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SYMPOSIUM REVIEW

Calcium signalling in salivary gland physiology and dysfunction

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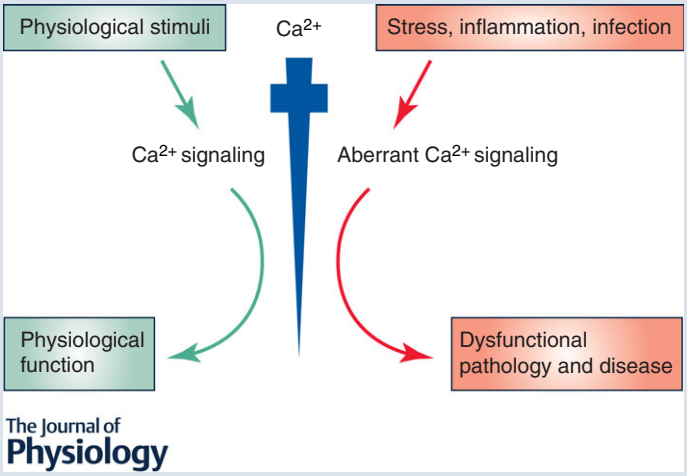

Abstract Studies over the past four decades have established that Ca^{2+} is a critical factor in control of salivary gland function and have led to identification of the critical components of this process. The major ion transport mechanisms and ion channels that are involved in fluid secretion have also been established. The key event in activation of fluid secretion is an increase in $[Ca^{2+}]_i$ triggered by inositol 1,4,5-trisphosphate (IP_3)-induced release of Ca^{2+} from ER via the IP_3 receptor (IP_3R). IP_3Rs determine the site of initiation and the pattern of the $[Ca^{2+}]_i$ signal in the cell. However, Ca^{2+} entry into the cell is required to sustain the elevation of $[Ca^{2+}]_i$ and fluid secretion and is mediated by the store-operated Ca^{2+} entry (SOCE) mechanism. Orai1, TRPC1, TRPC3 and STIM1 have been identified as critical components of SOCE in these cells. Cells finely tune the generation and amplification of $[Ca^{2+}]_i$ signals for regulation of cell function. An important emerging area is the concept that unregulated $[Ca^{2+}]_i$ signals in cells can directly cause cell damage, dysfunction and disease. Alternatively, aberrant $[Ca^{2+}]_i$ signals can also amplify and increase the rates of cell damage. Such defects in Ca^{2+} signalling have been described in salivary glands in conjunction with radiation-induced loss of salivary gland function as well as in the

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salivary defects associated with the autoimmune exocrinopathy Sjögren's syndrome. Such defects have been associated with altered function or expression of key Ca^{2+} signalling components, such as STIM proteins and TRP channels. These studies offer new avenues for examining the mechanisms underlying the disease and development of novel clinical targets and therapeutic strategies.

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Abstract figure legend Ca^{2+} signalling in cells is a double-edged sword: the tool kit that is involved in maintaining and regulating normal cell function can also be involved in pathophysiology. Normal physiological stimuli induce cytosolic Ca^{2+} signals by regulating the Ca^{2+} signalling toolkit, which includes various Ca^{2+} flux pathways as well as Ca^{2+}-regulated targets such as ion channels, enzymes and transcription factors. Under these conditions, Ca^{2+} homeostasis is maintained and cells display physiological responses. However, pathophysiological stimuli often generate unregulated and aberrant Ca^{2+} signals in cells due to modification of the same toolkit. This results in loss of Ca^{2+} homeostasis that can either cause or amplify the disease process.

Abbreviations $[Ca^{2+}]_i$, cytosolic $[Ca^{2+}]$; CaSR, calcium-sensing receptor; ER, endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol trisphosphate receptor; IR, irradiation; NFAT, nuclear factor of activated T cells; PIP_2, phosphatidylinositol 4,5-bisphosphate; PM, plasma membrane; pSS, primary Sjögren's syndrome; ROS, reactive oxygen species; SOCE, store-operated calcium entry; STIM1, stromal interacting molecule; TRP, transient receptor potential; TRPC, transient receptor potential canonical.

Introduction

Salivary glands secrete fluid and proteins in response to specific neurotransmitter-generated intracellular signals in acinar cells, which are the primary site of both types of secretion. Stimulation of β-adrenergic receptors leads to 3′,5′-cyclic adenosine monophosphate (cyclic AMP) generation, which activates exocytosis and protein secretion. In contrast, Ca^{2+} signals are induced in response to stimulation of plasma membrane (PM) receptors coupled to phospholipase C (PLC) activation and phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis, such as muscarinic cholinergic and α_1-adrenergic receptors, and result in activation of fluid secretion. The key trigger for stimulation of fluid secretion is an increase in cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$), which regulates ion channel activities that generate the appropriate osmotic gradient required to drive fluid secretion across the apical membrane (Melvin *et al.* 2005; Ambudkar, 2011, 2012). Physiologically, increase in $[Ca^{2+}]_i$ in salivary acinar cells is initiated in response to inositol 1,4,5, trisphosphate (IP_3)-mediated release of Ca^{2+} from the endoplasmic reticulum (ER) Ca^{2+} store(s) via the inositol trisphosphate receptor (IP_3R), a well characterized intracellular Ca^{2+}-release channel. The major subtypes of IP_3Rs found in salivary gland cells are IP_3R2 and -3, both of which are concentrated in the apical region of the acinar cells (Yule, 2001; Mikoshiba *et al.* 2008; Petersen & Tepikin, 2008). The apically localized IP_3Rs account for the initial increase in $[Ca^{2+}]_i$ detected in this region following stimulation. $[Ca^{2+}]_i$ then increases in a wave-like pattern from the apical to the basolateral areas of the cell (Yule, 2001; Melvin *et al.* 2005). This global increase in $[Ca^{2+}]_i$ is essential for the coordinated regulation of various ion channels and transporters located in the apical and basolateral membrane regions of the cell (Melvin *et al.* 2005; Ambudkar, 2011, 2012).

The critical contribution of extracellular Ca^{2+} and Ca^{2+} entry in the regulation of fluid secretion was established more than a decade ago (Putney, 1986; Lee *et al.* 1997; Melvin *et al.* 2005; Ambudkar, 2011, 2012, 2014). IP_3-mediated Ca^{2+} release via IP_3R, and resulting depletion of ER Ca^{2+}, is the main triggering event in the activation of Ca^{2+} entry, which is mediated by the store-operated Ca^{2+} entry (SOCE) mechanism (Putney, 1990). SOCE is achieved by the gating of plasma membrane Ca^{2+} channels, e.g. Orai1 and TRPC1, by STIM1, a Ca^{2+} binding protein that functions as the ER $[Ca^{2+}]$ sensor (Liou *et al.* 2005; Zhang *et al.* 2005; Zeng *et al.* 2008; Yuan *et al.* 2009; Hogan *et al.* 2010; Cheng *et al.* 2013; Prakriya, 2013). TRPC1 is an essential channel for salivary gland function, and lack of the channel results in significant loss of fluid secretion and SOCE (Liu *et al.* 2000, 2007; Hong *et al.* 2011). While Orai1 has been extensively studied, its exact role in salivary gland function is not yet known. One possible function of Orai1 in the gland could be to regulate TRPC1 function since studies with salivary gland cell lines have demonstrated that TRPC1 function is

completely dependent on Orai1 (Cheng *et al.* 2008, 2011; Lee *et al.* 2010; Choi *et al.* 2014).

$[Ca^{2+}]_i$ signals are a double-edged sword: while $[Ca^{2+}]_i$ increases are essential for the regulation of cell function, maintenance of Ca^{2+} homeostasis both in resting and stimulated cells is absolutely critical for cell survival. Unregulated increases in cytosolic Ca^{2+}, due to release from either ER or other intracellular stores, or due to Ca^{2+} entry, can be extremely deleterious to cells (Berridge, 2012). Deficits in, or aberrant, Ca^{2+} signalling have been associated with cellular and functional damage in a number of cell types, including neuronal cells and lymphocytes. Two major conditions result in loss of salivary gland function and tissue damage, although loss of fluid secretion does not always correlate with loss of glandular tissue. The first condition is radiation-induced xerostomia, or dry mouth condition, which occurs in patients who undergo radiation therapy for head-and-neck cancers that results in irreversible loss of salivary fluid secretion. This condition has been reproduced in several animal models, such as mouse, rats, mini-pigs and non-human primates. Interestingly, irradiation (IR) induces considerable loss of saliva flow in the absence of extensive tissue damage or loss of acinar cells. Fibrosis and loss of tissue occur subsequent to the functional loss, and the onset and severity of this phase of cellular damage differs among the various species. Irrespective of the progression of fibrosis, loss of salivary fluid secretion leads to xerostomia and associated complications such as difficulty swallowing, rampant dental caries, oral mucosal lesions and fungal infections that together severely affect the quality of life for the patients (Vissink *et al.* 2003; Delli *et al.* 2014). Thus, the mechanism involved in IR-induced loss of salivary gland function is a subject of great interest in the field, with clinical studies being directed towards assessing therapies targeted to recovery of cell function, prevention of functional loss, or regrowth of salivary glands.

A second condition associated with progressive loss of salivary gland function is primary Sjögren's syndrome (pSS), a chronic autoimmune disease involving lymphocytic infiltration and loss of secretory function in salivary and lacrimal glands (Delaleu *et al.* 2005). The pathogenesis of this disease has not yet been elucidated although viral, hormonal, environmental and neuronal factors have been implicated (Hansen *et al.* 2005; Nikolov & Illei, 2009; Mavragani & Moutsopoulos, 2014). A major question which remains to be addressed is the poor correlation between salivary flow and extent of inflammation or tissue damage (Fox & Stern, 2002; Nikolov & Illei, 2009). It has been reported that a large number of patients have low levels of inflammation within their salivary glands and yet display substantial loss of function. The molecular alterations underlying this secretory defect are not yet known.

In this review we will summarize the current knowledge regarding the role of Ca^{2+} signalling in salivary gland function, with discussion of animal models that display defects in fluid secretion. Further, we will discuss some current studies that describe the involvement of Ca^{2+} signalling in salivary gland dysfunction.

Ca^{2+} signalling in salivary gland function

Generation of cytosolic Ca^{2+} signals. In resting cells $[Ca^{2+}]_i$ is maintained at around 50–100 nM, which is lower than the threshold required to activate the ion flux systems that drive fluid secretion (Melvin *et al.* 2005; Yang *et al.* 2008; Ambudkar, 2014). Following stimulation, the increase in $[Ca^{2+}]_i$ activates the K^+ and Cl^- channels as well as the Na^+–K^+–$2Cl^-$ cotransporter and Na^+/H^+ and Cl^-/HCO_3^- exchangers (Manganel & Turner, 1990; Nguyen *et al.* 2004). Together, the concerted activities of these ion-flux mechanisms result in transepithelial flux of Cl^-. Importantly, the rise in $[Ca^{2+}]_i$ also triggers recruitment of aquaporin AQP5 water channels into the apical plasma membrane, thus substantially increasing fluid secretion (Ishikawa *et al.* 1998). The key molecular determinants of $[Ca^{2+}]_i$ signal generation include the G-protein-coupled receptor-signalling complex and the IP_3Rs (Ambudkar, 2012, 2014). Muscarinic–cholinergic receptors (M1/M3) are major receptors involved in the regulation of salivary gland fluid secretion (Proctor, 2006; Proctor & Carpenter, 2007) as shown by the almost complete loss of fluid secretion in mice lacking both receptors. In contrast, $M3^{-/-}$ mice have a secretory defect only at low pilocarpine levels and exhibit problems with chewing and ingesting dry food (Gautam *et al.* 2004; Nakamura *et al.* 2004). Consistent with this, carbachol-induced $[Ca^{2+}]_i$ increase was attenuated in submandibular gland cells from mice lacking M3 receptors and absent in those lacking both M1 and M3 receptors.

One of the best characterized Ca^{2+} channels is the IP_3R in the ER (Mikoshiba, 2007; Mikoshiba *et al.* 2008). IP_3R is regulated by both IP_3 and Ca^{2+}. Ca^{2+} regulation of IP_3R function displays a bell-shaped curve with stimulation at low and inhibition at high $[Ca^{2+}]$. Furthermore, an increase in IP_3 levels increases the $[Ca^{2+}]$ sensitivity of IP_3R. This exquisite regulation of IP_3R by IP_3 and $[Ca^{2+}]_i$ ensures that the channel is relatively more active when $[Ca^{2+}]_i$ is low and less active when $[Ca^{2+}]_i$ is high, thus protecting ER Ca^{2+} stores and regulating $[Ca^{2+}]_i$ within the physiological range required for the cell function (Mikoshiba, 2007). This feed-forward and feedback regulation of IP_3R can also explain the spread of the Ca^{2+} wave from the apical to the basal membrane in salivary gland cells (Lee *et al.* 1997; Yule, 2001; Melvin *et al.* 2005). Two major isoforms, IP_3R2 and IP_3R3, are

found in salivary gland acinar cells (Yule, 2001; Melvin *et al.* 2005; Mikoshiba *et al.* 2008), and mice lacking either subtype display decreases in $[Ca^{2+}]_i$ mobilization, while those lacking both receptors demonstrate complete loss of agonist-stimulated intracellular Ca^{2+} release as well as fluid secretion (Futatsugi *et al.* 2005). More importantly, these data suggest that in the absence of IP_3Rs there is no significant level of Ca^{2+} entry that can support elevation of $[Ca^{2+}]_i$ or fluid secretion. Thus, the Ca^{2+} entry mechanisms activated directly by PIP_2 hydrolysis appear to be minimal in these cells. Several cellular factors and proteins modulate IP_3R function including ATP, cAMP, RACK1 and the IP_3R-binding protein known as inositol 1,4,5-trisphosphate receptor-binding protein (IRBIT) (Ahuja *et al.* 2014) and contribute to modification of $[Ca^{2+}]_i$ increase as well as secretion (Ambudkar, 2011; Ando *et al.* 2014). cAMP-dependent phosphorylation of the IP_3R increases the sensitivity of the receptors for $[IP_3]$. One underlying mechanism that has been revealed is that cAMP-dependent phosphorylation of IP_3R induces release of IRBIT from IP_3R, which not only increases IP_3R function but also allows IRBIT-dependent activation of ion transport in the plasma membrane. Thus the cross talk between cAMP and IP_3R has important physiological implications as typically both cAMP and Ca^{2+} signalling can be simultaneously activated in acinar cells (Park *et al.* 2013; Ahuja *et al.* 2014).

Mechanisms and physiological relevance of store-operated Ca^{2+} influx. As noted above early studies established that Ca^{2+} influx is the primary determinant of sustained fluid secretion from salivary acinar cells (Ambudkar, 2014). It is now widely accepted that the primary mode of Ca^{2+} entry in acinar cells that is required for fluid secretion is mediated by SOCE. Physiologically SOCE can be activated by any stimulus that leads to depletion of ER Ca^{2+}, typically via IP_3-induced activation of IP_3R (Putney, 1997; Parekh & Putney, 2005). SOCE is a ubiquitous mode of Ca^{2+} influx that in addition to regulation of salivary gland fluid secretion is required for key physiological functions in other cell types: protein secretion in pancreatic acinar cells, platelet aggregation, endothelial cell permeability and migration, cell proliferation, T-lymphocyte activation and mast cell degranulation among others (Parekh & Putney, 2005; Ong *et al.* 2014).

The main molecular components involved in SOCE in salivary gland acinar cells have now been identified. Members of the transient receptor potential canonical (TRPC) family, TRPC1 and TRPC3, contribute to SOCE in dispersed acinar cell preparations as well as salivary gland cell lines (Ambudkar, 2014; Hong *et al.* 2014; Sun *et al.* 2015). These two TRPC channels generate relatively non-selective, Ca^{2+}-permeable channels that are activated downstream from receptor-stimulated PIP_2 hydrolysis. Mice lacking TRPC1 or TRPC3 show reduced SOCE and fluid secretion (Liu *et al.* 2007; Kim *et al.* 2009). Interestingly, the contribution of TRPC3 to SOCE is dependent on the presence of TRPC1 as $TRPC1^{-/-}$ mice do not display TRPC3-dependent SOCE (Lee *et al.* 2014). Thus, it has been proposed that either the channels are assembled as a store-operated heteromeric channel or that TRPC1 is required for store-dependent regulation of TRPC3. Indeed, TRPC3–TRPC1 interaction is necessary for STIM1 regulation of the channels in salivary gland ductal cells. The two TRPC channels coimmunoprecipitate following cell stimulation together with STIM1. Loss of TRPC1 eliminates the association of STIM1 with TRPC3 (Yuan *et al.* 2007; Lee *et al.* 2014). The role of TRPC3 in SOCE-independent regulation of salivary gland function has not yet been identified.

STIM1 and Orai have been identified as essential components of SOCE (Hogan *et al.* 2010; Cheng *et al.* 2013; Prakriya, 2013). STIM1, an ER Ca^{2+}-binding protein, responds to reductions in ER $[Ca^{2+}]$ and undergoes substantial conformational changes that result in multimerization of the protein, extension of its C-terminal domain, and translocation to the periphery of the cells where it clusters at specific ER–PM junctions. At these locations, STIM1 interacts with and activates Orai1 and TRPC1 (Wu *et al.* 2006; Liou *et al.* 2007; Luik *et al.* 2008; Muik *et al.* 2011; Zhou *et al.* 2013). While the SOAR domain of STIM1 activates Orai1, the C-terminal polybasic residues $^{639}KK^{640}$ of STIM1 are involved in activation of TRPC1 (Zeng *et al.* 2008; Yuan *et al.* 2009). Orai1 has been conclusively established as the main pore-forming component of store-operated calcium release activated Ca^{2+} (CRAC) channels, although its role in salivary gland function is yet to be determined (Prakriya *et al.* 2006, 2013). Orai1 function has been studied in two other exocrine glands, lacrimal and pancreatic. $Orai1^{-/-}$ mice display loss of lacrimal gland function and reduced SOCE in lacrimal gland acinar cells, another exocrine gland cell type (Xing *et al.* 2014). Further, knockdown of Orai1 in isolated pancreatic ductal cells also resulted in loss of SOCE and Ca^{2+}-activated ion channel activity similar to that seen in $TRPC1^{-/-}$ cells (Hong *et al.* 2011). This suggests that TRPC1 is unlikely to function in the absence of Orai1 and, further, Orai1 cannot compensate for the lack of TRPC1.

Detailed studies on the role of TRPC1, Orai1 and STIM proteins have been carried out in salivary gland cells lines, e.g. human salivary gland adenocarcinoma HSG cells. Knockdown of either Orai1 or STIM1 leads to a complete loss of SOCE while knockdown of TRPC1 induces a $> 50\%$ decrease in SOCE (Ong *et al.* 2007*a*; Cheng *et al.* 2008, 2011). These findings suggest that although TRPC1 is gated by STIM1, its function, and activation subsequent to ER-Ca^{2+} store depletion, is

dependent on Orai1. The critical mechanism underlying the functional interaction between TRPC1 and Orai1 was shown by a study (Cheng *et al.* 2011) demonstrating that Orai1-mediated Ca^{2+} entry triggers recruitment of TRPC1 to the plasma membrane. Thus, TRPC1 and Orai1 form separate channels that are activated by STIM1 following neurotransmitter simulation of salivary gland cells and contribute to the $[Ca^{2+}]_i$ increase seen in stimulated cells. Orai1 is the first channel to be activated while recruitment and activation of TRPC1 leads to amplification and modulation of $[Ca^{2+}]_i$ due to Orai1 function.

STIM2 is a second ER-localized Ca^{2+}-sensor protein that has been associated with SOCE and Ca^{2+} signalling, and it shares considerably sequence homology with STIM1. However, the Ca^{2+} sensitivity and activation kinetics of STIM2 differ from those of STIM1 (Stathopulos & Ikura, 2013). STIM2 has a relatively low affinity for Ca^{2+} and thus can detect small depletions of ER $[Ca^{2+}]$. STIM1 on the other hand, with a higher Ca^{2+}-affinity, responds only to substantial ER Ca^{2+} depletion. Like STIM1, STIM2 clusters and translocates to form puncta in ER–PM junctions, where it clusters with Orai1 and STIM1 (Brandman *et al.* 2007; Darbellay *et al.* 2010; Wang *et al.* 2014; Ong & Ambudkar, 2015). However, STIM2 is a poor activator of Orai1 and SOCE. The distinct responses of STIM1 and STIM2 to ER $[Ca^{2+}]$ are physiologically relevant since in the levels of stimulus exerted on cells *in vivo* tend to be low and induce less depletion of ER Ca^{2+} (Ong *et al.* 2007*b*). Evidence for a novel role of STIM2 in SOCE has been provided by a recent study that shows that STIM2 promotes STIM1 clustering in ER–PM junctions under conditions when there is less depletion of ER Ca^{2+} (Ong *et al.* 2015). This enhances assembly of the Orai1–STIM1 complex and activation of SOCE despite inability of STIM1 to detect the small change in ER $[Ca^{2+}]$. Knockdown of STIM2 in cell lines induced about 10-fold increase in the agonist concentration required for half-maximal activation of SOCE. Importantly, targeted knockout of STIM2 within salivary glands of $STIM2^{fl/fl}$ mice resulted in a reduction of fluid secretion at relatively low stimulus intensities, and acinar cells isolated from the glands display reduced $[Ca^{2+}]_i$ increases following stimulation with muscarinic receptor agonists. Together the findings suggest that STIM2 recruits STIM1 and facilitates formation of STIM1 puncta in ER–PM junctions at low stimulus intensities. This increases the agonist sensitivity of assembly of the STIM1–Orai1 channel complex and SOCE activation. (Ong *et al.* 2015). These findings demonstrate a critical role for STIM2 in regulating SOCE-mediated Ca^{2+} signalling and cell function. However, other studies indicate that involvement of STIM2 in SOCE might vary (Bird *et al.* 2009; Kar *et al.* 2012). Bird *et al.* have previously suggested that the oscillatory pattern of SOCE is primarily dependent on the mobilization of STIM1, not STIM2. Another study by Kar *et al.* showed that stimulation of cysteinyl leukotriene type I receptor in mast cells leads to mobilization of STIM1, while that of the high-affinity IgE receptor (FCεRI) involves mobilization of both STIM2 and STIM1.

Ca^{2+} signals generated due to Ca^{2+} entry via TRPC1 or Orai1 are decoded by cells for the regulation of distinct Ca^{2+}-dependent functions. In cells lines, TRPC1-mediated Ca^{2+} regulates nuclear factor-κB (NFκB), K_{Ca} and transmembrane member 16A (TMEM16A) activities. Studies with $TRPC1^{-/-}$ mice also suggest that the residual Orai1 in $TRPC1^{-/-}$ salivary gland acinar cells cannot sustain the activation of the ion channel mechanisms required for salivary secretion; e.g. K_{Ca} (Liu *et al.* 2007; Cheng *et al.* 2011). On the other hand, Orai1 function is sufficient for supporting nuclear factor of activated T cells (NFAT) activation, which is unaffected by knockdown of TRPC1, in salivary gland cell lines. Interestingly, the channels generate distinct patterns of Ca^{2+} signals in salivary gland cells lines, which might underlie the differential regulation of cell function (Ong *et al.* 2012). The exact nature of the Ca^{2+} signals generated by these two channels in salivary gland acinar cells and the Ca^{2+} sensors and effector proteins in the vicinity of the channels that determine the specificity of functional regulation remain to be elucidated (Fig. 1).

Aberrant $[Ca^{2+}]_i$ signals and salivary gland dysfunction

Reactive oxygen species and Ca^{2+} in irradiation-induced loss of salivary gland function. A debilitating side-effect of IR in head and neck cancer patients is xerostomia, or dry mouth, caused by irreversible loss of salivary gland function due to bystander effects of the treatment. The exact mechanisms underlying the persistent loss of salivary gland function are not yet fully understood, although it has been suggested that IR might damage progenitor cells within the adult salivary gland leading to compromised regeneration of acinar cells (Stephens *et al.* 1991; Vissink *et al.* 1992; Konings *et al.* 2005; Asari *et al.* 2009). However, significant decrease in salivary fluid secretion is detected before onset of overt damage, or loss, of the gland. In mice glandular loss and fibrosis are not seen until 4 months after radiation while loss of function is almost immediate and persists even after the reactive oxygen species (ROS) has been cleared from the system. Importantly, there are no adequate therapies to protect against, or reverse, IR-induced salivary hypofunction.

Radiation can induce DNA damage and membrane damage by generating intracellular ROS that can lead to long-term irreversible salivary gland dysfunction. However, a recent study has implicated the involvement of Ca^{2+} signalling in radiation-induced loss of salivary fluid secretion. While some calcium channels, including some

transient receptor potential (TRP) channel members, are modulated by ROS due to protein modification, transient receptor potential melastatin-like 2 (TRPM2), a Ca^{2+}-permeable non-selective cation channel, is activated by metabolites generated as a direct consequence of ROS production in the cell (Di *et al.* 2012). H_2O_2 and other agents or conditions that produce ROS (Sumoza-Toledo & Penner, 2011) trigger activation of poly-ADP ribose polymerase (PARP) resulting in generation of adenosine diphosphate ribose (ADPR), which is the intracellular ligand that binds to and gates TRPM2. Thus, TRPM2 serves as an endogenous ROS sensor. A recent study showed that TRPM2 is present in salivary gland cell lines and acinar cells and is activated in response to IR, leading to enhancement of Ca^{2+} entry in salivary gland cells (Liu *et al.* 2013). In a variety of cell types, Ca^{2+} entry via TRPM2 has been associated with increased cell death and apoptosis. Further, TRPM2 activity also leads to cytokine production in lymphocytes and other haematopoetic cells (Takahashi *et al.* 2011). While the consequence of TRPM2-mediated Ca^{2+} entry in acinar cells is still not known, unlike the persistent loss of saliva flow following IR of $TRPM2^{+/+}$ mice, $TRPM2^{-/-}$ mice show a transient loss of salivary gland function which recovers to $>$ 60% of the normal saliva flow within a month after treatment (Liu *et al.* 2013). Notably, treatment of WT mice with the PARP inhibitor 3AB or ROS scavenger Tempol induced significant protection of salivary gland function after IR. Together, these findings provide evidence that TRPM2 contributes to irreversible IR-induced salivary gland dysfunction. This study also showed that while acini from untreated mice undergo robust decrease in cell volume upon stimulation with agonist (a process associated with secretion of fluid from the cells), this volume decrease is attenuated in cells from irradiated mice. Importantly, $TRPM2^{-/-}$ acinar cells, which are protected from IR-induced loss of the secretory function, do not display deficits in cell volume decrease. These findings suggest that IR results in an inherent defect at the level of acinar cell function that can account for the loss of fluid secretion. What is significant is that this defect is associated with the presence and activation of TRPM2. Future studies should be directed towards delineating the intracellular targets of TRPM2-mediated Ca^{2+} influx that can account for loss of fluid secretion. Possible factors are activation or inactivation of transcription factors, disruption of cell metabolism, altered Ca^{2+} signalling or ion channel or transport functions required for saliva flow.

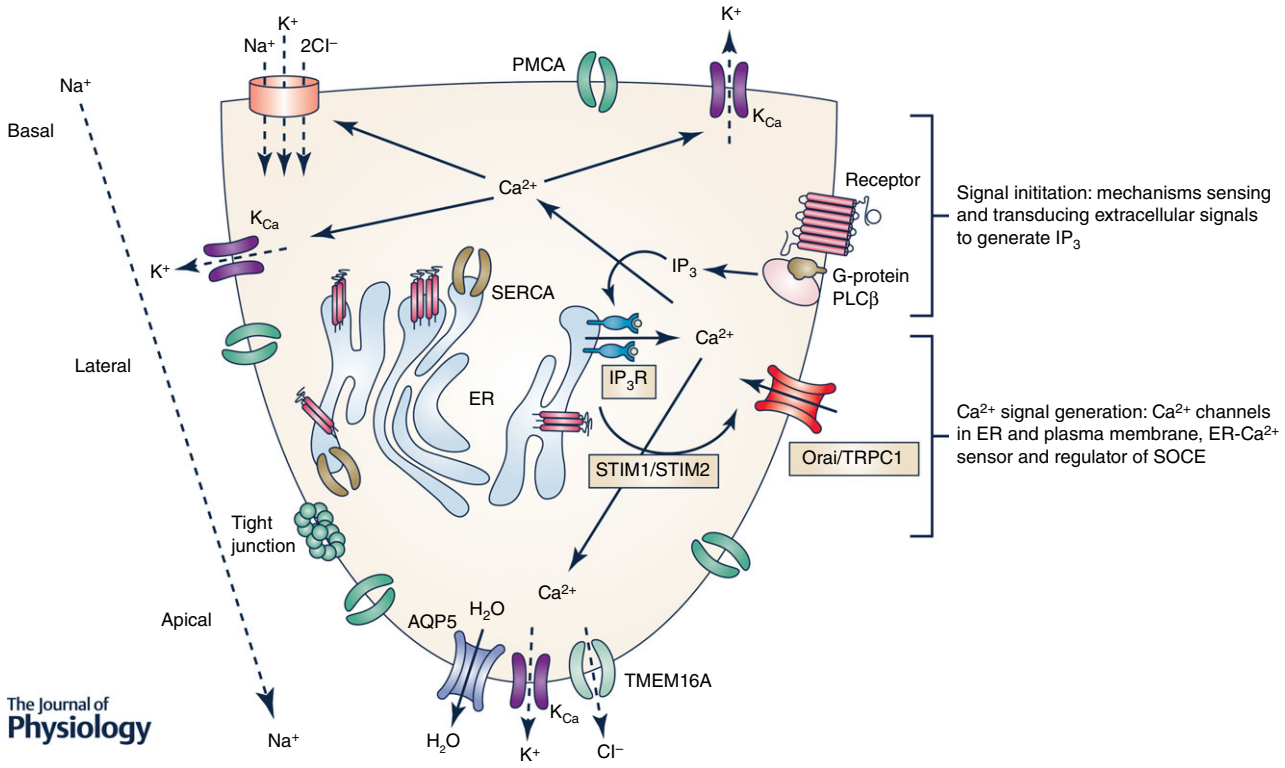

Figure 1. Schematic representation of Ca^{2+}-dependent regulation of fluid secretion in salivary gland acinar cells
Key molecular components involved in initiation and generation of $[Ca^{2+}]_i$ signals are indicated in the figure, as are ion channels and transporters regulated by $[Ca^{2+}]_i$ elevation.

Maximal activation of TRPC3 induces acinar cell toxicity. Aberrant increase in $[Ca^{2+}]_i$ in pancreatic acinar cells has long been considered to be the basis for pancreatitis. Recent studies have implicated TRPC3 and Orai1 activities in the onset and progression of acinar cell damage and disease (Kim *et al.* 2009; Kim *et al.* 2011; Gerasimenko *et al.* 2014). Conditions that abrogate channel function result in protection of tissue. While Ca^{2+} signalling does not directly regulate protein secretion in salivary gland acinar cells, unregulated or a very high level of Ca^{2+} influx that is maintained for long time periods could have several intracellular consequences including ROS generation, mitochondrial dysfunction and membrane damage. A recent study demonstrated that prolonged activation of salivary gland acinar cells with high levels of agonist results in cytotoxity that is associated with TRPC3-mediated Ca^{2+} entry (Kim *et al.* 2011). Similar conditions trigger TRPC3-mediated acinar cell damage in pancreas as well. It is unclear whether these are due to direct effects of Ca^{2+} on the secretory granules or other intracellular organelles, or as a result of activation of proteases. By monitoring the release of glucose 6-phosphate dehydrogenase (G6PD) and in the presence and absence of extracellular Ca^{2+}, Kim *et al.* reported massive cell damage in acinar cells stimulated with 1 mM carbachol for 30–40 min at 37°C in the presence of external Ca^{2+}. Removal of external Ca^{2+}, lack of TRPC3, or inhibition of TRPC3 prevented the cell damage. Most notably, cells lacking TRPC3 or pharmacological inhibition of TRPC3 in +/+ cells by the selective inhibitor pyrazole-3 (Pyr3) similarly protected salivary glands and the pancreas from Ca^{2+}-mediated cell toxicity.

It was suggested that Ca^{2+} entry in salivary gland acinar cells increases autophagosome formation as well as ER stress, both of which were reduced by eliminating TRPC3 function. Cell damage also resulted in an increased cellular ceramide content and accumulation of the lipid in the plasma membrane, which was also attenuated by blocking TRPC3. These studies demonstrate that under some conditions, TRPC3 activity can lead to acinar cell damage. Previous studies have shown that TRPC3 can be modified by ROS. Thus, further studies will be required to determine whether TRPC3 channels undergo further modification in cells stimulated at very high, non-physiologically relevant levels of agonist. Since TRPC3 and TRPC1 have been suggested to generate a heteromeric channel (Lee *et al.* 2014), it will also of interest to determine whether heteromeric TRPC3–TRPC1 or homomeric TRPC3 channels contribute to the cytotoxic effects seen in salivary gland acinar cells. Inhibition of Orai1 function provides significant protection against pancreatitis. It is not clear whether Orai1 also contributes to agonist-induced cytotoxicity in salivary gland acinar cells. However, since store-dependent activation of TRPC3–TRPC1 heteromeric channels is likely to be dependent on Orai1 in these two types of acinar cells (Ong *et al.* 2014), knockdown of Orai1 will also reduce TRPC1–TRPC3 function. Thus, there is fine regulation of various ion channel activities in the maintenance of cellular Ca^{2+} homeostasis, and either a gain or a loss of Ca^{2+} channel or transporter activity can result in substantial disruption of Ca^{2+} signalling and physiological function.

Role of Ca^{2+} signalling in ductal epithelium in exocrine dysfunction. Exocrine dysfunction and damage have been linked to ductal tissue in both salivary and pancreatic glands. The duct carries out critical secretory as well as absorptive functions (Larusch & Whitcomb, 2012). In addition, factors secreted by acinar cells trigger autocrine responses in ductal epithelium. Purinergic receptors (P2Y2, P2X4, and P2X7) are localized in the ductal luminal membrane and sense $[Ca^{2+}]$ and ATP in the secreted fluid, both of which are increased during cell injury. Activation of purinergic receptors by either of these factors triggers intracellular Ca^{2+} signals that lead to activation of the cystic fibrosis transmembrane conductance regulator (CFTR) and secretion of bicarbonate-rich fluid. Such secretion serves to flush the contents, such as mucins, out of the duct, thus decreasing the chance of ductal obstruction. Another receptor is the calcium-sensing receptor (CaSR), a G-protein-coupled receptor that senses $[Ca^{2+}]$ in the secreted fluid. It is coupled to activation of cyclic-AMP, which also leads to HCO_3^- secretion via increasing CFTR activity. When $[Ca^{2+}]$ in pancreatic juice increases, such as following acinar cell injury, there is risk of trypsinogen activation in the ductal epithelium, which could increase the rate and severity of pancreatitis. However, activation of CaSR protects ductal physiology by activating ductal secretion (Ravi Kanth & Nageshwar Reddy, 2014) Although salivary glands do not exhibit a phenomenon similar to pancreatitis, many key components of ductal epithelium are similar, e.g. CaSR, CFTR and purinergic receptors (Bandyopadhyay *et al.* 2012; Hong *et al.* 2014; Jung & Lee, 2014) Thus, it is reasonable to hypothesize that elevations of ATP and $[Ca^{2+}]$ in secretory fluid could similarly affect ductal secretion in salivary glands by activating purinergic receptors and CaSR. This would relieve any mucin accumulation in the ducts, protecting against sialoadenitis, which will be extremely beneficial for the maintenance of salivary gland function and integrity.

Ca^{2+} signalling defects in Sjögren's syndrome. Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease that results in lymphocytic infiltration and loss of secretory function in salivary and lacrimal glands (Nikolov & Illei, 2009). The pathogenesis of this disease has not yet been elucidated and neither has the basis for the loss of salivary fluid secretion been identified. While

extensive lymphocytic infiltration is always associated with destruction of the gland within the area of the infiltrate, salivary gland dysfunction is seen in patients with little or no overt inflammation (Shen *et al.* 2013; Xuan *et al.* 2013). Thus, the hallmark of the disease, xerostomia, is poorly correlated with the level of lymphocytic infiltration. There are two areas within the gland that have been studied in attempts to understand the loss of salivary gland function. The first is the area within and around the lymphocytic infiltrate. The infiltrate itself is made up of multiple cell types including T and B lymphocytes. Most studies have focused on the T-lymphocytes, although more is known about peripheral lymphocytes in the patients rather than the cells that populate the gland during the disease. It has been suggested that the infiltrating lymphocytes secrete cytokines and other factors that can alter the milieu and affect the epithelial cells in the vicinity. Some early studies therefore assessed the effects of Sjögren's syndrome-associated cytokines such as interferon-γ (IFNγ) and tumour necrosis factor-α (TNFα) on cell function. However, no clear mechanism has emerged to account for the loss of function. The second, and more physiologically important, area is the relatively large amount of apparently morphologically intact salivary gland tissue in glands from SS patients. The extent of functional loss in the patients suggests that this relatively intact tissue has aberrant function.

Disruption of SOCE has major immunological consequences. Interestingly, loss of Orai1 function due to mutation results in the severe combined immune deficiency (SCID) phenotype, while patients with STIM1 mutations display autoimmunity and lymphoproliferation (Feske *et al.* 2015). T lymphocyte-targeted deletion of STIM1 and STIM2 in mice resulted in loss of SOCE and SOCE-dependent cytokine production in T cells and a decrease in functional regulatory T cells (Tregs) (Oh-Hora *et al.* 2008). The mice displayed signs of autoimmunity, including dermatitis, blepharitis and lymphoproliferation, with lymphocytic infiltration in epithelial tissues, such as liver and lungs. Importantly, these mice developed spontaneous, rapid and progressive submandibular gland inflammation (Cheng *et al.* 2012). Within three months the glands in the mice resembled those of pSS patients, with severe salivary gland inflammation and damage. Evidence for development of SS in the mice was shown by the detection of major hallmarks of pSS: inflammation of salivary glands, loss of stimulated fluid secretion and elevated pSS-specific autoantibodies. This study further assessed peripheral blood mononuclear cells (PBMCs) from pSS patients. Remarkably, PBMCs from pSS patients showed deficits in levels of STIM1 and STIM2 proteins, as well as diminished SOCE. Together these data show an association between STIM1 and STIM2 deficiencies in T cells, and the consequent aberrations in T cell function, with onset and progression of salivary gland exocrinopathy in Sjögren's syndrome. The exact mechanism(s) that cause these defects in lymphocytes or trigger their infiltration into salivary glands is not known.

In addition to the possible role of purinergic receptors in salivary gland dysfunction discussed above, the P2X7 purinergic receptor (P2X7R) has been reported to have an essential role in the innate immune response. It is involved

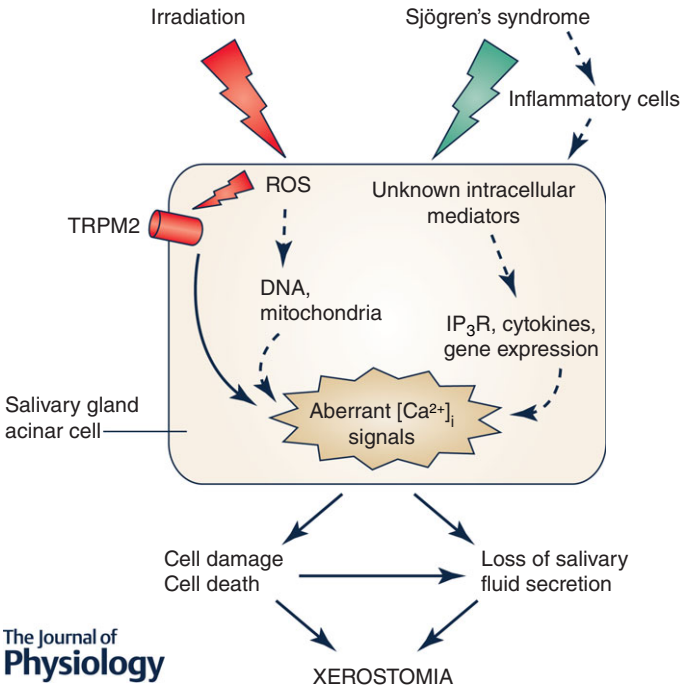

Figure 2. Ca^{2+} signalling in salivary gland dysfunction
Two conditions, IR and Sjögren's syndrome, induce salivary gland dysfunction that leads to xerostomia. TRPM2 has been identified as a target for IR-generated ROS in cells, and loss of TRPM2 function protects against IR-induced defect in salivary gland acinar cell function. IR-induced salivary gland dysfunction can include as yet unconfirmed effects on mitochondria and DNA (dashed arrows). Sjögren's syndrome also results in loss of salivary fluid secretion and xerostomia. While the condition causes inflammation in the gland, the intracellular mediators of the disease are not known. Decreases in IP_3R function, cytokine generation and changes in gene expression could contribute to initiation and the progress of the disease.

in the modulation of several critical cellular functions, such as growth and replication, as well as activation of the NLRP3 inflammasome that results in release of IL-1β and IL-18. Notably, serum and salivary glands of patients with pSS show increases in levels of IL-1β and IL-18 that have been proposed to exert proinflammatory effects. In salivary glands, periductal $CD68^+$ macrophages and ductal epithelial cells represent the major sources of IL-18. A recent study has shown that P2X7R is overexpressed in glands of patients with pSS. Importantly, this has been linked to the activation of the inflammasome complex and increased release of IL-18, suggesting a causative link between P2X7R, NLRP3 inflammasome activation and sialadenitis. Interestingly, inflammasome activation has also been described during acute pancreatitis, with the P2X7R upstream of the inflammasome and necessary for the development of pancreatic injury. In addition, P2X7R stimulation in salivary acinar cells has several deleterious consequences, including depolarization of the mitochondrial membrane and production of reactive oxygen species and cleavage and release of α-fodrin. It has been suggested that P2X7R is involved in mechanisms that initiate or maintain salivary gland inflammation in pSS. These observations in pSS provide a rationale for novel P2X7R-targeted therapies for this disease. (Woods *et al.* 2012; Baldini *et al.* 2013)

Recently, there has been more focus on the epithelial cell involvement in SS and it has been hypothesized that salivary gland acinar and ductal cells have functional defects that might account for the secretory dysfunction. Further, it has also been proposed that these cells synthesize and secrete cytokines that might contribute to disease progression. Some studies have shown that AQP5 localization is disrupted, which could account for the functional defect (Delporte, 2009; Soyfoo *et al.* 2012). Other studies suggested that pSS patients have auto-antibodies to muscarinic receptors, which might dampen endogenous neurotransmitter stimulated salivary gland secretion (Nikolov & Illei, 2009). Data are now emerging from studies that directly examine acinar cell function in salivary gland biopsies obtained from patients that provide definitive evidence that neurotransmitter-stimulated Ca^{2+} signalling and cell volume regulation are aberrant in acinar cells from pSS patients. A recent study links the decrease in Ca^{2+} signalling in acinar cells to loss of IP_3R2 and IP_3R3, two critical IP_3R subtypes in this gland (Teos *et al.* 2015). This represents an important advancement in our understanding of the mechanism underlying the loss of saliva flow in pSS. However, further studies are required to establish the exact mechanism that initiates loss of the IP_3Rs as well triggers P2X7–inflammasome activation. Such knowledge is likely to provide novel targets for therapy.

Conclusions

$[Ca^{2+}]_i$ signals are a double-edged sword: while $[Ca^{2+}]_i$ increases are essential in the regulation of cell function, maintenance of Ca^{2+} homeostasis in both resting and stimulated cells is absolutely critical for cell survival. The key trigger for stimulation of fluid secretion is an increase in $[Ca^{2+}]_i$ that occurs in response to stimulation of cells by neurotransmitters and that leads to regulation of ion channel activities and generation of the appropriate osmotic gradient required to drive fluid secretion. Key steps that determine the increase in cytosolic $[Ca^{2+}]$ are IP_3-mediated release of Ca^{2+} from the ER via the IP_3R, and Ca^{2+} entry via plasma membrane SOCE-channels (Putney, 1990). Critical components of SOCE in acinar cells include Orai1, STIM1, STIM2 and TRPC1. Thus, the major mechanisms and key players associated with regulation of Ca^{2+} signals in salivary gland function have now been elucidated. However, further studies are required to determine their assembly in the cells and delineate the Ca^{2+} sensors and other regulatory components involved in decoding the Ca^{2+} changes for regulation of specific cell functions.

Deficits and aberrant Ca^{2+} signalling have been associated with salivary gland dysfunction that results in xerostomia, or dry mouth condition, in patients. Consequences of this include difficulty swallowing, rampant dental caries, oral mucosal lesions, and fungal infections that together severely affect the quality of life for the patients (Fig. 2). The first is an irreversible loss of salivary gland function due to by-stander effects of head and neck irradiation. This is a subject of great interest in the field with clinical studies being directed towards assessing therapies targeted to recovery of cell function, prevention of functional loss, or regrowth of salivary glands. Novel targets such as TRPM2 or mitochondrial or cytosolic ROS modulators could provide effective strategies for treatment. However, further studies are required to fully understand the mechanism underlying the loss of fluid secretion in the irradiated gland. The second is the progressive loss of salivary gland function associated with primary Sjögren's syndrome, a chronic autoimmune disease. Notably, there is poor correlation between salivary flow and extent of inflammation or tissue damage with a large number of patients displaying low levels of inflammation within their salivary glands with substantial loss of function. The molecular alterations underlying this secretory defect are not yet known. Emerging studies implicate Ca^{2+} signalling and functional defects in both lymphocytes as well as salivary gland acinar cells from patients diagnosed with this disease. Further studies are required to validate these initial observations and identify possible novel components that can be used to improve diagnosis or treatment for this disease.

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Additional information

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The author declares that there are no conflict of interests.

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