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Pancreatitis and Calcium Signalling:

Report of an International Workshop

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Abstract

Pancreatitis and Calcium Signalling was an international research workshop organized by the authors and held at the Liverpool Medical Institution, Liverpool, United Kingdom, from Sunday 12th to Tuesday 14th November 2006. The overall goal of the workshop was to review progress and explore new opportunities for understanding the mechanisms of acute pancreatitis with an emphasis on the role of pathological calcium signaling. The participants included those with significant interest and expertise in pancreatitis research and others who are in fields outside gastroenterology but with significant expertise in areas of cell biology relevant to pancreatitis. The workshop was designed to enhance interchange of ideas and collaborations, to engage and encourage younger researchers in the field, and promote biomedical research through the participating and supporting organizations and societies. The workshop was divided into 8 topic-oriented sessions. The sessions were: (1) Physiology and pathophysiology of calcium signaling; (2) Interacting signaling mechanisms; (3) Premature digestive enzyme activation; (4) Physiology Society Lecture: Aberrant Ca^{2+} signaling, bicarbonate secretion, and pancreatitis; (5) NFJB, cytokines, and immune mechanisms; (6) Mitochondrial injury; (7) Cell death pathways; and (8) Overview of areas for future research. In each session, speakers presented work appropriate to the topic followed by discussion of the material presented by the group.

The publication of these proceedings is intended to provide a platform for enhancing research and therapeutic development for acute pancreatitis.

PHYSIOLOGICAL AND PATHOLOGICAL Ca^{2+} SIGNALING

Overview of Acinar Cell Ca^{2+} Signaling

Ole Petersen (Liverpool, UK) began by acknowledging the major contribution to understanding cell signaling made by patch-clamp technology, introduced by the 1991 Nobel laureates Bert Sakmann and Erwin Neher for their discoveries concerning the function of single ion channels in cells.¹ After secretagogue receptor activation, the second messengers inositol trisphosphate (IP_3), cyclic adenosine diphosphate ribose (cADPR), and

nicotinic acid adenine dinucleotide phosphate (NAADP) all induce release of Ca^{2+} through their respective receptor (IP_3R and RyR) Ca^{2+} channels from internal stores into the apical cytosol (Fig. 1).^{1–5} The Ca^{2+} waves can only be initiated within the apical pole and are usually confined there by the perigranular mitochondrial buffer zone, where the Ca^{2+} signals initiate stimulus-metabolism coupling. The Ca^{2+} signals originate from terminals of the endoplasmic reticulum (ER) (via IP_3 , NAADP, and cADPR) and an acidic store that recent data indicate includes both the lysosomes (via NAADP) and zymogen granules (ZGs) (via IP_3 and cADPR).^{2,3} Globalization of Ca^{2+} signals results from combinatorial action of the 3 second messengers, as well as Ca^{2+} -induced Ca^{2+} release, which acts particularly on the RyR . All Ca^{2+} signals are the result of the interaction of second messenger-mediated release, which produces agonist-specific signatures. Second-messenger effects from acetylcholine (ACh) stimulation are largely mediated through IP_3 -mediated Ca^{2+} release, whereas those from cholecystokinin (CCK) are predominantly through NAADP and cADPR. When adenosine triphosphate (ATP) supplies fail, Ca^{2+} cannot be cleared from the cytosol into the ER by the sarcoendoplasmic reticulum ATPase Ca^{2+} pump (SERCA) or out of the cell through the plasma membrane ATPase Ca^{2+} pump (PMCA).⁴ This is especially dangerous for the pancreatic acinar cell, which lacks a $\text{Na}^+/\text{Ca}^{2+}$ exchanger.^{1,5}

In the discussion of Dr Petersen's presentation, it was emphasized that Ca^{2+} influx is as important for maintenance of signal generation as is release of Ca^{2+} from the ER at least in part because the Ca^{2+} influx is necessary for recharging the ER with calcium.

Ca^{2+} Entry in Health and Disease

Anant Parekh (Oxford, United Kingdom) introduced store-operated Ca^{2+} entry channels (SOCs), a family of plasma membrane Ca^{2+} channels that open in response to active (eg, second messenger-mediated) or passive (eg, SERCA pump inhibition) ER Ca^{2+} depletion.⁶ The best described and most widely distributed is the Ca^{2+} release-activated Ca^{2+} channel (CRAC), characterized electrophysiologically by patch-clamp measurements of the whole-cell Ca^{2+} current, I_{CRAC} . The CRAC channels are critical not only to store refilling, but also to ATP production, exocytosis, and cell proliferation. Furthermore, CRAC channel abnormalities are implicated in human disease. The I_{CRAC} is highly selective for Ca^{2+} , with 10,000 times greater relative permeability to that of Na^+ ; single-channel conductance is low, and of the order of 8000 CRAC, channels are estimated to be present in the plasma membrane of a single cell. RNA interference knockdown approach has demonstrated that stromal interaction molecule 1 (STIM-1), which spans the ER membrane, is a sensor that upon ER Ca^{2+} depletion migrates to puncta below the plasma membrane to trigger CRAC opening. The Orai1/CRACM1 is a plasma membrane protein, the selectivity of which to Ca^{2+} is changed by site-directed mutagenesis, revealing that Orai1 is the CRAC channel pore.⁷ To simulate physiological CRAC channel opening in isolated cells, mitochondria are energized with a cocktail of metabolic substrates, by means of which they become hyperpolarized. This has permitted demonstration that mitochondria activate the CRAC channel and reduce Ca^{2+} -dependent inactivation of I_{CRAC} .⁸ In addition, paired patch-clamp recordings from 2 cells in close proximity have demonstrated that a cytoplasmic rise in Ca^{2+} after I_{CRAC} activation in one cell results in a paracrine signal from that cell which activates I_{CRAC} in the second cell. Subsequent work on rat mast cells has shown that this signal is

leukotriene C₄ (LTC₄), the release of which follows the initiation of a signaling cascade by Ca²⁺ via protein kinase C (PKC) α and β1, cytosolic phospholipase A2, the mitogen-activated protein kinase MEK/ERK pathway and then 5-lipoxygenase, converting arachidonic acid into LTC₄.^{9,10} The relevance of this pathway to human disease is currently under exploration in human mast cells extracted from tissues affected by allergy.

The discussion of Dr Parekh's presentation added that coordination of Ca²⁺ entry occurs via receptor-mediated mechanisms rather than gap junctions.

Critical Subcellular Compartments

Rosario Rizzuto (Ferrara, Italy) described how the transfection of targeted aequorin and of ATP luciferase had identified rapid large increases in mitochondrial Ca²⁺, which occur immediately after cytoplasmic Ca²⁺ signals, and which regulate mitochondrial function.^{11,12} When accompanied by oxidative and/or toxic stress, these changes induce cell death,¹³ discussed later (see Mitochondrial Injury later). The regulatory mechanisms are not all clear because the molecular identities of the Ca²⁺ uniporter, Na⁺/Ca²⁺ exchanger and H⁺/Ca²⁺ exchanger on the inner mitochondrial membrane remain unknown. Close contacts between the ER and mitochondria have been identified and characterized.^{14,15} Again using targeted aequorins and Ca²⁺-sensitive green fluorescent proteins, the voltage-dependent anion channel (VDAC) was found to be an essential part of this closely interacting micro-domain, allowing rapid transfer of Ca²⁺ from the ER through the outer mitochondrial membrane, which then enters the matrix through the Ca²⁺ uniporter.¹⁵ VDAC overexpression in HeLa cells was found to enhance mitochondrial Ca²⁺ uptake, confirming that transfer of Ca²⁺ from the mouth of the IP₃R on the ER membrane into mitochondria is critically dependent on the VDAC repertoire. To find regulatory components of the VDAC complex, yeast 2-hybrid screens were conducted using VDAC as bait, identifying a strong interaction with the chaperone glucose-regulated protein 75 (GRP75, also known as mitochondrial heat shock protein 70 [HSP70] or mortalin). Recent work examining the effects on VDAC function of the outer mitochondrial membrane binding domain of the IP₃R and GRP75 has shown that the binding domain of the IP₃R increases Ca²⁺ entry through VDAC, and that coupling of the IP₃R on the ER and VDAC on the outer mitochondrial membrane depends on the presence of GRP75, further defining the VDAC complex.

During discussion of the presentation, Dr Rizzuto stated that the capacity of mitochondria to cope with Ca²⁺ is considerable, but matters go awry when combined with another stress, suggesting that Ca²⁺ is permissive to injury. Further discussion raised the possibility that mitochondria have a role by removing Ca²⁺ from the cytosol to prevent pathology, which could be one reason for their close interaction with the ER. Other discussions raised possibilities for roles for PKCs and HSPs in the regulation of VDAC. There was an open and unresolved debate as to whether different types of mitochondria exist in the same cell, or whether apparent differences arise from different signals and microdomains occurring within different parts of the same cell.

INTERACTING SIGNALING MECHANISMS

PKC, Alcohol, and Pancreatitis

Stephen Pandol discussed his group's original findings that acinar cells produce and respond to cytokines, underscoring the role of inflammation in acinar cell injury and pancreatitis. The role of PKC isoforms remains a particularly important focus for their work.^{16–20} Both CCK hyperstimulation and tumor necrosis factor- α (TNF- α) activate nuclear factor- κ B (NF- κ B) via phosphatidylinositol-specific phospholipase C (PI-PLC, by CCK only) and phosphatidylcholine-specific PLC (PC-PLC, by both CCK and TNF- α), forming 1,2 diacylglycerol (nota bene activated PI-PLC also produces IP₃), which activates PKCs that induce NF- κ B activation.^{16,17} Dissection of the PKC isoforms responsible showed that it is the combination of the novel isoforms PKC- δ and PKC- ϵ that induces NF- κ B activation. Further work showed that ethanol sensitizes the pancreas to inflammation from CCK stimulation by up-regulating PKC- ϵ because without ethanol, 100 pM CCK induced PKC- δ but not NF- κ B, whereas with ethanol, 100 pM CCK induced both PKC- δ and PKC- ϵ , and subsequently activated NF- κ B.^{18,19} Very recent work has implicated PKCs in pathological basolateral exocytosis mediated by the syntaxin-binding protein Munc18c, consequent upon ethanol and CCK stimulation of pancreatic acinar cells.²⁰

The discussion of the presentation by Dr Pandol indicated that it is not known whether PKC activation alone or Ca²⁺ release alone was sufficient to induce acinar cell injury. Furthermore, the effects on PKC activation of secretin, vasoactive intestinal polypeptide, JMV180 (with or without ethanol), or bile salts have not been determined. Nor was it known whether the effects of PKC were structural or enzymatic, but fatty acid ethyl esters (FAEEs) and acetaldehyde had been found to down-regulate PKC.

Phosphatidylinositide 3 Kinases in Pancreatitis

Anna Gukovskaya (Los Angeles, Calif) outlined relationships among phosphoinositides and the 3 classes of mammalian phosphatidylinositide 3 kinases (PI3Ks), classes IA and IB, II, and III, which phosphorylate by inducible (class I and II) and constitutive (class III) means. Receptor tyrosine kinases and G protein-coupled receptors initiate class I PI3K activation of the AKT (protein kinase B [PKB], a serine-threonine protein kinase belonging to the protein kinase A, G, and C superfamily) signaling pathway that has complex effects on protein synthesis, metabolism, and cell survival and cycling. Class III PI3K also contributes to vesicle sorting and autophagy. Whereas class I PI3K activation of AKT occurs via phosphorylation of phosphatidylinositol-4,5-bisphosphate (hydrolysis of which by PI-PLC forms IP₃ and diacylglycerol) to phosphatidylinositol-3,4,5-trisphosphate, phosphatase and tensin homologue deleted on chromosome ten (PTEN) (a tumor-suppressor gene) inhibits AKT by reversing this reaction. The PTEN itself is inhibited by oxidation, which destabilizes PTEN, and phosphorylation, which stabilizes PTEN. The role of many signaling molecules in pancreatitis has been investigated using CCK hyperstimulation, sometimes supplemented with ethanol administration.^{21,22} Previous work with this model had not implicated class I PI3Ks in the pathogenesis of pancreatitis, but had shown that class III PI3K induces formation of phosphatidylinositol phosphate, which may impair lysosomal trafficking and contribute to trypsinogen activation. Further evidence for this effect was

derived from the actions of wortmannin, a PI3K inhibitor, which reduced phosphatidylinositol phosphate levels and ameliorated both cerulein-induced and taurocholate-induced acute pancreatitis. Subsequent experiments, however, have indicated that AKT activation occurs early in the course of cerulein-induced acute pancreatitis, as well as during ethanol feeding, an effect attributed to PTEN inhibition. A more specific approach was adopted through the use of mice deficient in subunit γ of class IB PI3K, implicated in signaling from G protein-coupled receptors in tissues that include the exocrine pancreas; both in cerulein-induced and choline-deficient ethionine-supplemented diet-induced acute pancreatitis, such mice developed less trypsinogen activation, pancreatic neutrophil infiltration, and acinar cell necrosis, with more acinar cell apoptosis.²³ In addition, in contrast to the effects of wortmannin, lack of γ resulted in less NK- κ B activation.

Significant parallel work has shown that PI3K regulates Ca^{2+} signaling in isolated pancreatic acinar cells through an inhibitory effect on SERCA after hyperstimulation²⁴ or bile salt exposure²⁵; knockout of PI3K γ prevented cytosolic overloading of Ca^{2+} and trypsinogen activation with these toxins.^{24,25}

Discussion of Dr Gukovskaya's presentation revealed that in acute pancreatitis, the relative importance of AKT activation versus trypsinogen activation is not known. Furthermore, the mechanisms that relate PI3K and PTEN activity to trypsinogen activation are not known. It is of note, however, that both PI3K inhibition with wortmannin or LY-294002 and γ deletion reduced Ca^{2+} mobilization and influx, indicating that PI3K inhibits SERCA pump activity and promotes Ca^{2+} mobilization into the cytosol, which in conditions of stress (eg, hyperstimulation) would tend to induce acinar cell injury and trypsinogen activation. Elucidation of the roles of different PI3Ks should be a topic of future research.

Novel Mechanisms of Nitric Oxide Release in Pancreatic Acinar Cells

Alexei Tepikin (Liverpool, United Kingdom) presented work investigating the role of nitric oxide (NO) generation and nitrosative stress in pancreatic acinar cells,^{26,27} building on novel techniques using fluorescent measurement of subcellular responses to toxins and cell stress.^{28–30} Nitric oxide was found to be released in isolated mouse pancreatic acinar cells hyperstimulated with either 10 μM ACh or 10 nM CCK. Nitric oxide responses were also seen after more representative, quasiphenological secretagogue concentrations (50 nM ACh, 10 pM CCK), although in fewer cells, so most experiments were conducted with hyperstimulation.²⁷ This work was conducted using intracellular dialysis with whole-cell patch clamp, measuring intracellular NO release with the fluorescent dye 4-amino-5-methylamino-2',7'-difluorofluorescein, most probably because this approach removed mobile intracellular nitrosation scavengers. 1,2-Bis(*o*-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA) inhibited ACh-induced responses of 4-amino-5-methylamino-2',7'-difluorofluorescein, indicating that the NO release was dependent on cytosolic Ca^{2+} rises, and reduced glutathione and NO scavengers suppressed ACh-induced NO responses. Further analysis of the source of NO release, notably through the combination of HgCl_2 (which catalyzes *S*-NO bond cleavage and has been used to quantify *S*-nitrosothiols) and ionomycin, showed intracellular *S*-nitrosothiols to be the primary source, with NO synthase

(NOS) activity contributing to subsequent NO production. Interestingly, NO responses were found to be independent of calmodulin, PKC, and transition metals, but were inhibited by calpain antagonists, μ -calpain and m -calpain, having previously been established as present in the exocrine pancreas. Putatively, calpain-induced partial proteolysis of *S*-nitrosylated proteins may promote thiol denitrosylation.

Comment during the discussion of Dr Tepikin's presentation indicated that, from evidence to date, NO does not appear important in the early acinar cell events of acute pancreatitis but may be important in vascular events. The pancreatic acinar cell contains all 3 types of NOS (neuronal NOS, endothelial NOS [eNOS], and inducible NOS), which are activated by Ca^{2+} . Previous experiments show that during the first 8 hours of cerulein-induced acute pancreatitis, there is evidence of an increase in eNOS activity; and eNOS deletion impairs the increase in pancreatic blood flow that occurs during cerulein hyperstimulation, resulting in more severe pancreatitis.

PREMATURE DIGESTIVE ENZYME ACTIVATION

The Granule, an Intracellular Ion Oscillator: Implications for the Pathology of Secretion

Pedro Verdugo (Washington) explained that within secretory granules in many cell types, there is a polymer matrix network that behaves as an ion exchange resin and which determines how the signaling of secretion is coordinated. The polymer matrix (eg, heparin in mast cell granules, chromogranin in chromaffin cell granules) holds the secretory product (eg, histamine from mast cells, adrenaline from chromaffin cells), together with Ca^{2+} and H^+ ions as well as ATP in the granules. The polymer matrix undergoes a phase transition, depending on local physicochemical factors, notably a change in granular Ca^{2+} concentration; with a small decrease in concentration, a large increase in volume occurs.³¹ The transition is dependent on the second power of the valence of this ion. Thus, during passage through the signal transduction domain of the apical secretory pole, granules hold secretory products tightly in their polymer matrix, but during exocytosis, Na^+ and K^+ replace Ca^{2+} , and the matrix expands to release the secretory product.³² Image deconvolution studies of isolated granules from many cell types have shown oscillations induced by IP_3 and cADPR through Ca^{2+} , K^+ , and H^+ channels in the membrane of granules, thought to prime effector mechanisms in secretion and related processes.^{33–35}

The discussion of Dr Verdugo's work centered on the amount of Ca^{2+} present in ZGs, and whether granular Ca^{2+} reloading occurs. The concentration of Ca^{2+} is 10 mM in the granular matrix and 50 μM in the granular solution; high levels of stimulation cause a drop in total granular Ca^{2+} . In addition, the release of Ca^{2+} from the ZGs is followed by reloading; and Ca^{2+} oscillations occur and are probably dependent on reloading.

Determinants of Activation and Activity: Ca^{2+} Regulation of Trypsinogen Activation and Degradation

Miklos Sahin-Toth (Boston, Mass) focused on the effects of Ca^{2+} within the context of various interacting digestive enzymes that may contribute to, or protect from, acinar cell damage and thus sporadic or hereditary pancreatitis.^{36–40} The concentration of Ca^{2+} alters

the speed at which trypsin activates trypsinogen (the rapid phase of the S-shaped curve of autoactivation) and degrades itself (as well as trypsinogen).³⁸ Ca^{2+} does not, however, significantly affect the activation of trypsinogen by enterokinase, the activation of other zymogens by trypsin, or the digestion of dietary proteins by trypsin. Concentrations of Ca^{2+} in pancreatic juice and in the duodenum are normally greater than 1 mM, at which there is plentiful trypsin activity and robust trypsinogen activation, but little trypsin degradation. At lower Ca^{2+} concentrations (10–100 μM or less), autoactivation of human cationic trypsinogen (PRSS1, the major human trypsinogen) is less; notably, the Ca^{2+} binding site of trypsinogen (Glu75 to Glu 85) has a dissociation constant of 25 to 50 μM . Chymotrypsin C (formerly known as enzyme Y) accelerates trypsinogen activation through a Ca^{2+} -independent effect on Asp218, which otherwise maintains an electrostatic interaction with the activation peptide.³⁹ This effect of chymotrypsin C is further enhanced by the A16V mutation of the PRSS1 gene that causes about 5% of cases of hereditary pancreatitis. Chymotrypsin C also accelerates trypsin (and trypsinogen) degradation by cleaving the Ca^{2+} binding loop (Glu75 to Glu85), an effect that is inversely proportional to the Ca^{2+} concentration, near maximal at 25 μM and minimal at greater than 1 mM. The degradative action of chymotrypsin C is trypsin dependent because the R122H mutation of PRSS1 (at the trypsin cleavage site) that causes about 70% of cases of hereditary pancreatitis⁴⁰ is resistant to its action, as is R122A produced by site-directed mutagenesis. If significant trypsinogen activation occurs within ZGs as a result of (in part) a loss of granular Ca^{2+} , chymotrypsin C activation would be likely, leading to chymotrypsin C-enhanced degradation of trypsin, a potential line of defense against premature intracellular digestive enzyme activation.

During discussion of his presentation with the audience, Dr Sahin-Toth stated that there are no known mutations of chymotrypsin C, although this enzyme has been found in all mammalian pancreata examined, and it is more similar to elastase than trypsin.

Onset Events in the Apical Pole

Ashok Saluja (Minneapolis, Minn) advocated the view derived from many experimental data that pancreatitis begins with an acinar cell insult that blocks secretion, inducing colocalization of lysosomes and ZGs.^{41–45} These data include comparison of the effects of cerulein, which induces premature intracellular digestive enzyme activation, secretory block, apical-to-basolateral F-actin redistribution, and pancreatitis, with those of CCK-JMV180 and bombesin, which induce digestive enzyme activation without secretory block, F-actin redistribution, or pancreatitis.⁴⁵ Admixture of the content of lysosomes and ZGs leads to trypsinogen activation by lysosomal cathepsin B, which if inhibited, markedly reduces trypsinogen activation, plasma amylase (prognostic in rodents), pancreatic edema, pancreatic necrosis, and myeloperoxidase activity. Results indicate that colocalization is a result of an abnormal elevation of the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) produced by a toxic insult, the effects of which can be prevented or reduced by Ca^{2+} chelators (eg, BAPTA), heat shock proteins, PI3K inhibitors (eg, low concentrations of wortmannin) or cathepsin B inhibition.^{42–44} The secretory block induced by hyperstimulation or L-arginine can be overcome by protease-activated receptor 2 activation, for example, with the peptide SLIGRL, which ameliorates pancreatitis, confirming the importance of secretory block in pathogenesis.

Further information shared during the discussion of Dr Saluja's presentation brought up the point that the actin coating of ZGs may be upset by a persistently high $[Ca^{2+}]_i$, which may induce secretory block that could augment the zymogen activation in the cell. There are substantial data to support the view that trypsinogen activation occurs in condensing vacuoles, subsequently spreading elsewhere. Considering that there are two stages to secretion-fusion of granules with the apical plasma membrane followed by pore expansion, there are very elegant data showing that without pore expansion, no secretion occurs. The absence of pore expansion may be important in the pathogenesis of acute pancreatitis.

Other discussions focused on whether enzyme activation is the first event in acinar cell injury and the fact that colocalization has never been shown to occur before trypsinogen activation, a weakness in the colocalization hypothesis. It is not known how the pH at which cathepsin B activates trypsinogen is achieved in vivo, that is, with a maximum effect at pH 4.0 and no effect above pH 5.0. Nevertheless, 80% of pancreatic trypsinogen activation is lost during hyperstimulation-induced pancreatitis in the cathepsin B-knockout mouse, suggesting a direct effect, and overcoming a problem of experiments with CA-074me, which also inhibits cathepsin L, a lysosomal enzyme that degrades trypsinogen. The mechanism by which protease-activated receptor 2 activation restores secretion is not known.

Early Extracellular Events

Markus Lerch (Griefswald, Germany) provided a perspective with rhetorical questions and answers, complementing the focus of the workshop on intracellular events, notably Ca^{2+} ⁴⁶ and cathepsin B,⁴⁷ up to this point. The extravasation of fluid and recruitment of leukocytes, notably polymorphonuclear (PMN) leukocytes (neutrophils), are important early events in acute pancreatitis associated with a breakdown of intercellular adhesion that depends on disruption of cadherin/catenin adherens complexes.^{48–50} Neutrophil recruitment depends on rolling on capillary endothelia, tight binding via intercellular adhesion molecules (ICAMs), diapedesis, and migration. Why is cleavage of E-cadherin observed so early during the onset of acute pancreatitis? The answer is provided by the effects of PMN elastase, which contributes to disease severity, with PMN elastase levels being prognostic.⁵⁰ During the first hour of hyperstimulation-induced rodent acute pancreatitis, CD45-positive (leukocyte common antigen positive) leukocytes infiltrate the pancreatic parenchyma, and PMN elastase and myeloperoxidase activity are elevated. Furthermore, when neutrophil recruitment is prevented by an absence of essential components of leukocyte integrins essential for ICAM binding, as in the CD18-knockout mouse, milder acute pancreatitis results in reduced pancreatic trypsin activity at 1 and 8 hours. It is concluded, therefore, that neutrophils contribute to early trypsinogen activation, elastase activation, and the severity of pancreatic injury in acute pancreatitis by an unknown mechanism.

The ensuing discussion of the presentation by Dr Lerch indicated that elastase is present in other inflammatory cells such as macrophages; and that breakdown of the intercellular adherens junctions may account for some of the changes of pancreatitis including inhibition of secretion. The discussion raised the possibility that neutrophil activation and/or elastase could perhaps be inhibited by a number of compounds to reduce the severity of pancreatitis. In addition, inhibition of the early production of chemokines by acinar cells themselves,

which may account for neutrophil activation in pancreatitis, is a possible strategy for therapeutic intervention. Another point raised is that there is no need for colocalization of lysosomes and ZGs, an early event in pancreatitis, for neutrophil activation to occur; and it remains a challenge to explain why neutrophils cause and/or exacerbate trypsinogen activation.

PHYSIOLOGY SOCIETY LECTURE: ABERRANT Ca^{2+} SIGNALING, BICARBONATE SECRETION, AND PANCREATITIS

Shmuel Muallem (Texas) elaborated the view that abnormal Ca^{2+} signaling initiates pancreatitis,⁵¹ with a variety of factors, including protease-activated receptors (notably protease-activated receptor type 2 [PAR2]) modulating systemic impact.⁵² Injury occurs via abnormal elevations of $[\text{Ca}^{2+}]_i$, maintained by Ca^{2+} influx into the cell, without which noxious effects are not observed (Fig. 2). Thus, the identity, localization, and regulation of pancreatic acinar cell Ca^{2+} influx channels, notably CRAC and SOCs, are critical.^{53–56} Scaffolding proteins regulate Ca^{2+} entry and Ca^{2+} release within the cytosol, whereas the proposed SOC may be canonical transient receptor potential channels (TRPC), implicated from several studies to be SOCs and found as TRPC-1, TRPC-3, and TRPC-6 in the pancreatic acinar cell.⁵³ Upon stimulation of the cell and cytosolic Ca^{2+} release leading to Ca^{2+} store depletion, TRPC-3 translocates from vesicles to the apical plasma membrane, allowing Ca^{2+} entry by an IP_3 – and Homer 1–dependent mechanism.⁵⁴

Another recently identified regulator of CRAC and SOC Ca^{2+} entry is a member of the stromal interaction molecule family (STIM-1). The molecular basis for the function of STIM-1 has been unraveled using a screening assay based on the translocation of a transcription factor, nuclear factor of activated T cells, which translocates from the cytosol to the nucleus in response to a sustained elevation of $[\text{Ca}^{2+}]_i$.⁵⁵ These studies have shown that STIM-1 activates SOC, CRAC, and TRPC channels by a mechanism that relies on ezrinradixin-moesin (ERM)-dependent binding and lysine-rich region–dependent activation of the channel from its position within the ER. Ca^{2+} depletion leads to translocation and clustering of the proteins at sites of close apposition between the plasma membrane and the ER, and promotes the action of the lysine-rich region of STIM-1 on channel opening. It is clustering of STIM-1 within the ER near the membrane in response to store depletion of Ca^{2+} that is the critical event for channel opening; subsequently, STIM-1 binds TRPCs and determines their function.⁵⁶ The E α -helix-loop-F α -helix hand domain of STIM-1 residing in the ER senses ER Ca^{2+} concentrations, and functions to inhibit STIM-1 activity when the stores are filled.⁵⁵ Both the ERM domain and the lysine-rich tail of STIM-1 are crucial for the activation of endogenous SOC, I_{CRAC} , and TRPC channels.

The focus then turned to the enormous output of bicarbonate secreted by the pancreas,^{57–60} which both ethanol and bile increase. In exploring the mechanism of bicarbonate secretion including the role of the cystic fibrosis transmembrane conductance regulator (CFTR), an ABC chloride ion transporter, the actions of the solute carrier protein 26 transporter (SLC26A) family were discussed.^{58–60} This family of epithelial anion transporters have currently taken center stage in determining how epithelial cells transport a variety of ions

including Cl^- , bicarbonate, I^- , oxalate, formate, and sulfate. SLC26A3 and SLC26A6 exchange Cl^- and bicarbonate in the pancreatic duct, and CFTR regulates these SLC26A types, although SLC26A transporters modulate the activity of CFTR also. Interestingly, cerulein pancreatitis is significantly more severe in SLC26A6-knockout mice than in wild-type animals. These data led to the suggestion that acute pancreatitis may be ameliorated by stimulation of pancreatic ductal secretion, in part to flush out activated enzymes and toxic mediators (Fig. 2).

In the discussion of Dr Muallem's presentation, it was revealed that TRPC-1, TRPC-3, and TRPC-6 are situated predominantly in the lateral membrane of the pancreatic acinar cell, but there is 100% overlap of TRPC and IP_3R . Ca^{2+} influx is necessary to sustain secretion because without Ca^{2+} in the external medium, secretion is sustained for 5 minutes only. In addition, cystic fibrosis requires 90% loss of CFTR activity, although with a less severe loss, pancreatic exocrine insufficiency may result.

NF- κ B, CYTOKINES, AND IMMUNE MECHANISMS

NF- κ B in Pancreatitis

Ilya Gukovsky (Los Angeles, Calif) reviewed the role of NF- κ B, particularly in pancreatitis, which features two peaks of NF- κ B activation early in its pathogenesis.^{61–65} This molecule is activated in canonical (classical) and noncanonical (nonclassical) pathways, with an atypical pathway, for example, via DNA damage, all of which may occur within pancreatic acinar cells. NF- κ B is activated by bile salts⁶³ and FAEs,^{64,65} and alongside p38 mitogen-activated protein kinase, contribute to cytokine up-regulation.⁶² In hyperstimulation-induced acute pancreatitis, NF- κ B is activated within 5 minutes, although bombesin, carbachol, and JMV180 do not induce this, and JMV180 prevents cerulein-induced activation.⁶¹ When NF- κ B activation is prevented or reduced, as in knockout of TNF- α R (which activates NF- κ B) or interleukin-1 receptor, hyperstimulation-induced pancreatitis is less severe. Similarly, P50 (a subunit of NF- κ B)-knockout mice develop less severe pancreatitis. The relative roles of $[\text{Ca}^{2+}]_i$ elevations, PKC isoforms (δ and ϵ), PI3K (γ), trypsinogen activation, and reactive oxygen species (ROS) are unclear, but it appears that Ca^{2+} and PKC- δ and/or - ϵ may activate NF- κ B independently. It remains to be determined what the mechanisms of upstream signaling are in pancreatitis and the role of NF- κ B in cell death responses.

Dr Gukovsky's presentation prompted considerable discussion, initially on the possible pathways, which all depend largely if not entirely on external stimuli affecting the cell. For example, the second peak of NF- κ B activation is caused by inflammatory infiltrate. A role for LTC_4 was suggested. Further debate continued on the relative roles of enzyme activation versus cytokine release in determining the severity of pancreatitis. Several lines of experimentation were suggested, including experiments with BAPTA, and investigation as to whether adenoviral transfer of P65 into the pancreas induces actual acinar cell injury and elevation of serum amylase. Cytokines are critical to the systemic inflammatory response syndrome and the acute respiratory distress syndrome. It was noted that in cardiomyocyte necrosis, ischemia is a key trigger, then a secondary phase occurs during reperfusion with generation of ROS and elevated $[\text{Ca}^{2+}]_i$. The relative contribution of inflammatory mediators in acinar cell injury and pancreatitis requires clarification because it is unclear why in

producing inflammatory mediators, acinar cells appeared to be overdosing themselves with what should be a protective response. The group pondered whether the relative roles of NF- κ B, cytokines, neutrophils, ROS, and Ca^{2+} could be determined. There is a paradox in that the volume of the pancreas and death of tissue in necrotizing pancreatitis is out of proportion to the systemic effect, and much greater than expected from the death of a similar volume of other tissues.

Inflammation and the Severity of Pancreatitis

Stephen Pandol explored the effects of the inflammatory response in acinar cell injury and pancreatitis.^{66–70} The production of cytokines by acinar cells,⁶⁶ neutrophil infiltration and activation,⁶⁷ stellate cell activation,⁶⁸ and vascular permeability changes⁶⁹ all have an effect on disease severity⁷⁰ and progression to chronicity.^{68,70} Antineutrophil serum decreases enzyme activation and necrosis in acute pancreatitis, with an accompanying increase in apoptosis. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase knockdown has the same effect.⁶⁷ Using measurements of Western blot analysis, generation of ROS and immunocyto-chemistry, NADPH oxidase activity in pancreatitis was demonstrated to be in neutrophils and not pancreatic acinar cells. Guamerin-derived synthetic peptide is a novel specific inhibitor of neutrophil elastase that also inhibits the production of ROS by neutrophils and has been found to reduce the severity of hyperstimulation-induced pancreatitis. Neutrophils seem to convert apoptosis into necrosis, but neutrophil myeloperoxidase (MPO) seems to have no part in this because MPO knockdown was not found to reduce the severity of pancreatitis.⁶⁷

The discussion of Dr Pandol's presentation was focused on the sequence of processes from inflammation signaling to necrosis. The mechanisms connecting 1 process to the next in sequence are not determined. Intercellular adhesion molecule 1 is expressed on hyperstimulated cells, which may account for the targeted action of neutrophils, but how apoptosis is converted into necrosis is not known, although the inflammatory response contributes. It was suggested to be a misfortune that evolution has resulted in PMN leukocytes causing trypsinogen activation.

The Role of Neutrophils in Pancreatitis

Ashok Saluja examined questions concerning the stimuli and actions of neutrophils in pancreatic acinar cell death and pancreatitis.^{71–75} Less severe acute pancreatitis results from hyperstimulation in ICAM-1–knockout mice compared with wild-type animals.⁷² Similarly, deficiencies of P-selectin or E-selectin, which bind leukocytes to endothelia, result in fewer neutrophils infiltrating the pancreas and less severe pancreatitis. Neutrophil infiltration begins after trypsinogen activation starts but also causes further trypsinogen activation. There are a number of unsolved puzzles. Does Toll-like receptor 4 (TLR-4), a pattern recognition receptor, have a role? This is certainly suggested from experiments in TLR-4–knockout mice, where MPO activity is reduced in both the pancreas and lungs after the induction of acute pancreatitis by hyperstimulation or L-arginine.⁷⁵ A similar reduction has been found in mice with knockout of CD14, another pattern recognition receptor and coregulator with TLR-4 of responses to endotoxin. No lipopolysaccharide, however, has

been found in the experimental animals anywhere, so what activates TLR-4 remains unknown.

The discussion of Dr Saluja's presentation addressed whether inflammatory signaling alone is sufficient to account for all the processes that occur in pancreatitis. The answer is not evident. In addition, there was a discussion as to why PAR2 has apparently different roles in the pancreas and elsewhere. That is, in the pancreas, PAR2 seems to be protective, whereas in the lung tissue, it seems to worsen damage.

MITOCHONDRIAL INJURY

Mitochondrial Targets and Mediators of Cell Injury

Michael Duchon (London, United Kingdom) pointed to the role of mitochondria in the life and death of cells as an energy source, replete with synthetic enzymes, and containing maternally inherited DNA necessary for respiration.^{76–80} Mitochondria accumulate Ca^{2+} , integrating the spatiotemporal patterning of cytosolic Ca^{2+} signals into an energy supply for the rest of the cell.⁸⁰ Central to mitochondrial function is maintenance of a mitochondrial membrane potential (ψ_M) of 150 to 180 mV across the inner mitochondrial membrane, negative inside, produced by pumping protons into the intermembrane space. Some ROS generation is normal, and their role/effect on ψ_M is unclear, but there are innumerable mitochondrial targets of injury, for example, complexes from excessive ROS generation.^{77–79} The part played by uncoupling proteins, including uncoupling protein 2, remains unclear, but in uncoupling, protons leak back across the inner membrane.

Mitochondrial dysfunction is the primary cause of some diseases, whereas mitochondrial damage is the final determinant of irreversible cell injury in others.⁸⁰ Thus, principal questions are how mitochondrial function is compromised, and what happens when this occurs. Tools to dissect mechanisms include oligomycin, which inhibits mitochondrial ATPase; bongkrekate, which inhibits adenine nucleotide translocase (ANT) and thereby ATP exchange from the cytoplasm for mitochondrial ADP; 2-deoxy-D-glucose, which inhibits glycolysis; and pyruvate, on which mitochondrial ATP generation is entirely dependent.^{76–80} Mitochondria, however, can also be ATP consumers, an important feature of mitochondrial dysfunction, as demonstrated in studies of cardiac myocytes using the mitochondrial dye TMRM. Thus, with laser light application, the ψ_M is lost, but oligomycin prevents the rigor (and cell shortening) that normally results from mitochondrial membrane depolarization. The combination of Ca^{2+} loading and oxidative and/or nitrosative stress is particularly damaging (Fig. 3), as shown in studies where dyes are oxidized to a fluorescent product, with A amyloid causing a large irreversible mitochondrial depolarization dependent on Ca^{2+} entry^{77,79} and NADPH oxidase activation via PKC.⁷⁹ The effects of Ca^{2+} loading and oxidative and/or nitrosative stress can be prevented by exclusion of Ca^{2+} from the medium, blockage of the Ca^{2+} uniporter (which transports Ca^{2+} from the cytosol into the mitochondrial matrix), using Ru_{360} (μ -oxo) bis (*trans*-formatotetramine ruthenium), cyclosporine A (CyA, which inhibits the mitochondrial permeability transition pore [MPTP]), or knockout of cyclophilin D (CyP-D) (part of the MPTP).^{77–79} It can also be prevented by antioxidants,⁷⁹ including 2,2,6,6-tetramethylpiperidyl-1-oxyl radicals and catalase.

Questions raised in the discussion of Dr Duchen's presentation led him to comment that the principal determinant of ATPase reversal is its thermodynamic equilibrium, that inhibition of respiration increases proton leak, and that the inhibitor protein IF1 and pharmacological agents prevent ATPase reversal. It was also noted that ROS are more important than Ca^{2+} in the behavior of the MPTP, that reversibility is maintained in vitro as long as glucose is present in the medium, and the extent to which the MPTP is open or closed determines outcome. In vivo, both glycolytic (cytoplasmic) and aerobic (mitochondrial) metabolism may occur, but Dr Duchen stated that caution is required in the interpretation of experiments based on transformed cell lines because these are heavily dependent on glycolysis; damaged cells may also be.

The Role of the MPTP in Apoptotic and Necrotic Cell Death

Andrew Halestrap (Bristol, United Kingdom) continued the mitochondrial theme by recounting how the inner membrane is impermeable to all but a few ions. Toxins or hypoxia causes the inner mitochondrial membrane to become permeable to all solutes less than 1500 d, and triggered by a high Ca^{2+} in the matrix, mitochondria swell, leak, and become uncoupled.^{81–85} Sensitization to such damage occurs as a result of oxidative stress. CyP-D is the main target of CyA, which inhibits the MPTP; in CyP-D-knockout mice, there is impaired formation of the MPTP.⁸⁵ High Ca^{2+} overcomes the protection of CyA, sanglifehrin A and Cyp-D knockout.⁸³ The MPTP is inhibited by ADP, and the potency of different nucleotides to inhibit the MPTP is dependent on their affinity as substrates of the ANT.⁸¹ Nevertheless, mice with a double ANT knockout in their liver mitochondria still show a CyA-sensitive MPTP, although it is less sensitive to Ca^{2+} and is not blocked by ADP or activated by carboxyatractylide (a specific inhibitor of the ANT). There is also an interesting question in that it is unclear how the double ANT knockout can survive—there must presumably be another mechanism of mitochondrial ATP export.⁸⁵

Using the “hot-dog” technique for measuring MPTP opening, where the amount of [3H]-2-deoxy-D-glucose-6-P in mitochondria is used as an indicator of pore opening, ischemic preconditioning has been shown to inhibit MPTP opening.⁸² Preconditioning protection develops during the second round of ischemia, by decreasing Ca^{2+} -dependent swelling of mitochondria and attenuating mitochondrial protein oxidation, and may inhibit MPTP indirectly by decreasing oxidative stress. Because necrosis probably results from sustained MPTP opening, whereas apoptosis results from transient MPTP opening, preconditioning reduces the likelihood of necrosis.^{82,85}

Discussion of Dr Halestrap's presentation centered on the role of ANT4, which he commented was unclear, of CyP-D, which has an enzymic action, and fatty acids (FAs), which require ATP to be used, whereas deleterious effects of FAs may be attributable to direct effects on the MPTP. Fatty acids also may have direct uncoupling effects, which would be easy to test. Dr Halestrap noted that swelling via the MPTP leading to rupture is common to all cells.

Menadione-Induced Oxidative Stress Activates 2 Apoptotic Pathways in Pancreatic Acinar Cells

Oleg Gerasimenko (Liverpool, United Kingdom) presented recent work examining mechanisms of calcium signaling and apoptosis in pancreatic acinar cells subjected to a variety of stresses.^{86–90} Previous work had shown the oxidative stressor menadione to induce repetitive cytosolic Ca^{2+} spikes, partial mitochondrial depolarization, cytochrome c release, and apoptosis.⁸⁶ The induction of apoptosis was found to be dependent on opening of the MPTP, blocked by bongkrekic acid, and resulted in activation of caspases 9 (within 5 minutes) and 3 via the intrinsic pathway. Approximately 10% to 15% of the induction of apoptosis, however, was not prevented by specific inhibition of caspase 9, suggesting an alternative mechanism, investigation of which formed the body of the presentation.^{89,90} By selective use a range of fluorescent dyes, Ca^{2+} chelators, agents that destroy acid compartments (eg, lysosomes) and specific inhibitors, investigation showed that menadione also induces extrinsic activation of caspase 8 that is Ca^{2+} independent but relies on the release of cathepsins D and E from lysosomes, and occurs over a longer time course (30–40 minutes). When pancreatic acinar cells are exposed to menadione and caspase 9 is inhibited, activation and activity of caspase 8 are increased, suggesting a compensatory mechanism if the intrinsic apoptotic pathway fails to be activated under conditions of cell injury.⁹⁰ This may be particularly helpful to the exocrine pancreas where necrotic cell death is potentially lethal to the individual.⁸⁹

The discussion of Dr Gerasimenko's work focused on the possible uses of animals with genetic knockouts of caspase 8, cathepsin B, and cathepsin L to establish their contribution to the death mechanism. Dr Gerasimenko also commented that his findings were independent of changes in ADP or ATP; and that in the continuous presence of menadione, there was recovery of ψ_{M} , as measured by the TMRM probe. The possibility of arylation and/or thiol group formation by menadione was raised.

CELL DEATH PATHWAYS

Mechanisms and Consequences of Apoptosis

Rosario Rizzuto appraised the role of several molecules in cell death, notably Bcl-2, peroxisome proliferator-activated receptor γ coactivator (PGC)-1 α , PKC, Shc/p66, and IP_3R ,^{91–95} studying their effects on Ca^{2+} signaling and the likelihood of preventing or inducing cytosolic and/or mitochondrial Ca^{2+} overload, a key cell death trigger. After IP_3 production and Ca^{2+} release into the cytosol, mitochondria take up Ca^{2+} and mitochondrial metabolism is stimulated, a pattern that may be perturbed in various ways, and depends on close ER-mitochondrial and nuclear-mitochondrial interactions.^{91,92} Ca^{2+} overload and/or release of caspase cofactors from the mitochondria sensitize the cell to apoptosis. A somewhat rhetorical question was whether Bcl-2 is an ion channel because Bcl-2 reduces Ca^{2+} uptake into the ER, Ca^{2+} release from the ER into the cytosol, and Ca^{2+} uptake into mitochondria. The effects of Bcl-2 on Ca^{2+} flux, however, are independent of the putative MPTP, and likely to be mediated by direct regulation of the IP_3R Ca^{2+} channel. Key underlying questions concerning Bcl-2, Ca^{2+} , and apoptosis are how mitochondrial Ca^{2+} uptake is directly modulated by physiological and/or pathological conditions, how this might

contribute to apoptosis, what the exact role of Bcl-2 is and whether the anti-apoptotic actions of Bcl-2 are explained by its effects on Ca^{2+} signaling. The apoptotic agent ceramide was used to dissect out these effects, showing that when cytosolic Ca^{2+} signals and mitochondrial Ca^{2+} uptake were reduced by Bcl-2, apoptosis was rendered less likely.

Attention was then turned to the role of PGC-1 α in adaptive thermogenics, focused on mitochondrial effects.⁹⁴ Via adrenergic stimulation and cyclic AMP, PGC-1 α induces proteomic changes, including up-regulation of uncoupling proteins NRFs and mtTFA, as well as mitochondrial biogenesis, simultaneously reducing mitochondrial Ca^{2+} uptake. The importance of PGC-1 α is well shown in PGC-1 α -knockout mice, which have defective adaptive energy metabolism.

As alluded to earlier (see PKC, Alcohol, and Pancreatitis), PKC isoforms are important players in cellular responses to injury. Classical (α , β I, β II, γ), novel (δ , ϵ , η , τ), and atypical (ζ , λ) isoforms are variously produced in response to IP_3 and diacylglycerol second-messenger production, and inhibit mitochondrial Ca^{2+} entry (eg, α , β) and/or cytosolic Ca^{2+} release (eg, α), or increase mitochondrial Ca^{2+} entry (ζ). Among the actions of PKCs are those on p66^{Shc} (66 kd alternatively spliced isoform of the growth factor adapter Shc that is phosphorylated in response to oxidative stress), which accumulates within mitochondria, inducing increased mitochondrial ROS production that can initiate apoptosis.^{93,95} Of note, knockout of p66^{Shc} (–/–) extends life span, and the molecule has been proposed to be an integration point for signaling pathways that control longevity and cell death. Work with p66^{Shc}-transfected mouse embryonic fibroblast cells has shown that in response to oxidative stress (H_2O_2), PKC- β modifies Ca^{2+} homeostasis via p66^{Shc}, which as a result inhibits mitochondrial Ca^{2+} import. Despite the reduction in Ca^{2+} entry, mitochondrial import of p66^{Shc}, facilitated by propyl isomerase 1-dependent isomerization, increases intramitochondrial ROS production and induces opening of the MPTP.⁹⁵

The discussion of Dr Rizzuto's presentation raised questions about transport and localization of p66^{Shc} into the mitochondrial matrix. The only information available is that p66^{Shc} is distributed at a low level in all mitochondria. In addition, these mitochondrial studies provide another example of the sensitivity of ER-mitochondrial interactions because such small changes in ER Ca^{2+} activity can have profound effects on mitochondrial function and cell fate; and that different PKC isoforms regulate mitochondrial ψ_M in different ways.

Determinants of Death and Survival in Neurons

Pierluigi Nicotera (Leicester, United Kingdom) postulated that the same molecular actors that are responsible for cell death are also responsible for cell survival.^{96–100} Developing the theme, apoptosis can be seen as directly related to necrosis, as part of the same series of events. Thus, if during apoptosis the PMCA is cleaved or ψ_M and ATP supplies are impaired, necrosis results.^{96,99,100} Contributors to Ca^{2+} entry, induced by glutamate accumulation at synaptic or extrasynaptic terminals (which leads to neural excitotoxicity) include *N*-methyl-D-aspartate receptors, transient receptor potential cation channel, subfamily M, member 7, and acid-sensing ion channels.^{97,98} Deregulated Ca^{2+} homeostasis inducing cell death depends on “closed doors.” After *N*-methyl-D-aspartate receptor, transient receptor potential cation channel, subfamily M, member 7, acid-sensing ion

channels, and voltage-gated channels allow excessive Ca^{2+} entry, calpains are activated, cleaving the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and PMCA (closing the doors), resulting in prolonged, excessive $[\text{Ca}^{2+}]_i$.^{96,99,100} Differing mitochondrial affinity for Ca^{2+} and Ca^{2+} -buffering capacity may distinguish signals leading to normal function from those leading to cell death. Thus, in neurons, mitochondria at synaptic clefts have a higher affinity for Ca^{2+} than extrasynaptic mitochondria and may be more able to cope with Ca^{2+} signals, although it is not clear whether this results from complex signal differences rather than intrinsic differences between mitochondria in different subcellular locations.

The discussion of Dr Nicotera's presentation focused on the question of whether there are differences between mitochondria in different subcellular regions, particularly with respect to microdomains of NOS activation. This question remained unanswered. It was hypothesized, however, that differences may be related to the distance of mitochondria from the plasma membrane or to mitochondrial NO handling that occur at the receptor level.

Mechanisms Mediating Cell Death Responses in Pancreatitis

Anna Gukovskaya drew attention to the inverse correlation between apoptosis and necrosis in various models of pancreatitis, depending on species and severity.^{101–105} In the mouse, high levels of X-linked inhibitor of apoptosis protein are found that inhibit apoptosis, unlike in the rat.¹⁰⁵ When pancreatitis is mild, more apoptosis and less necrosis are found, whereas in severe pancreatitis, there is less apoptosis and more necrosis; a distinction can perhaps be drawn between Baccidental necrosis[(mediated by loss of ψ_M and/or loss of ATP production) and Bprogrammed necrosis[(mediated by death receptors and receptor-interacting protein). Because mitochondrial permeabilization is a key cell death mechanism, the mechanisms contributing to permeabilization of pancreatic acinar mitochondria require clarification—hyperstimulation, for example, induces early cytochrome *c* release and loss of ψ_M .^{102,103,105} Isolated pancreatic acinar mitochondrial experiments have shown that micromolar concentrations (up to 40 μM) of Ca^{2+} induce loss of ψ_M (change in TMRM fluorescence) proportional to the Ca^{2+} concentration, whereas cytochrome *c* release is maximal at 1 to 2 μM Ca^{2+} . At that Ca^{2+} concentration, bongkreikic acid or ADP restored ψ_M fully, CyA restored this partially. Reactive oxygen species, however, had no effect. The partial effect of CyA suggested that Ca^{2+} -induced depolarization is not mediated through the classical MPTP in pancreatic acinar mitochondria. Further experiments indicated that for cytochrome *c* release from pancreatic mitochondria, ψ_M must be maintained, as well as ROS generation—which is negatively regulated by depolarization. Superimposed Ca^{2+} entry will then induce either apoptosis or necrosis, depending on the severity of the stress to the cell, the loss of ψ_M , and the loss of ATP production.

The discussion of Dr Gukovskaya's presentation focused on the possibility that the MPTP could contribute to cytochrome *c* release caused by Ca^{2+} overload in pancreatic acinar cell mitochondria because bongkreikic acid blocked the Ca^{2+} -induced depolarization, and little mitochondrial swelling was seen on the scattergrams. In addition, the MPTP can open and close without being detectable in this experimental system.

Pancreatic Acinar Cell Necrosis

Robert Sutton (Liverpool, United Kingdom) gave the last presentation on causes, mechanisms, and effects of pancreatic acinar cell necrosis.^{106–110} Pancreatic necrosis makes a major contribution to the severity of clinical acute pancreatitis, its morbidity, and resulting mortality.^{107,110} In the face of a lack of biological explanations for premature digestive enzyme activation, acinar cell dysfunction, and cell death for more than a decade, the Ca^{2+} hypothesis¹⁰⁶ has gained more substance and thrown up many important leads.¹¹⁰ Thus, causes of pancreatitis (passage of gallstones through the ampulla of Vater resulting in bile entering the pancreatic duct; ethanol and its metabolites; hyperlipidemia; hyperstimulation; etc) induce excessive Ca^{2+} release from intracellular stores, that when sustained by continued Ca^{2+} entry, initiates cell damage and death.^{108,109} Globalization of elevated $[\text{Ca}^{2+}]_i$ beyond the perigranular mitochondrial firewall is a consistent feature, at least in part, the result of inhibition of ATP production and/or use. More than 2 decades ago, FAEEs were identified as nonoxidative metabolites of alcohol formed by the combination of ethanol with intracellular FAs through the action of synthase enzymes, present in high concentration within the pancreas, whereas their products, FAEEs, were found in high concentrations within the pancreata of individuals who were acutely intoxicated with ethanol at the time of death. Recent work has shown that FAEEs induce massive Ca^{2+} release via IP_3 receptors from ER Ca^{2+} stores, sustained by Ca^{2+} entry and resulting in mitochondrial depolarization, failure of ATP production, and cellular necrosis.¹⁰⁹ The FAEEs bind within mitochondria and undergo hydrolysis to release FAs and ethanol, which are subsequently oxidized. In fact, it is the FAs that are mostly responsible for impaired ATP production because inhibition of FAEE hydrolysis prevents mitochondrial impairment, whereas FAs themselves induce sustained global elevations of pancreatic acinar $[\text{Ca}^{2+}]_i$, mitochondrial impairment, and cellular necrosis, rescued by patch-pipette delivery of intracellular ATP.¹¹⁰ Thus, among many potential avenues of new prophylactic or therapeutic intervention for pancreatitis, either inhibition of Ca^{2+} release or inhibition of FAEE hydrolysis could find clinical application.

OVERVIEW OF AREAS FOR FUTURE RESEARCH

Organelle Dysfunction

The link between the initial cellular insult, its immediate effects, and subsequent events in subcellular organelles requires significant further research. Hypotheses should be developed for experimental testing with a high potential for clinical application. As an example, do the effects of secretory block lead to intracellular enzyme activation and/or mitochondrial death cascades? What are the mechanisms connecting these processes? Testable hypotheses relating the combination of high $[\text{Ca}^{2+}]_i$ and ROS generation to mitochondrial and cell death are very relevant. Many questions exist concerning mitochondria, including whether there is any role for mitochondrial genomic degradation, if there is a minimal MPTP sequence, and what the role of ANT, sensitivity factors (eg, VDAC), or protective factors (eg, PKC) might be. Particularly, productive hypotheses will be those designed to test mechanisms to prevent or reverse deleterious mitochondrial changes.

Disordered Acinar Secretion

Persistently high $[Ca^{2+}]_i$ destroys the normal cellular machinery caused in part by decreased cellular ATP, resulting in disassembly of normal cellular functions especially affecting zymogens and their secretion. The mechanisms of secretion are only partly understood. There should be significant effort to enhance our understanding of the molecular machinery of secretion, including soluble *N*-ethylmaleimide sensitive factor attachment receptors (SNARES), to better determine strategies to prevent and/or reverse the secretory blockade of pancreatitis.

Role of Ductal Secretion

Secretion from the pancreatic ductal tree may be important in pancreatitis, to wash away activated enzymes. Ductal secretion may be promoted by modification of ion transporters that are CFTR independent, but the relative importance of intraductal versus intraparenchymal enzyme activity is undetermined. In pancreatitis, disordered secretion includes basolateral exocytosis at the expense of apical secretion, so maintenance of normal acinar cell secretion may be equally important.

New Approaches

As well as new technologies, more in vivo data are required and recognition of the uses and limitations of appropriate models. Alongside the many sophisticated bioscience tools, more human data are needed, both in prophylactic and therapeutic scenarios. Significant impact on human acute pancreatitis may require cocktails of medications, so it is likely that many more clinical trials will be required.

Therapeutic Targets

At present, it would seem that if a specific SOC or CRAC were to be identified on the plasmalemma of the pancreatic acinar cell, Ca^{2+} entry might be prevented and so injury dependent on an elevated $[Ca^{2+}]_i$ could be prevented. It is appreciated that there are many other signaling mechanisms that can mediate injury alone or in combination with Ca^{2+} . There are several windows of opportunity that could alter the natural history of pancreatitis (prevent the disease entirely, modify the early course of severe acute pancreatitis, or prevent progression to chronic disease), which further research along these and other lines offers prospects of exploitation.

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FIGURE 1.

Ca^{2+} release mechanisms in the pancreatic acinar cell. **A**, Cross-sectional representation of Ca^{2+} pools, with the secretory pole to left (high sensitivity) where cytosolic Ca^{2+} signals begin, in response to the second messengers IP_3 , cyclic ADP ribose, or NAADP acting on their calcium channel receptors (IP_3R , RyR , and/or NAADPR). Experimental application (via patch pipette) of a single second messenger (IP_3 or RyR or NAADPR) elicits Ca^{2+} signals localized to the apical pole by the perigranular mitochondrial firewall. **B**, Combinations of second messengers evoke global cytosolic Ca^{2+} signals that spread beyond the mitochondrial firewall throughout the cell (into the low-sensitivity basolateral area), amplified by Ca^{2+} -induced Ca^{2+} release. Ca^{2+} -ATPase pumps clear Ca^{2+} from the cytosol back into the ER and out of the cell. Toxic Ca^{2+} release occurs when the cytosol is overloaded and Ca^{2+} is not cleared, for example, as a result of mitochondrial failure. **C**, Ca^{2+} release mechanisms in the high sensitivity apical pole, where ER, endolysosomal and ZG Ca^{2+} stores interact. IP_3 and cADPR elicit Ca^{2+} release from ER terminals and ZGs, whereas NAADP elicits Ca^{2+} release particularly from lysosomes, with amplification by Ca^{2+} -induced Ca^{2+} release. Different second messengers predominate after stimulation by different secretagogues, for example, IP_3 after ACh, NAADP after CCK.

FIGURE 2.

Maintenance and effects of toxic cytosolic Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) in pancreatic acinar cells. Toxic $[\text{Ca}^{2+}]_i$ builds up in response to cell insult from bile salts, nonoxidative ethanol metabolites, or FAs, which induce excessive release from intracellular stores and inhibit Ca^{2+} clearance mechanisms. Premature activation of trypsinogen and other digestive enzymes occurs, with basolateral secretion of some activated enzymes into the interstitial space, contributing to cell injury and pancreatitis. This sequence depends on continued Ca^{2+} entry into the cell, without which toxicity does not occur. Ductal secretion of HCO_3^- offers one means of protection, whereby clearance of activated enzymes may be hastened. GPR indicates G protein-linked receptors; CFTR, cystic fibrosis transmembrane conductance regulator; SLC26, solute carrier protein 26, ion transporters that interact to control pancreatic ductal $\text{Cl}^-/\text{HCO}_3^-$ exchange.

FIGURE 3.

Factors controlling cell death in response to pancreatic acinar cell stress. Sustained Ca^{2+} release induced by toxins results in mitochondrial impairment, which causes release of cytochrome *c*, more likely if initiator caspases are activated or ROS generation is excessive. Cytochrome *c* activates effector caspases, which are inhibited by NF- κ B, but in turn inhibit necrosis. With marked mitochondrial impairment, however, ATP production is insufficient to support apoptosis, and necrosis results, hastened through the activation of PARP. Activation of cathepsin B and trypsinogen in lysosomes and ZGs contributes to cell injury and deregulated necrotic cell death. PARP indicates poly ADP-ribose polymerase.