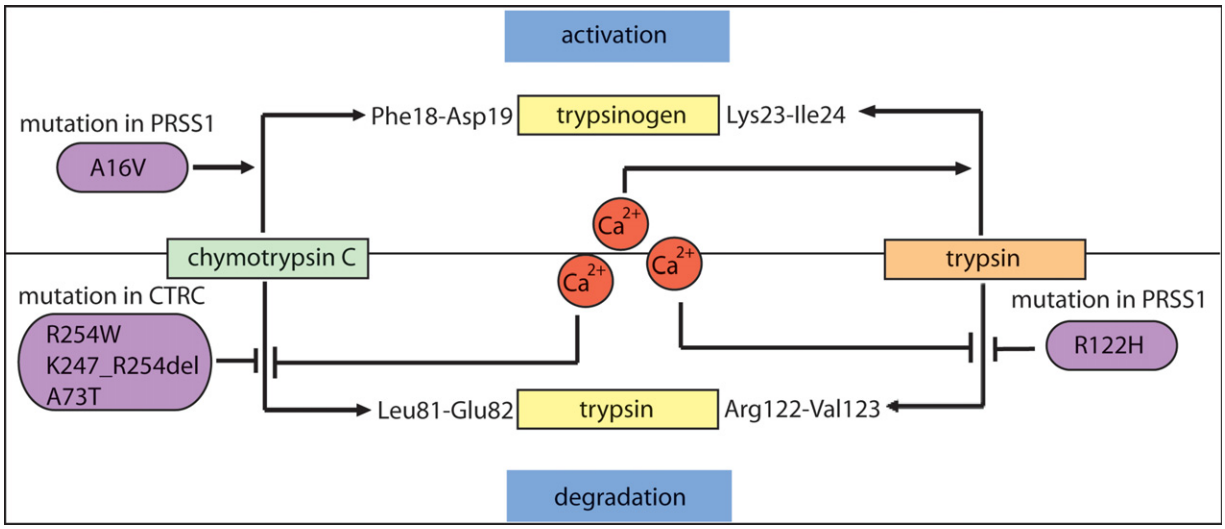

Figure 21.2 Chymotrypsin C has different functions in the processing of trypsin or trypsinogen. The major role in pancreatitis is degrading active trypsin. This function is disturbed by mutations within the *CTRC* gene or by high levels of Ca^{2+}. The activation of trypsinogen to trypsin can also be mediated by chymotrypsin C. Trypsin itself has the capability to autoactivate or to self-degrade. The R122H mutation results in the decreased autolysis of active trypsin.

the ER [63]. Two types of Ca^{2+} channels are expressed in the ER of acinar cells, inositol-triphosphate-receptors (IP_3) and ryanodine receptors. Both of these channels are required for apical Ca^{2+} peaks. ACh activates phospholipase C (PLC) and initiates the Ca^{2+} release via the intracellular messenger IP_3 [64,65], whereas cholecystokinin does not activate PLC but increases the intracellular concentration of nicotinic acid adenine dinucleotide phosphate (NAADP) in a dose-dependent manner [66]. The higher density of Ca^{2+} channels in the apical part of the ER explains the initiation of Ca^{2+} signals in the granule part of the cytoplasm. The apical, zymogen granule-enriched part of the acinar cell is surrounded by a barrier of mitochondria which absorb released calcium and prevent higher Ca^{2+} concentrations from expanding beyond the apical part of acinar cells [67]. The spatially limited release of Ca^{2+} at the apical pole also prevents an unregulated chain reaction across gap junctions, which would affect neighboring cells. The mitochondrial Ca^{2+} uptake leads to increased metabolism and generation of ATP [68,69]. ATP is required for the reuptake of Ca^{2+} in the ER via the sarcoplasmatic endoplasmatic reticulum calcium ATPase (SERCA), and for exocytosis across the apical membrane [70]. Thus, Ca^{2+} homeostasis plays a crucial role for maintaining signal-secretion coupling in pancreatic acinar cells (Figure 21.3).

Elevated Ca^{2+} concentrations in the extracellular compartment or within acinar cells is known to be a risk factor for the development of acute pancreatitis [71–74]. Disturbances in the Ca^{2+} homeostasis of pancreatic acinar cells occur early in the secretagogue-induced model of pancreatitis. An attenuation of Ca^{2+} elevation in acinar cells results from exposure to the cytosolic Ca^{2+} chelator BAPTA-AM, which also prevents zymogen activation, proving that Ca^{2+} is essential for zymogen activation. The sustained elevation that follows the initial intracellular Ca^{2+} spike induced by supramaximal concentrations of cerulein is also attenuated in the complete absence of intracellular calcium and appears to depend on extracellular Ca^{2+}. In the absence of extracellular Ca^{2+}, the activation of trypsinogen induced by supramaximal doses of cerulein is also attenuated, suggesting that the initial and transient rise in Ca^{2+} caused by the release of calcium from the internal stores is not sufficient to permit trypsinogen activation. In contrast, interference with high calcium plateaus by the natural Ca^{2+} antagonist magnesium or a Ca^{2+} chelator *in vivo* abolishes trypsinogen activation as well as pancreatitis [62,75–77].

Acute pancreatitis is characterized by the pathologic activation of zymogens within pancreatic acinar cells. The process requires a rise in cytosolic Ca^{2+} from undefined intracellular stores. Zymogen activation is thereby mediated by ryanodine receptor-regulated Ca^{2+} release, and early zymogen activation takes place in a supranuclear compartment that overlaps in distribution with the ryanodine receptor. Furthermore, *in vivo* inhibition of the ryanodine receptor results in a loss of zymogen activation. Therefore, Ca^{2+} release from the ryanodine receptor mediates zymogen activation but not enzyme secretion [78].

Recent reports have shown that metabolites of alcohol metabolism can have a pathological effect on acinar cell Ca^{2+} homeostasis, suggesting a possible pathogenic mechanism in alcoholic pancreatitis. Nonoxidative metabolites like fatty acid ethyl esters (FAEE) and fatty acids (FA) can cause Ca^{2+}-dependent acinar cell necrosis [79]. It was previously demonstrated that FAEEs are generated in acinar cells incubated with clinically relevant concentrations of ethanol [79]. FAEEs activate the IP_3 receptor after which Ca^{2+} is released from the ER [80]. In contrast, FAs do not activate Ca^{2+} channels but decrease ATP levels in the cytoplasm, which results

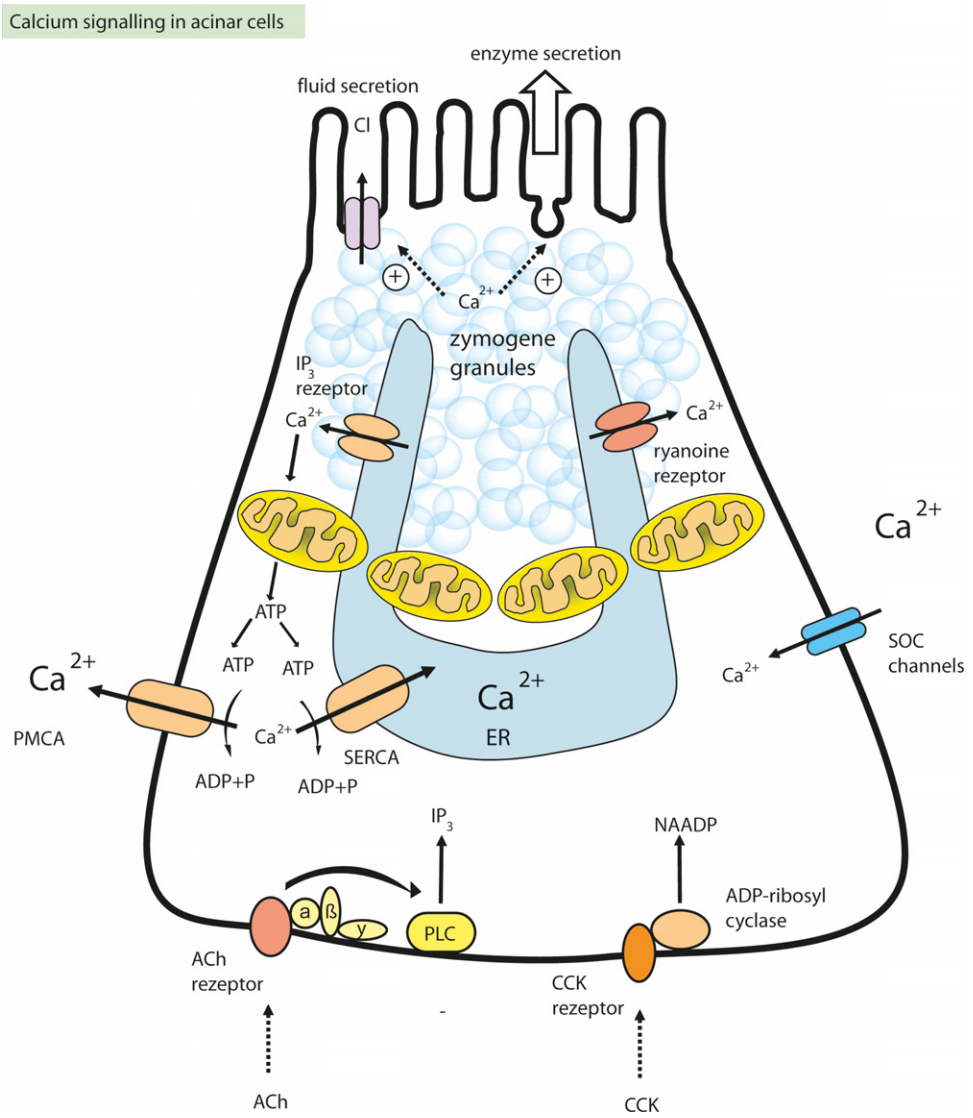

Figure 21.3 Calcium signaling in acinar cells. Intracellular Ca^{2+} homeostasis regulates the secretion of zymogens and fluid. Acetylcholine (ACh) regulates via the second messenger inositol-3-phosphate the apical Ca^{2+} influx from intracellular stores (ER). Choleocystekinin leads to the production of nicotinic acid adenine dinucleotide phosphate (NAADP), which interacts with ryanodine receptor in the ER membrane and also regulates the apical Ca^{2+} influx. The plasma membrane calcium ATPase (PMCA) or the sarcoplasmatic endoriticulum calcium ATPase (SERCA) regulates cytoplasmatic Ca^{2+} decrease. A mitochondrial barrier inhibits a global Ca^{2+} increase by absorbing free Ca^{2+} from the apical zymogen-enriched part of the acinar cell. Interferences in the calcium homeostasis lead to a global Ca^{2+} increase in the cytoplasm, which results in premature activation of zymogens.

in impaired Ca^{2+} reuptake into the ER. Subsequently, increased intracellular Ca^{2+} levels contribute to premature zymogen activation.

Ca^{2+} not only is an important second messenger, but has a direct effect on the activation, activity, and degradation mechanisms of trypsin. Experiments with purified human anionic and cationic trypsinogen in the absence of Ca^{2+} show markedly reduced autoactivation compared to high Ca^{2+} concentration [81]. Moreover, in cytoplasmic vacuoles developing upon supramaximal cholecystokinin stimulation in acinar cells, Ca^{2+} concentrations decreases rapidly to levels which are much lower than optimal for trypsinogen autoactivation [82]. This mechanism could represent a protective mechanism of the endosomes to prevent damage from premature trypsinogen activation.

CHRONIC AND HEREDITARY PANCREATITIS

Chronic pancreatitis is clinically defined as recurrent bouts of a sterile inflammatory disease characterized by persistent and often progressive and irreversible

morphological changes, typically causing pain and permanent impairment of pancreatic function. Chronic pancreatitis histologically represents a transformation of focal necrosis into perilobular and intralobular fibrosis of the parenchyma, pancreatic duct obstruction by pancreatic stones and tissue calcification, and the development of pseudocysts. In the course of the disease, progressive loss of endocrine and exocrine function can be monitored. It should be noted that the clinical distinction between acute and chronic pancreatitis is becoming pathophysiologically ever more blurred, and similar or identical onset mechanisms may play a role. These mechanisms include premature and intracellular activation of digestive proteases. Much of our present knowledge about this process has been generated since a genetic basis for pancreatitis was first reported in 1996. Hereditary pancreatitis represents a genetic disorder associated with mutations in the cationic trypsinogen gene and presents with a disease penetrance of up to 80%. Patients with hereditary pancreatitis suffer from recurrent episodes of pancreatitis which do progress in the majority to chronic pancreatitis. The disease usually begins in early childhood, but onset can vary from infancy to the sixth decade of life [83].

Mutations Within the *PRSS1* Gene

Hereditary pancreatitis is associated with genetic mutations in the cationic trypsinogen gene, suggesting that mutations in a digestive protease (such as trypsin) can cause the disease. Hereditary pancreatitis typically follows an autosomal dominant pattern of inheritance with an 80% disease penetrance. The gene coding for cationic trypsinogen (*PRSS1*) is approximately 3.6 kb in size, is located on chromosome 7, and contains 5 exons. The precursor of cationic trypsinogen is a 247 amino acid protein, the first 15 amino acids of which represent the signal sequence; the next 8 amino acids, the activation peptide; and the remaining 224 amino acids form the backbone and catalytic center of the digestive enzyme. Presently, there are two known mutations within the same codon in the *PRSS1* gene: (i) His-122-trypsinogen shows increased autoactivation, and (ii) Cys-122 trypsinogen has reduced activity. The first of these mutations was discovered in 1996, exactly one century after Chiari proposed his theory on autodigestion of the pancreas as a pathogenic mechanism of pancreatitis. Whitcomb et al. reported a mutation in exon 3 of the cationic trypsinogen gene (*PRSS1*) on chromosome 7 (7q36) [84–86] that was strongly associated with hereditary chronic pancreatitis. This single point mutation (CGC to CAC) causes an arginine to histidine (R to H) substitution at position 22 of the cationic trypsinogen gene (R122H). The amino acid exchange is located in the hydrolysis site of trypsin and can prevent the autodegradation of active trypsin [87] (Figure 21.2). Once trypsin has been activated intracellularly, the R122H mutation interferes with the elimination of active trypsin by autodegradation [88]. This conclusion was derived from *in vitro* data using recombinant R122H mutated trypsinogen. Using the same *E. coli* based system, Sahin-Toth and coworkers showed that the R122H mutation leads to an increase in trypsinogen autoactivation [89]. Therefore, the R122H mutation represents a dual gain of function mutation which facilitates intracellular trypsin activity and results in a higher stability of R122H-trypsin [88,90]. The direct pathogenic role of the R122H mutations for the development of pancreatitis was confirmed by the group of Bar-Sagi. These investigators generated a transgenic mouse in which the expression of the murine *PRSS1* mutant R122H (R122H_mPRSS1) was targeted to pancreatic acinar cells by fusion to the elastase promoter. Pancreata from transgenic mice displayed early-onset acinar cell injury and inflammatory cell infiltration. With progressing age, the transgenic mice developed areas of pancreatic fibrosis and displayed acinar cell dedifferentiation [91,92]. Interestingly, no increased trypsin activity was found in these animals under experimental supramaximal stimulation.

Shortly after the identification of the R122H mutation a second mutation was reported in kindreds with hereditary pancreatitis. The R122C mutation is a single amino acid exchange affecting the same codon as the R122H mutation [93,94]. In contrast to the R122H mutation, the R122C mutation causes a decreased trypsinogen autoactivation, and biochemical studies demonstrated a 60%–70% reduced activation in the Cys-122 trypsinogen mutant induced by either enterokinase or cathepsin B activation. The amino acid exchange of the R122C mutation leads to altered cysteine-disulfide bonds and consequently a misfolded protein structure with reduced catalytic activity. In recent years several other attempts have been made to elucidate the pathophysiological role of trypsinogen in the onset of pancreatitis, but the issue is still a matter of intense research [95].

Since the initial discovery, several other mutations (24 to date) in the trypsinogen gene have been reported, but the R122H mutation is still the most common. In addition to the R122H mutation, five other mutations in different regions of the *PRSS1* gene associated with hereditary pancreatitis have been biochemically characterized: A16V [96], D22G [97], K23R [98], E79K [99], and N29I [100]. It has been suggested that these mutations may have different structural effects on the activation and activity of trypsinogen.

The nucleotide substitution from A-T in exon 2 of the *PRSS1* gene (AAC to ATC) in codon 29 of cationic trypsinogen results in an asparagine to isoleucine amino acid change (N29I). This amino acid substitution affects the protein structure of trypsinogen, and seems to stabilize the enzyme [101]. The N29I mutation does not affect the autoactivation of trypsinogen but impairs enzyme degradation *in vivo* [102,103]. The N29I mutation causes a slightly milder course of hereditary pancreatitis compared to the R122H mutation, the onset of disease occurs somewhat later, and the need for in-hospital treatment is lower [83,104].

The A16V mutation results in an amino acid exchange from alanine to valine in the signal peptide of trypsinogen. This mutation is rare [96] and in contrast to R122H and N29I mutations, which are burdened with an 80% penetrance for chronic pancreatitis, develops only in one out of seven carriers of the A16V mutation. Two other mutations were found in the signal peptide of cationic trypsinogen, D22G and K23R. Both mutations result in an increase of autoactivation of trypsinogen [105] but are resistant to cathepsin B activation [106]. Furthermore, in contrast to wild-type trypsinogen, expression of active trypsin and mutated trypsinogens (D22G, K23R) reduced cell viability of AR4–2J cells [107]. This suggests that mutations in the activation peptide of trypsinogen play an important role in premature protease activation, but the biochemical mechanisms remain unsolved.

The EUROPAC-1 study compared genotype to phenotype characteristics of hereditary pancreatitis patients. This study confirmed the importance of *PRSS1* mutations associated with chronic pancreatitis. In a multilevel proportional hazard model employing data obtained from the European Registry of Hereditary Pancreatitis, 112 families in 14 countries (418 affected individuals) were collected [83]: 58 (52%) families carried the R122H mutation, 24 (21%) carried the N29I mutation, and 5 (4%) carried the A16V mutation, while 2 families had rare mutations, and 21 (19%) had no known *PRSS1* mutation. The median time to the start of symptoms for the R122H mutation is 10 years of age (8 to 12 years of age, 95% confidence interval), 14 years of age for the N29I mutation (11 to 18 years of age, 95% confidence interval), and 14.5 years of age for mutation negative patients (10 to 21 years of age, 95% confidence interval; $P = 0.032$). The cumulative risk at 50 years of age for exocrine pancreas failure was 37.2% (28.5%–45.8%, 95% confidence interval), 47.6% for endocrine failure (37.1%–58.1%, 95% confidence interval), and 17.5% for pancreatic resection for pain (12.2%–22.7%, 95% confidence interval). Time to resection was significantly reduced for females ($P < 0.001$) and those with the N29I mutation ($P = 0.014$). Pancreatic cancer was diagnosed in 26 (6%) of 418 affected patients (Table 21.1).

The remaining *Prss1* mutations are very rare and are for the most part detected only in a single family or a single patient. Mutations like P36R, K92N, or G83E were each found in only one patient with idiopathic chronic pancreatitis [108]. The biochemical consequences of these mutations do not result in increased stability or autoactivation of trypsinogen compared to the R122H or N29I mutations [109]. This observation causes difficulties explaining the underlying pathogenic mechanism for these rare mutations.

Mutations Within the *PRSS2* Gene

The fact that mutations in the *PRSS1* gene encoding for cationic trypsinogen are associated with hereditary pancreatitis could suggest that genetic alterations of the anionic trypsinogen gene (*PRSS2*) could also be associated with chronic pancreatitis. The E79K mutation (related to a G to A mutation at codon 237) reduces autoactivation of cationic trypsinogen by 80%–90% but leads to a 2-fold increase in the activation of anionic trypsinogen, suggesting a potential role of *PRSS2* [99]. A direct link between the development of chronic pancreatitis and mutations in the *PRSS2* gene was not established until 2006 [110]. Genetic analysis of the *PRSS2* gene in 2466 chronic pancreatitis patients and 6459 healthy individuals revealed an increased rate of a rare

Table 21.1 Most Common Mutations Associated with Pancreatitis

Gene	Mutation	Comments	Frequency
PRSS1	R122H	increased autoactivation and decreased autolysis of cationic trypsin	most common mutation (>500)
	R122C	decreased autoactivation of trypsinogen, decreased autolysis of trypsin also decreased trypsin activity	5 affected carriers
	N29I	increased autoactivation of trypsinogen	second most common mutation (>160)
	A16V	increased autoactivation of trypsinogen	25 affected carriers
	D22G	increased autoactivation of trypsinogen	rare, 2 carriers
	K23R	increased autoactivation of trypsinogen	rare, 2 carriers
	E79K	increased activation of anionic trypsinogen decreased autoactivation of trypsinogen	8 affected carriers
SPINK1	N34S	no functional defect reported	
	R65Q	60% loss of protein expression	
	G48E	nearly complete loss of protein expression	
	D50E	nearly complete loss of protein expression	
	Y54H	nearly complete loss of protein expression	
	R67C	nearly complete loss of protein expression	
	L14P and L14R	rapid intracellular degradation of SPINK1	
CTRC	R254W	decreased activity of chymotrypsin C	
	K247_R254del	loss of function of chymotrypsin C	
	A37T	decreased trypsin degradation of chymotrypsin C	
CFTR	various >1000	decreased fluid secretion from acinar cells	

mutation in the anionic trypsinogen gene in control subjects. A variant of codon 191 (G191R) was present in 220/6459 (3.4%) control subjects, but in only 32/2466 (1.3%) affected individuals [111]. Biochemical analysis of the recombinantly expressed G191R protein showed a complete loss of tryptic function after enterokinase or trypsin activation, as well as a rapid autolytic proteolysis of the mutant. This was the first report of a loss of trypsin function (in *PRSS2*) that has a protective effect on the onset of pancreatitis.

Mutations in the Chymotrypsin C Gene

Because trypsin degradation is thought to represent a protective mechanism against pancreatitis, Sahin-Toth and coworkers hypothesized that loss-of-function variants in trypsin-degrading enzymes would increase the risk of pancreatitis. Since they knew that chymotrypsin can degrade all human trypsins and trypsinogen isoforms with high specificity, they sequenced the chymotrypsin C gene (*CTRC*) in a German cohort suffering from idiopathic and hereditary pancreatitis. They detected two variants in the *CTRC* gene in association with hereditary and idiopathic chronic pancreatitis. Mutation of codon 760 (C to T) resulted in an R254W variant form of the protein that occurred with a frequency of 2.1% (19/901) in affected individuals compared to 0.6% (18/2804) in healthy controls. In addition, a deletion mutation (c738_761del24) resulting in a K247–R254del variant protein was found in 1.2% of affected individuals compared to 0.1% in controls [112]. In a confirmative cohort of different ethnic background, the authors detected a third mutation in affected patients with a frequency of 5.6% (4/71) compared to 0% (0/84) in control individuals. This mutation leads to an amino acid exchange at the position 73 (A73T) resulting from a G to A mutation at codon 217. The assumed pathogenic mechanism of *CTRC* mutations is based on lowered enzyme activity in the R254W variant and a total loss of function in the deletion mutation (K247–R254del) and the A73T variant (Figure 21.2). Thus, chymotrypsin C is an enzyme that can counteract the disease-causing effect of trypsin, and loss of function mutations impair its protective role in pancreatitis by letting prematurely activated trypsin escape its degradation.

Mutations in Serine Protease Inhibitor Kazal-Type 1

Shortly after the identification of mutations in the trypsinogen gene in hereditary pancreatitis, another important observation was made by Witt et al. [113]. This group found that mutations in the *SPINK-1* gene (the pancreatic secretory trypsin inhibitor or PSTI, OMIM 167790) can be associated with idiopathic chronic pancreatitis in children. *SPINK-1* mutations can be frequently detected in cohorts of patients who do not have a family history but also have none of the classical risk factors for chronic pancreatitis [114,115]. The most common mutation is found in exon 2 of the *SPINK-1* gene (AAT to AGT), which leads to an asparagine to serine amino acid change (N34S) [114]. Homozygote and heterozygote N34S mutations were detected in 10%–20% of patients with pancreatitis compared to 1%–2% of healthy controls, suggesting that *SPINK-1* is a disease-modifying factor [116–118]. Tropical pancreatitis is a common form of pancreatitis in Africa and Asia. This form of pancreatitis is characterized by abdominal pain, intraductal pancreatic calculi, and diabetes mellitus in young nonalcoholic patients. Tropical pancreatitis is associated with an even higher frequency of N34S mutations in the *SPINK-1* gene, representing up to 30% of affected individuals [119]. Structural modeling of SPINK-1 predicted that the N34S region near the lysine41 residue functions as the trypsin-binding pocket of SPINK-1 [120] and that the N34S mutation changes the structure of the trypsin-binding pocket of SPINK-1, resulting in decreased inhibitory capacity of SPINK-1. In contrast to the computer-modeled prediction, *in vitro* experiments using recombinant N34S *SPINK-1* and wild-type *SPINK-1* demonstrated identical trypsin inhibitory activities [116]. To study the role of SPINK-1 *in vivo,* experimenters generated a knockout mouse mode. The murine *SPINK-3* is the functional homolog to human *SPINK-1*. *SPINK-3*-deficient mice show a perturbed embryonic development of the pancreas and die within 2 weeks after birth [121]. No increase in trypsin activity was observed in *SPINK3-/-* mice. This suggests that other yet unknown disease factors are involved in the antiprotease/protease balance, contributing to the disease phenotype, or that trypsin-SPINK-1 interactions differ between murine and human isoforms. Nevertheless, targeted expression of *PSTI* in pancreas of transgenic mice increased endogenous trypsin inhibitor capacity by 190% ($P < 0.01$) compared to control mice. Cerulein administration to transgenic *PSTI* mice produced significantly reduced histologic severity of pancreatitis. There was no difference in trypsinogen activation between cerulein-treated transgenic and wild-type mice. However, trypsin activity was significantly lower in transgenic mice receiving cerulein compared with nontransgenic mice [122]. Recently, two novel *SPINK-1* variants affecting the secretory signal peptide have been reported. Seven missense mutations occurring within the mature peptide of PSTI associated with chronic pancreatitis were analyzed for their expression levels. The N34S and the P55S mutation neither results in a change of PSTI activity nor in a change of expression. The R65Q mutation involves substitution of a positively charged amino acid by a noncharged amino acid, causing ~60% reduction of protein expression. G48E, D50E, Y54H, and R67C mutations, all of which occur in strictly conserved amino acid residues, cause nearly complete loss of PSTI expression. As the authors had excluded the possibility that the reduced protein expression may have resulted from reduced transcription of unstable mRNA, they concluded that these missense mutations probably cause intracellular retention of their respective mutant proteins [123]. In addition, two novel

mutants have been described recently. A disease associated codon 41 T to G alteration was found in two European families with an autosomal dominant inheritance pattern, whereas a codon 36 G to C variant was identified as a frequent alteration in subjects of African descent. L14R and L14P mutations resulted in rapid intracellular degradation of the protein and thereby abolished SPINK-1 secretion, whereas the L12F variant showed no such effect [124]. The discovery of *SPINK-1* mutations in humans provides additional support for a role of active trypsin in the development of pancreatitis. SPINK-1 is believed to form the first line of defense in inhibiting prematurely activated trypsinogen in the pancreas.

CFTR Mutations: A New Cause of Chronic Pancreatitis

Cystic fibrosis is an autosomal-recessive disorder with an estimated incidence of 1 in 2500 individuals and is characterized by pancreatic exocrine insufficiency and chronic pulmonary disease. The extent of pancreatic involvement varies between a complete loss of exocrine and endocrine function, to nearly unimpaired pancreatic function. In 1996, Ravnik-Glavac et al. were the first to report mutations in the cystic fibrosis gene in patients with hereditary chronic pancreatitis [125]. Analysis of larger cohorts revealed recurrent episodes of pancreatitis in 1%–2% of patients with cystic fibrosis and normal exocrine function, and rarely in patients with exocrine insufficiency as well. Compared to an unaffected population, 17%–26% of patients who suffer from idiopathic pancreatitis carry mutations in *CFTR*. Chronic pancreatitis now represents, in addition to chronic lung disease and infertility due to vas deferens aplasia, a third disease entity associated with mutations in the *CFTR* gene. It is important to note that pancreatic exocrine insufficiency in patients with cystic fibrosis is a different disease entity and not to be confused with chronic pancreatitis in the presence of *CFTR* mutations [126–130]. CFTR is a chloride channel, regulated by 3′,5′-cAMP and phosphorylation [131,132] and is essential for the control of epithelial ion transport. The level of executable protein function determines the type and the severity of the disease phenotype. *CFTR* knockout mice show a more severe form of experimental pancreatitis induced by supramaximal cerulein stimulation compared with wild-type animals. The underlying hypothesis of the *CFTR*-related pancreatic injury is a disrupted fluid secretion, which leads to impaired secretion of pancreatic digestive enzymes in response to stimulation [133]. Today more than 1000 mutations within the *CFTR* gene are known, and several of them have been reported in direct association with chronic pancreatitis [126,127]. For healthy subjects who are heterozygous carriers of *CFTR* mutations, the risk of developing pancreatitis is about 2-fold [134].

SUMMARY

Recent advances in cell biological and molecular techniques have permitted investigators to address the intracellular pathophysiology and genetics underlying pancreatic diseases in a much more direct manner than was previously considered possible. Studies that have employed these techniques have changed our knowledge about the disease onset. Pancreatitis has long been considered an autodigestive disorder in which the pancreas is destroyed by its own digestive proteases. Under physiological conditions, pancreatic proteases are synthesized as inactive precursor zymogens and stored by acinar cells in zymogen granules. Independent of the pathological stimulus that triggers the disease, the pathophysiological events that eventually lead to tissue destruction begin within the acinar cells and involve premature intracellular activation of proteases. Cell injury subsequently induces a systemic inflammatory response. Much of our present understanding of the underlying pathogenic mechanism comes from genetic studies, which support a crucial role of trypsinogen activation. Different mutations within the *PRSS1* gene (like the R122H mutation), in genes coding for endogenous inhibitors of active trypsin (such as *SPINK-1*), or in trypsin-degrading enzymes (such as *CTRC*), have all been found in association with different varieties of pancreatitis. Nevertheless, the molecular mechanisms that regulate the balance between proteases and antiproteases, as well as the role of individual digestive enzymes in the proteolytic cascades that precede cell injury, still need to be defined by further experimental studies.

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