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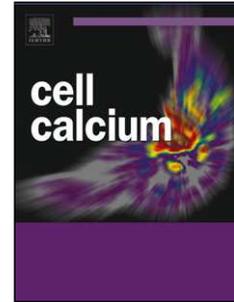
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## Accepted Manuscript

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## Ca<sup>2+</sup> signalling underlying pancreatitis

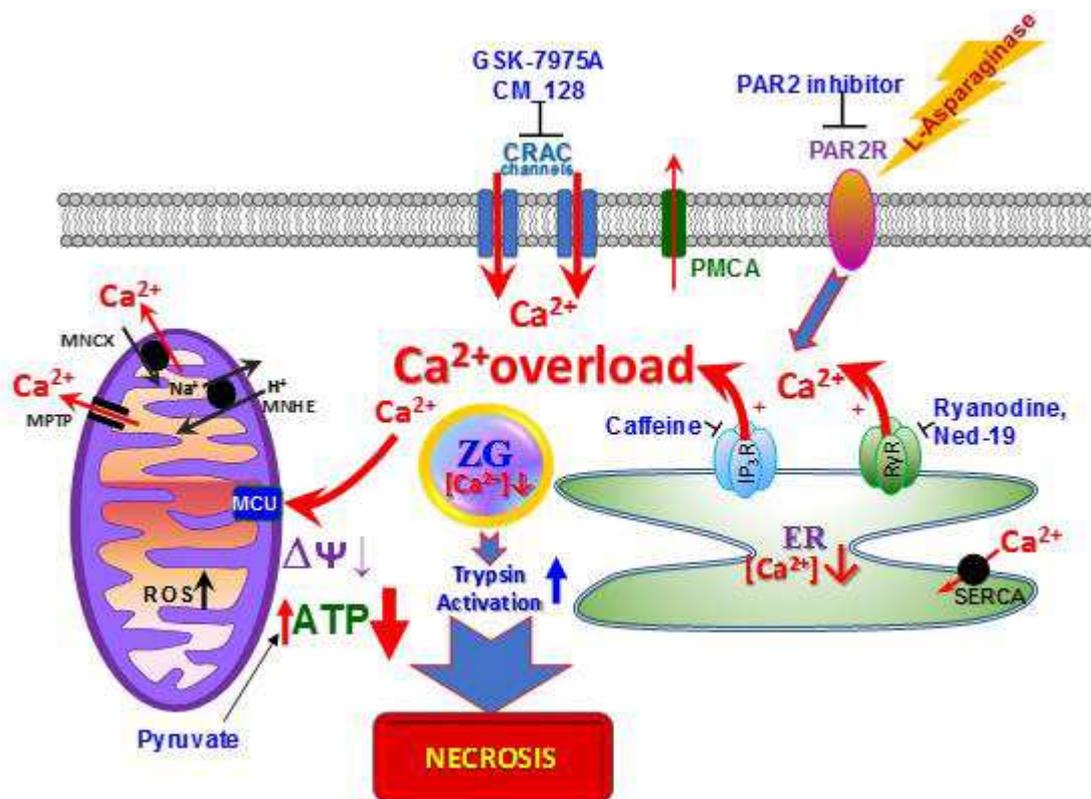
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### Graphical abstract



### Highlights

- Ca<sup>2+</sup> overload is responsible for initiation of pancreatic pathology in AP
- Mechanism of Asparaginase-induced AP is similar to alcohol- and bile- induced AP
- Pharmacological interventions in pancreatic stellate cells provide additional opportunities for developing of AP treatments

### Abstract

In spite of significant scientific progress in recent years, acute pancreatitis (AP) is still a dangerous and in up to 5% of cases deadly disease with no specific cure. It is self-resolved in the majority of cases, but could result in chronic pancreatitis (CP) and increased risk of pancreatic cancer (PC). One of the early events in AP is premature activation of digestive pro-enzymes, including trypsinogen, inside pancreatic acinar cells (PACs) due to an excessive rise in the cytosolic  $\text{Ca}^{2+}$  concentration, which is the result of  $\text{Ca}^{2+}$  release from internal stores followed by  $\text{Ca}^{2+}$  entry through the store operated  $\text{Ca}^{2+}$  channels in the plasma membrane. The leading causes of AP are high alcohol intake and biliary disease with gallstones obstruction leading to bile reflux into the pancreatic duct. Recently attention in this area of research turned to another cause of AP - Asparaginase based drugs – which have been used quite successfully in treatments of childhood acute lymphoblastic leukaemia (ALL). Unfortunately, Asparaginase is implicated in triggering AP in 5-10% of cases as a side effect of the anti-cancer therapy. The main features of Asparaginase-elicited AP (AAP) were found to be remarkably similar to AP induced by alcohol metabolites and bile acids. Several potential therapeutic avenues in counteracting AAP have been suggested and could also be useful for dealing with AP induced by other causes. Another interesting development in this field includes recent research related to pancreatic stellate cells (PSCs) that are much less studied in their natural environment but nevertheless critically involved in AP, CP and PC. This review will attempt to evaluate developments, approaches and potential therapies for AP and discuss links to other relevant diseases.

## Introduction

Acute pancreatitis (AP) is a disease usually caused by alcohol abuse or bile reflux due to gallstones. Other causes include some type of antibiotics, chemotherapy, infections, certain rare conditions and others [1]. One of the complications that can result from AP is chronic pancreatitis (CP) that significantly increases risk of pancreatic cancer [2-5]. AP is a severe disease and has a significant

mortality of about 5% [1, 3]. However, in severe cases, the mortality rate can rise to 30% [6], with significant pancreatic acinar cell (PAC) necrosis followed by a damaging inflammatory response. The leading causes of AP have been identified as gallstone biliary disease and high alcohol intake, while abnormality in calcium signalling in PACs was found to be one of the first events in the initiation of AP [3].

### **Physiological calcium signalling in PACs**

Calcium signalling plays a fundamental role in regulation of digestive enzymes and fluids secretion by the exocrine pancreas [7]. A range of endogenous stimuli such as the neurotransmitter acetylcholine (ACh) [8] produced by the vagal nerve endings and the hormone cholecystokinin (CCK) secreted by enteroendocrine I cells of the small intestine [9,10] serve as triggers in the activation of the calcium signalling machinery. Binding of ACh to the muscarinic receptor type 3, which is a G-protein-coupled receptor, results in activation of phospholipase C and production of the second messenger inositol trisphosphate (IP<sub>3</sub>) that induces Ca<sup>2+</sup> release from the internal stores through IP<sub>3</sub> receptors [11]. In fact, in the absence of IP<sub>3</sub> receptors or type 2 and 3 (double-knockout), carbachol-induced secretion is completely abolished in PACs [12]. Similarly, Ca<sup>2+</sup> release in PACs elicited by physiological doses of CCK, also in the human pancreas [13,14], is primarily mediated by nicotinic acid adenine dinucleotide phosphate (NAADP). In this case the action depends on functional ryanodine receptors (RyRs) and two-pore channels (TPCs) and also involves acid Ca<sup>2+</sup> stores [15,16,17,18]. Calcium response then further amplified by calcium induced calcium release (CICR) from the acid stores and the ER [17,18]. While the exact mechanism of the NAADP production and action remains elusive, in PACs NAADP-induced calcium responses are linked to both ER and acidic stores, and is highly depend on RyR type 1 and TPC type 2 [18]. A hypothesis that takes into account practically all published data suggests a mechanism involving 3 stores: an initial, virtually undetectable, NAADP-elicited Ca<sup>2+</sup> release via TPCs from endosomes/lysosomes triggers the detectable Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release via RyR1 and RyR3 occurring from the granules and the ER. Both pathways result in feeding of Ca<sup>2+</sup> to the cytosol and subsequent exocytosis of zymogen granule

content into the acinar lumen that, together with fluid supplemented with bicarbonate, is transported via the pancreatic duct system to the duodenum under physiological conditions [17,18].

### **Pathological calcium signalling in PACs**

In AP, the pancreatic proenzymes such as trypsinogen become prematurely activated intracellularly, resulting in the molecular cannibalism that digests pancreas and its surroundings [3,6]. There is overwhelming evidence, collected during last two decades, implicating abnormal cytosolic  $\text{Ca}^{2+}$  overload in the initiation and development of AP [3,19-23]. The most damaging are the sustained elevations in  $[\text{Ca}^{2+}]_i$ , from high concentrations of some secretagogues, as well as from ductal hypertension, alcohol, hypoxia, hypercalcaemia, hyperlipidaemia, viral infection, and various drugs—all factors known to precipitate acute pancreatitis [19, 24]. These factors cause either excessive release of acinar  $[\text{Ca}^{2+}]_i$ , or damage to the integrity of mechanisms that restore low resting levels of  $[\text{Ca}^{2+}]_i$ , and the consequent calcium toxicity become the key trigger of acute pancreatitis [19,20].

Aberrant calcium signalling, as the main initiation event in AP, has been proposed more than two decades ago and became the widely accepted mechanistic explanation [19,20,25]. Pathological stimuli, such as bile and alcohol, are capable of triggering massive  $\text{Ca}^{2+}$  release from intracellular stores through  $\text{IP}_3\text{Rs}$  and  $\text{RyRs}$  followed by excessive  $\text{Ca}^{2+}$  entry through  $\text{Ca}^{2+}$  release activated  $\text{Ca}^{2+}$  (CRAC) channels that are the most important mechanisms of  $\text{Ca}^{2+}$  overload in pancreatic acinar cells [3,26].

It has been shown that long lasting  $\text{Ca}^{2+}$  responses with a development of a sustained elevated cytosolic  $\text{Ca}^{2+}$  plateau component result in destabilisation of the secretory zymogen granules and conversion of them into empty looking vacuoles [27]. Intracellular vacuolisation [4], causes another calcium-dependent process, intracellular protease (trypsin) activation [5]. As a result, the inactive pancreatic pro-enzymes stored in zymogen granules (ZG) become active enzymes inside the PACs [21,27], inducing mitochondrial malfunction [25, 28-30], cell necrosis, digestion of pancreas and its surroundings [19-21].

AP is known to involve reactive oxygen (ROS) and reactive nitrogen (RNS) species, that together with calcium overload leads to abnormal mitochondrial  $\text{Ca}^{2+}$  uptake [25,30] and opening of the mitochondrial permeability transition pore (MPTP) [32,33] resulting in reduction of ATP production. The lack of ATP together with calcium overload and protease activation leads to acinar cells necrosis generating the damaging inflammatory response [5].

### **Asparaginase-induced pancreatitis**

Recently another form of AP has been studied in detail, a well-known complication of the treatment of childhood acute lymphoblastic leukaemia (ALL). The incidence of AP following childhood ALL treatment is between 7- 18% [34]. Whilst numerous anti-leukemic medications have been reported, the most important are based on Asparaginase [34]. The development of AP is one of the commonest causes for stopping Asparaginase treatment, because re-exposure is associated with recurrence of pancreatitis [35]. However, stopping the scheduled Asparaginase treatment because of previous pancreatitis has been linked to an increased relapse rate [36]. While significant progress has been made in characterizing the effects of alcohol and bile acids on pancreas [5,23,37,38], the Asparaginase-induced pancreatic pathology was largely unknown.

Findings presented recently [39] provide the first mechanistic insight into the process by which Asparaginase treatment of ALL may cause Asparaginase-induced AP (AAP). Pancreatic acinar cells can respond to a very low dose (0.1IU/ml, Fig. 2A) and in practically all cases to higher doses of Asparaginase (Fig.2B). The most accessible therapeutic target in Asparaginase-elicited toxicity is the  $\text{Ca}^{2+}$  release activated  $\text{Ca}^{2+}$  (CRAC) channel in pancreatic acinar cells (PACs) [23,40]. The Asparaginase-induced  $\text{Ca}^{2+}$  elevations (plateau) depend on CRAC channels and were markedly diminished by the inhibitor GSK-7975A [39]. Consequently, Asparaginase-induced necrosis was dramatically reduced by GSK-7975A to near control levels (Fig. 3A). The protective effects of CRAC channel inhibitors against alcohol-induced pancreatitis in isolated pancreatic acinar cells [23,26] and in pancreatic stellate cells [41,42] have been confirmed by *in vivo* studies [43]. Therefore, this approach is also likely to succeed against AAP [39] and the next step would be to test the effectiveness of CRAC channel blockade against AAP using an *in vivo* mouse model.

The AAP mechanism is apparently fundamentally different from the therapeutic action of Asparaginase on lymphoblastic cells in ALL [44]. The Asparaginase effect on cancer cells relies on depletion of asparagine, which the malignant cells cannot produce themselves, in contrast to normal cells [44], whereas the side-effect of Asparaginase, namely AAP, is owing to activation of a signal transduction mechanism involving PAR2 (Fig. 3A,C) but independent of asparagine [39]. Hence, several potential intervention points are available for treating the side effect of Asparaginase (Fig.3C). The key initiation site of Asparaginase action on PACs seems to be PAR2. This receptor has previously been implicated in AP, although its exact role is still debated [45,46]. Blocking PAR2 has inhibited both the pathological  $[Ca^{2+}]_i$  elevations and the Asparaginase-induced necrosis (Fig.3A) [39], suggesting that PAR2 inhibitors could be a useful tool to supplement Asparaginase ALL treatment in AAP cases. Both  $Ca^{2+}$  entry and extrusion were significantly affected by Asparaginase while sustained elevation of  $[Ca^{2+}]_i$  is responsible for the necrosis [39]. The simplest explanation for this is the reduction in the intracellular ATP level, limiting energy supply and, therefore, inhibiting plasma membrane  $Ca^{2+}$  ATPase [39]. Restoring energy supply by the addition of pyruvate (Fig.3A) provided a high degree of protection against pancreatic necrosis. The established mechanism of action of Asparaginase (Fig.3B) has been confirmed for several sources of Asparaginase, including the drug ELSPAR and PEG-Asparaginase and including Asparaginase from both *E Coli* and *Erwinia chrysanthemi* (Fig. 3B). These findings should allow the start of designing new treatments for AAP.

### **How to save PACs from $Ca^{2+}$ overload and necrosis?**

Interestingly, AAP and AP induced by other more common causes are not so much different [39]. Calcium overload, loss of ATP and massive necrosis are also the main features of AP induced by alcohol and bile. Therefore, current developments in AP field are undoubtedly applicable to AAP [39]. It has been shown previously that pharmacological inhibition of  $IP_3$ Rs in pancreatic acinar cells or knock out of  $IP_3$ Rs of type 2 and 3 in mice significantly diminished POAEE-elicited intracellular trypsin activity rise [37]. These data indicate that these intracellular channels are involved in critical steps of cytosolic  $Ca^{2+}$  overload, necrosis and AP progression. Attempts to protect acinar cells against

Ca<sup>2+</sup> overload by inhibition of Ca<sup>2+</sup> release from the internal stores are problematic due to the lack of specific inhibitors of IP<sub>3</sub>Rs and RyRs.

The intracellular Ca<sup>2+</sup> sensor calmodulin is known as a regulator of many intracellular targets including intracellular Ca<sup>2+</sup> receptor channels and this ability can be exploited to reduce Ca<sup>2+</sup> overload [38]. Calmodulin activator Ca<sup>2+</sup>-like peptides 3 (CALP-3) at a concentration of 100 μM was shown to be effective in regulation of alcohol-induced Ca<sup>2+</sup> release and cell necrosis [38] without affecting the physiologically relevant ACh and CCK-induced oscillations [38,47]. A much more potent analogue of CALP-3 was recently developed [48], which is effective at much lower concentrations starting from 0.1 μM and therefore, has a good potential for cytosolic Ca<sup>2+</sup> regulation.

Another recently published finding is based on the ability of high concentrations of caffeine to inhibit IP<sub>3</sub>Rs [49]; however, this development is still in its early stages. Alternatively, Bcl-2 family proteins have been shown to regulate Ca<sup>2+</sup> release via binding to the intracellular Ca<sup>2+</sup> channels, inhibiting Ca<sup>2+</sup> release and regulating Ca<sup>2+</sup> homeostasis. These Bcl-2 proteins therefore provide an additional therapeutic avenue in controlling intracellular Ca<sup>2+</sup> overload in acute pancreatitis. The BH4 domain of Bcl-2 was shown to inhibit IP<sub>3</sub>Rs and RyRs at low concentrations [50,51]. Inhibition of NAADP-induced signalling, e.g. Ned-19 is also worth considering particularly for bile acid induced AP [52].

While inhibition of Ca<sup>2+</sup> release is rather difficult to utilise and none of the directions mentioned above is in the clinical trial stage, inhibition of store operated Ca<sup>2+</sup> entry as a potential tool for AP therapy is much more developed [26, 43]. CRAC channel blockers GSK7579A (GlaxoSmithKline, UK) and CM\_128 (CalciMedica, US) have been remarkably successful in *in vitro* and *in vivo* models of AP, inhibiting all major disease hallmarks (Fig. 3C) [26,43]. Therefore, the developments of CRAC channel blockers might lead to a first specific therapy for AP.

Early observation that cells supplied with ATP can tolerate even high stress from AP-related pathologies [28], could eventually become an important part in future AP treatment. A systematic review by Mosztbacher et al 2017 [53] concluded that restoration of energy level can help in some forms of AP. While pyruvate is very effective for *in vitro* experiments in protecting acinar cells

against L-Asparaginase-triggered damage [39], a simple derivative of pyruvic acid, namely, ethyl pyruvate has been found useful for treatment of bile-induced AP in a rat model [54]. Ethyl pyruvate is also known as a ROS scavenger and, in addition, has other anti-inflammatory effects [55]. The role of ROS in AP remains controversial [30, 56,57, 58], while a recent study has shown an important contribution of nitric oxide [31] in pancreatic pathology, justifying the use of NO synthase inhibitors as a potential AP treatment [59,60]. In spite of a number of developments, some more advanced than others and it is possible that combination of drugs will have to be used eventually while their target might not be limited to the PACs.

### **Potential role of pancreatic stellate cells in AP**

PSC research has recently received increasing attention due to our growing understanding of this cell type's involvement in pancreatic health and disease [41,42,61,62].

The first discovery of PSCs 35 years ago, [63] was followed by the development of PSC culturing techniques in the 90's [64]. Recent pioneer studies using pancreatic lobules preparation have introduced new possibilities for investigating PSCs in their natural environment [41,42,61]. The advantage of lobule research is the unique opportunity to study the contribution and communication of different pancreatic cell types in response to physiological and pathological stimuli.

It has been generally accepted that under physiological conditions PCSs show characteristics of the normal quiescent state (qPSCs); however, their function in the pancreas is largely unknown. It has been suggested that qPSCs regulate the normal architecture of pancreatic tissue by regulating the synthesis and degradation of extracellular matrix (ECM) by the production of matrix metalloproteinase (MMPs) and tissue inhibitors of MMPs (TIMP) [65]. qPSCs can be recognised as small elongated cells located in the peri-acinar space with retinol/vitamin A-containing lipid droplets that can be visualized by intrinsic multiphoton fluorescence [41]. They also express a cytoskeletal protein desmin (Fig. 4A) and glial fibrillary acid protein (GFAP) [41,61].

During pancreatic injury, or culturing, qPSCs can be transformed to their activated state (aPSCs) that is highly implicated in fibrosis and desmoplastic reactions in chronic pancreatitis (CP) and pancreatic cancer, respectively [66-69].

However, our recently published data have disputed the accepted general concept of the quiescence of normal PSCs [41]. On the contrary, this cell type shows an extraordinary sensitivity to very small changes in the blood pressure-lowering nonapeptide bradykinin (BK) while PACs are completely insensitive to BK (Fig. 4B, C) [41].

BK was discovered for the first time in the late 40's and described as a plasma protein that induced a slow delayed muscle contraction in isolated guinea pig ileum (*brady* means *slow*; *kinein* means *to move, to stimulate* in Greek) [70,71]. Along with other kinins, BK belongs to a family of bioactive peptides produced from plasma protein kininogens by endogenous proteolytic enzymes kallikreins of kallikrein-kinin system (KKS) that controls many physiological and pathological functions. The BK normal plasma level was found within the range of 40-70 pM [72,73].

It has been shown previously that in pancreatic tissue kallikrein is mainly stored as the pro-enzyme pro-kallikrein in the acinar cell zymogen granules [74]. In acute pancreatitis, kallikrein would leak from damaged necrotic cells leading to a rise in BK production from the basal level in plasma to up to ~140 pM [72,73].

We have found that application of BK at concentrations from as low as 100 pM to a maximum effect at 1 nM reliably evoked cytosolic  $Ca^{2+}$  signals in PSCs by activating bradykinin type 2 receptors (B2 receptors) [41]. These data demonstrate that any increase in the BK plasma level above the normal range would induce a robust  $Ca^{2+}$  response in PSCs that has a direct link with acute pancreatitis development.

Recently published data have shown that BK-induced  $Ca^{2+}$  signals in PSCs do not evoke any change in  $[Ca^{2+}]_i$  in neighbouring PACs (Fig. 4B,C) [41]. There are, of course, possibilities for the involvement of alternative pathways. The beneficial effect of pharmacological blockade of the B2 receptor to suppress acute pancreatitis has been shown previously [73,75-78]. Using the model of isolated

pancreatic lobules, it was shown that B2 receptor blockade markedly reduced the level of acinar necrosis induced by major pancreatitis-inducing agents such as ethanol, FAEEs or bile acids [41]. However, the exact pathway(s) that link the functions of PACs and PSCs in pancreatic health and disease remains unclear.

### **Conclusion**

There are very promising developments in this area. Several approaches are now competing, but in the end, a combination of approaches might be needed to successfully treat AP. On the other hand, different treatments might be better suited for different causes of AP, rather than one for all. Further research in this area is necessary to utilise the findings and move to the development of potential drugs.

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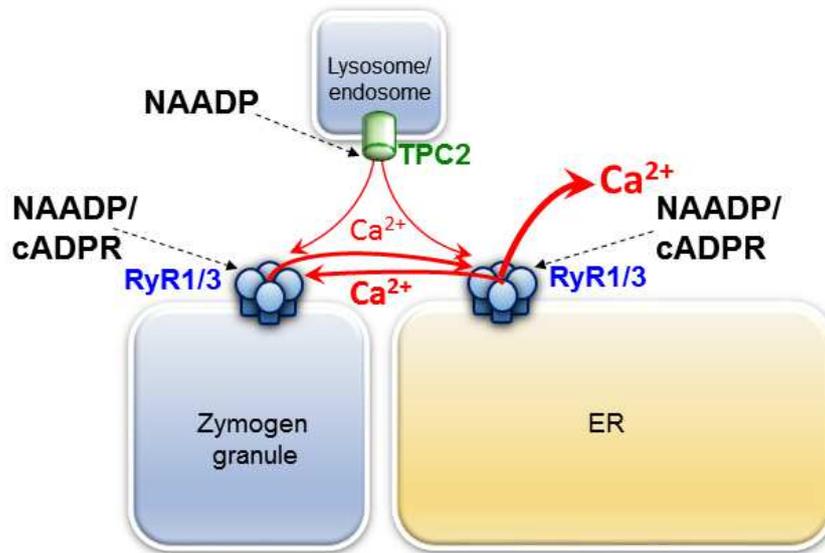
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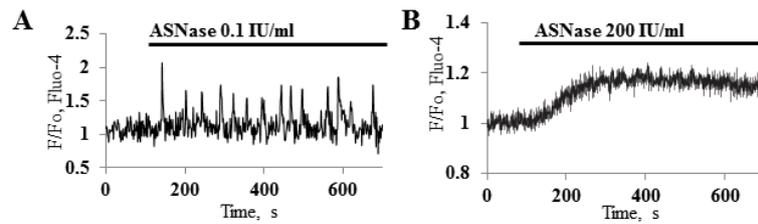
**Figure legends**

**Figure 1.** Model of the NAADP-induced  $\text{Ca}^{2+}$  release in pancreatic acinar cells. A very small primary  $\text{Ca}^{2+}$  release from an acid store (endosomes, lysosomes) being amplified very substantially by further  $\text{Ca}^{2+}$  release from both larger acid stores (zymogen granules) and the ER (Modified from [16]).



**Figure 2. Asparaginase induces calcium responses in PACs in dose-dependent manner. A.**

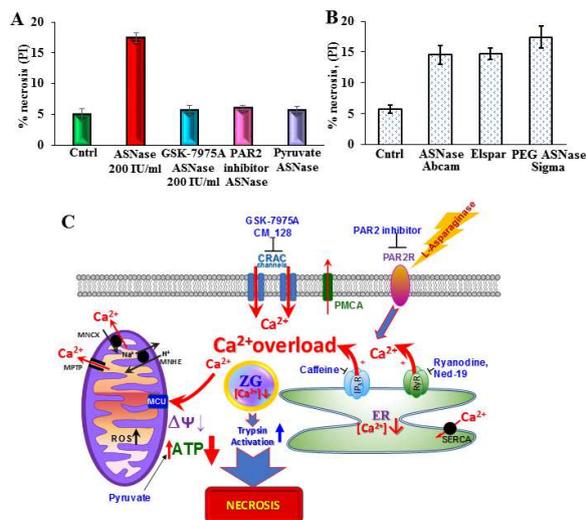
Representative trace of Asparaginase (0.1IU/ml)-induced calcium spikes in PACs. **B.** The average trace of 200IU/ml Asparaginase-induced cytosolic calcium plateau response. Traces were averaged and shown with error bars. Cells were loaded with Fluo-4 AM.



**Fig. 3. Asparaginase-induced necrosis can be inhibited using three different approaches. A.**

Asparaginase (200 IU/ml) induces a substantial level of necrosis in PACs. The CRAC channel inhibitor GSK-7975A (10  $\mu$ M) essentially abolished Asparaginase-induced necrosis to the control level ( $p>0.24$ ), but significantly ( $p<0.0001$ ) lower than that caused by 200 IU/ml of Asparaginase alone. PAR2 inhibitor (10  $\mu$ M FSLLRV-NH<sub>2</sub>) significantly blocked the Asparaginase-induced necrosis ( $p<0.0001$ ). Pyruvate (1 mM) also significantly ( $p<0.0001$ ) reduced the Asparaginase-induced necrosis to practically control level [34]. **B.** Asparaginase from different sources induce comparable level of necrosis. Abcam (number of tested cells  $n=1124$ ), Elspar ( $n=1708$ ) and PEGylated Asparaginase ( $n=1317$ ), all induce significant necrosis as compared to control ( $p<0.0001$  for all three sources). **C.** Schematic diagram illustrating the effects of Asparaginase on PACs.

Potential sites for therapeutic intervention (PAR2, calcium release, calcium entry, and ATP depletion) are also indicated.



**Figure 4. A.** The representative image of staining of pancreatic lobule with desmin antibody, detected by conjugated secondary antibody IgG-CruzFluor 594 (CFL594), overlaid with transmitted light image and with DAPI stained nuclei. **B.** Fluorescent image of freshly isolated mouse pancreatic cluster loaded with Fluo-4 in AM form. PSCs are shown as small elongated cells highlighted with red arrow; blue arrow is pointing to location of a neighbouring PAC. **C.** A representative cytosolic  $\text{Ca}^{2+}$  traces from PSC and PAC indicated in B: red trace from the PSC and blue trace from the PAC. Application of 1 nM BK results in a typical biphasic  $\text{Ca}^{2+}$  elevation in the PSC but not in the neighbouring PAC. Subsequent additions of 10  $\mu\text{M}$  ACh and 10  $\mu\text{M}$  CCh induce  $\text{Ca}^{2+}$  signals only in the PAC with no effect on PSCs. (Modified from [36]).

