Genetic and Pharmacologic Inhibition of the Ca^{2+} Influx Channel TRPC3 Protects Secretory Epithelia From Ca^{2+}-Dependent Toxicity

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BACKGROUND & AIMS: Excessive Ca^{2+} influx mediates many cytotoxic processes, including those associated with autoimmune inflammatory diseases such as acute pancreatitis and Sjögren syndrome. Transient receptor potential (canonical) channel (TRPC) 3 is a major Ca^{2+} influx channel in pancreatic and salivary gland cells. We investigated whether genetic or pharmacologic inhibition of TRPC3 protects pancreas and salivary glands from Ca^{2+}-dependent damage. **METHODS:** We developed a Ca^{2+}-dependent model of cell damage for salivary gland acini. Acute pancreatitis was induced by injection of cerulein into wild-type and *Trpc3*$^{-/-}$ mice. Mice were also given the Trpc3-selective inhibitor pyrazole 3 (Pyr3). **RESULTS:** Salivary glands and pancreas of *Trpc3*$^{-/-}$ mice were protected from Ca^{2+}-mediated cell toxicity. Analysis of Ca^{2+} signaling in wild-type and *Trpc3*$^{-/-}$ acini showed that Pyr3 is a highly specific inhibitor of Tprc3; it protected salivary glands and pancreas cells from Ca^{2+}-mediated toxicity by inhibiting the Trpc3-mediated component of Ca^{2+} influx. **CONCLUSIONS: TRPC3-mediated Ca^{2+} influx mediates damage to pancreas and salivary glands. Pharmacologic inhibition of TRPC3 with the highly selective TRPC3 inhibitor Pyr3 might be developed for treatment of patients with acute pancreatitis and Sjögren syndrome.**

Keywords: Ca^{2+} Influx; Inflammation; Cell Death; Therapeutics.

Calcium signaling regulates virtually all cell functions, and thus aberrant Ca^{2+} signaling is associated with many diseases. In particular, impaired Ca^{2+} signaling is the underlying cause in diseases that involve cell stress, endoplasmic reticulum (ER) stress, oxidative stress, and inflammation, which lead to autophagy and cell death.[1–4] Prominent gastrointestinal diseases associated with Ca^{2+} stress are acute pancreatitis[5,6] and Sjögren syndrome.[7] These are multifactorial diseases that are caused by generation of toxins within the pancreas and salivary glands in response to inflammatory insults. In the pancreas, this results in mistargeting of digestive enzymes to lysosomes that damage the pancreatic parenchyma.[8] In salivary glands, the inflammatory mediators, which include cytokines[9] and nitric oxides,[10] induce apoptotic and necrotic cell death,[11,12] which are associated with activation of Ca^{2+} signaling. In addition, patients with Sjögren syndrome express anti-M3 muscarinic receptors, which have a profound effect on the acute and chronic damage in salivary gland cells. Because so many key functions are regulated by Ca^{2+}, impaired Ca^{2+} signaling is intimately associated with acute pancreatitis[13] and likely salivary gland dysfunction.

The receptor-evoked Ca^{2+} signal involves Ca^{2+} release from the ER, which leads to activation of the store-operated Ca^{2+} influx channels (SOCs) at the plasma membrane.[14] ER Ca^{2+} is rapidly exhausted if it is not replenished by the SOCs, which also sustain the physiologic Ca^{2+} oscillations to determine their amplitude and frequency.[14,15] In fact, Ca^{2+} influx provides most of the Ca^{2+} that regulates exocytosis, fluid secretion, and gene regulation.[14,16–18] This scenario changes under cell stress, at which the cells are continuously overstimulated, resulting in a pathologic Ca^{2+} signal. The persistent strong stimulus depletes the ER Ca^{2+} store, resulting in an uncontrolled activation of the SOCs and a prolonged increase in free cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$)[6,13,14,19] that is responsible for the pathologic effects of Ca^{2+}.

The SOC in pancreatic and salivary gland cells is mediated by transient receptor potential (canonical) channel (TRPC) 1, TRPC3, and TRPC6[2,20] as well as the Orai channels.[21–23] The role of the native Orais in these cells has not yet been examined. The TRPC channels mediate part of the receptor-stimulated Ca^{2+} influx in many cells.[24–26] Indeed, deletion of Trpc1[27] and Trpc3[15] in mice strongly reduces receptor- and store-mediated Ca^{2+} influx in salivary glands and pancreatic acini.

Abbreviations used in this paper: $[Ca^{2+}]_i$, free cytoplasmic Ca^{2+}; ER, endoplasmic reticulum; G6PD, glucose 6-phosphate dehydrogenase; PERK, PKR-like endoplasmic reticulum kinase; Pyr3, pyrazole 3; SMG, submandibular glands; SOC, store-operated Ca^{2+} influx channel; TRPC, transient receptor potential (canonical) channel.

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In a recent work, we showed that part of the pathologic effect of excessive Ca^{2+} influx is mediated by the Trpc3,[15] with deletion of Trpc3 in mice reducing the severity of acute pancreatitis. These findings raised several important questions: Does Trpc3 have a role in the function of other secretory glands, like the salivary glands? Does excessive Ca^{2+} influx by Trpc3 contribute to Ca^{2+} toxicity in other secretory glands? Can pharmacologic inhibition of Trpc3 reduce the severity of Ca^{2+}-dependent cell stress and damage toward developing treatment for acute pancreatitis and Sjögren syndrome? To address these questions, we developed a cell model of Ca^{2+} toxicity in salivary glands based on intense and persistent stimulation of receptor-mediated Ca^{2+} signaling. We then used the salivary gland Ca^{2+} toxicity and acute pancreatitis models to show that Trpc3 has a prominent role in salivary gland function and dysfunction. Most notably, deletion of Trpc3 in mice and pharmacologic inhibition of Trpc3 by the selective Trpc3 inhibitor pyrazole 3 (Pyr3) similarly protected salivary glands and the pancreas from Ca^{2+}-mediated cell toxicity. These findings establish Ca^{2+} influx by Trpc3 in the function and pathogenesis of Ca^{2+} in secretory cells. Because Pyr3 given both short-term and long-term is well tolerated by mice with no known toxicity,[28] our findings describe a promising drug for the treatment of Ca^{2+} toxicity as observed in acute pancreatitis and salivary gland damage.

Materials and Methods

Detailed methods are given in Supplementary Materials and Methods. Only key methods are described in detail here.

Induction of Acute Pancreatitis in Mice

Mice fasted overnight were injected hourly in the abdominal cavity over 4 hours with cerulein (40 ng/g body wt), with or without Pyr3 (0.1 μg/g body wt), as indicated.[15] Collection and processing of blood, measurement of serum amylase levels, H&E staining, and evaluation of edema were by standard methods and are detailed in Supplementary Materials and Methods.

Determination of Intracellular Trypsin Activation and $[Ca^{2+}]_i$

Intracellular trypsin activity was measured using the cell permeate synthetic substrate rhodamine 110-$(CBZ\text{-}Ile\text{-}Pro\text{-}Arg)_2$,[8] and $[Ca^{2+}]_i$ was determined with the Ca^{2+}-sensitive dye Fura2, as described before[15] and in Supplementary Materials and Methods.

Western Blot

Protein levels were analyzed by standard Western blot analysis, as detailed in Supplementary Materials and Methods. Proteins were probed with a 1:500 dilution of phospho–PKR-like ER kinase (PERK) and 1:1000 dilutions of β-actin.

Immunohistocytochemistry

Tissue sections and acini were stained by incubation with anti-LC3 (1:100), α-amylase (1:100), LAMP2 (1:100), and ceramide (1:50) antibodies as detailed in Supplementary Materials and Methods.

Measurement of Whole Saliva Secretion

Before the experiment, the body weight of each mouse was measured. To collect the whole saliva, mice were anesthetized with a mixture of 75 mg/kg body wt ketamine and 1 mg/kg body wt dexmedetomidine. The mice underwent endotracheal intubation to maintain an open airway path. Salivation was stimulated by injection of pilocarpine (10 mg/kg body wt) intraperitoneally, and saliva was collected through a tube placed close to the duct exist into the oral cavity that was under minimal vacuum to ensure collection of all the saliva. Saliva was collected every 5 minutes for 35 minutes, and salivary secretion was determined by its weight. Salivation was normalized and presented as salivary weight per body weight. To determine the effect of Pyr3 on salivation, Pyr3 (0.1 μg/g body wt) was injected into the abdominal cavity for 1 hour before anesthesia and measurement of salivary secretion. After anesthetizing the mice, they were injected with a mixture of pilocarpine and Pyr3 to induce salivation.

Determination of Cytotoxicity

Freshly prepared acini from the pancreas and salivary glands were stimulated with the indicated agonist for 20 minutes at 37°C in a shaking water bath. The acini were spun down for 20 seconds at 3000*g*, and the supernatants were collected. Cell damage was assayed with the Vybrant Cytotoxicity Assay Kit (Molecular Probes, Eugene, OR) following the manufacturer's instructions. Briefly, glucose 6-phosphate dehydrogenase (G6PD) released by damaged cells was determined in each sample. Samples were incubated with a lyophilized mixture of diaphorase, glucose 6-phosphate, oxidized nicotinamide adenine dinucleotide phosphate, and resazurin in 0.5 mol/L Tris buffer (pH 7.5) for 15 minutes at 37°C. Resazurin fluorescence was measured at an excitation wavelength of 545 nm and emission wavelength of 590 nm. Fluorescence intensity in each sample was calculated as percentage of total fluorescence released by lysing the cell.

Cell damage was also assayed by determining accumulation of ceramide. Pancreatic and salivary gland acini stimulated with supramaximal agonist concentration were fixed by incubation with cold MeOH and processed for staining with anti-ceramide antibodies as described previously in this report.

Statistics

Results are expressed as mean ± SEM of the indicated number of observations obtained from the indicated number of independent experiments and mice. Statistical significance was determined by analysis of variance.