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Dissection of Calcium Signaling Events in Exocrine Secretion

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Abstract

The secretion of fluid and electrolytes by salivary gland acinar cells requires the coordinated regulation of multiple ion channel and transporter proteins, signaling components, and water transport. Importantly, neurotransmitter stimulated increase in the cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) is critical for the regulation of salivary gland secretion as it regulates several major ion fluxes that together establish the sustained osmotic gradient to drive fluid secretion. The mechanisms that act to modulate these increases in $[Ca^{2+}]_i$ are therefore central to the process of salivary fluid secretion. Such modulation involves membrane receptors for neurotransmitters, as well as mechanisms that mediate intracellular Ca^{2+} release, and Ca^{2+} entry, as well as those that maintain cellular Ca^{2+} homeostasis. Together, these mechanisms determine the spatial and temporal aspects of the $[Ca^{2+}]_i$ signals that regulate fluid secretion. Molecular cloning of these transporters and channels as well as development of mice lacking these proteins has established the physiological significance of key components that are involved in regulating $[Ca^{2+}]_i$ in salivary glands. This review will discuss these important studies and the findings which have led to resolution of the Ca^{2+} signaling mechanisms that determine salivary gland fluid secretion.

Keywords

Calcium homeostasis; Calcium influx; Ion channels; Fluid secretion; Salivary gland cells; Physiology; Knockout-mouse models

Introduction

Ca^{2+} has a pivotal role in the physiological function of both excitable and non-excitable cells [1, 2]. It has been conclusively established that Ca^{2+} is the primary intracellular factor that regulates secretion in exocrine gland cells, including fluid secretion in salivary and lacrimal glands and protein secretion in pancreas [3–5]. Unlike in the pancreas, protein secretion in salivary glands is primarily controlled by β-adrenergic stimulation and mediated by cAMP-dependent activation of protein kinase-A. Moreover, the exact mechanism involved in protein secretion, i.e. granule fusion, in salivary gland cells is not yet understood. Although the contributions of various ion transporters to electrolyte and fluid secretion are also quite distinct in both gland types, there is remarkable similarity in the basic Ca^{2+} signaling mechanism(s) that regulate protein secretion in pancreas and fluid secretion in salivary glands. This review will primarily focus on our current understanding of the regulation of Ca^{2+} signaling in salivary gland cells and the role of Ca^{2+} in fluid secretion. We will briefly discuss aspects which have been noted to be distinct in the two types of glands.

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Historically salivary glands have been widely used in studies to determine the mechanism(s) of fluid secretion and Ca^{2+} signaling. These early studies demonstrated that under normal physiological conditions, salivary glands maintain a continuous low level of saliva flow, often referred to as "resting" or "basal" secretion, which is dramatically increased upon demand, i.e. during a meal or other masticatory and gustatory stimuli. The regulation of secretion in the salivary glands is achieved by autonomic sympathetic and parasympathetic stimuli via a coordinated sequence of signal transduction and intracellular signaling events. These events include activation of membrane receptors and associated signal transduction proteins in the plasma membrane, generation of intracellular second messengers, calcium mobilization, and stimulation of ion transport pathways.

Salivary gland cells have receptors for a number of neurotransmitters which are localized in the plasma membrane which when stimulated lead to increases in fluid and protein secretion [3, 4]. Major receptors associated with salivary gland fluid secretion are muscarinic, α-adrenergic, and purinergic receptors. Receptor activation leads to activation of $G_{\alpha q/11}$ family of G-proteins and phosphatidylinositol 4, 5, bisphosphate (PIP_2)-specific phospholipase C (PLC), which results in hydrolysis of the membrane bound phospholipid, PIP_2, and generation of inositol 1, 4, 5, trisphosphate (IP_3) and diacylglycerol. IP_3 diffuses into the cytosol and binds to the IP_3 receptor (IP_3R) localized on the endoplasmic reticulum (ER) membrane. This induces release of Ca^{2+} from the ER Ca^{2+} store(s) via the IP_3R. Three subtypes of the IP_3 receptor have been described (IP_3R1, IP_3R2 and IP_3R3), of which IP_3R2 and 3 are the major subtypes found in exocrine gland cells [4–10]. These are concentrated in the apical pole of the cells. The localization of IP_3Rs in the acinar cells have important functional consequences. In pancreatic acinar cells, internal Ca^{2+} release originates in the luminal region of the cell and at lower levels of stimulation this signal is restricted to this region of the cell [6, 9–11]. At higher levels of stimuli the signal subsequently propagates to the basal regions of the cell. In salivary gland acinar cells the first signal is initiated in the apical region but unlike the pancreatic cells, the signal propagates to the basal region at either low or high levels of stimuli [12, 13]. The net result of the IP_3-induced release of Ca^{2+} from the ER is an increase in the $[Ca^{2+}]_i$, which is the triggering event for the stimulation and sustained regulation of fluid secretion in salivary gland acini and protein secretion in pancreatic acini [4, 5]. While intracellular Ca^{2+} release is sufficient to trigger fluid secretion, sustained secretion is dependent on Ca^{2+} influx. In salivary gland acini, this influx is primarily mediated by store-operated calcium entry (SOCE) [4, 14]. Remarkably, this Ca^{2+} entry is activated by the intracellular Ca^{2+} release process [15, 16]. Thus, the coordinated activation and regulation of intracellular Ca^{2+} release and Ca^{2+} entry result in generating optimal $[Ca^{2+}]_i$ signals that are required to drive sustained fluid secretion and also for regulation of other critical cellular functions.

A Historical Perspective of Calcium Signaling in Salivary Acinar Cells

Studies reported almost three decades ago show that Ca^{2+} is involved in the regulation of fluid secretion in the salivary gland [17, 18]. Experiments with perfused salivary gland preparations demonstrated that sustained fluid secretion was achieved only when Ca^{2+} was present in the external medium; while in the absence of external Ca^{2+}, fluid secretion was only stimulated transiently. Similar effect of external Ca^{2+} was obtained in measurements of agonist stimulated Rb^+ efflux in salivary gland cells (Rb^+ is used as a surrogate for K^+, a critical cation in fluid secretion). Increase of Rb^+ efflux from the cells was biphasic with an initial rapid transient increase which was independent of external Ca^{2+} and a lower sustained release which was completely dependent on the external $[Ca^{2+}]$. More direct involvement of Ca^{2+} in salivary gland physiology was demonstrated by studies showing a flux of Ca^{2+} following stimulation of salivary gland cells with muscarinic and α_1-adrenergic receptor agonists [18]. The rates of Ca^{2+} uptake as well as that of Ca^{2+} release were increased. Thus,

it was suggested that intracellular Ca^{2+} homeostasis was altered and the Ca^{2+} permeability of the cell membrane was increased following agonist stimulation of cells. Furthermore, the release of Ca^{2+} from the cell was temporally correlated with the activation of K^+ efflux and increase in fluid secretion, while Ca^{2+} uptake was associated with prolonged K^+ channel activity and fluid secretion. Experiments examining uptake of isotopic Ca^{2+} into the cell suggested the involvement of an internal Ca^{2+} store into which Ca^{2+} was sequestered, or released from following stimulation. Further, use of a permeabilized cell system demonstrated the presence of an ATP-dependent Ca^{2+} uptake mechanism and a Ca^{2+} store in the ER of exocrine gland cells which was proposed to be the likely agonist-sensitive Ca^{2+} store. A landmark study in the field of Ca^{2+} signaling was the demonstration that IP_3 induced the release of Ca^{2+} from ER Ca^{2+} store(s) [19]. This important finding linked the increase in inositol lipid turnover following receptor stimulation to intracellular Ca^{2+} mobilization and enzyme secretion in pancreatic acinar cells. This study has been critical to our present understanding of the receptor-stimulated Ca^{2+} signaling since a similar mechanism is present in all non-excitable cells.

The introduction of fluorescent probes for measuring $[Ca^{2+}]_i$ in the early 1980s revolutionized the field of Ca^{2+} signaling. The use of these dyes not only confirmed previous suggestions which were made regarding Ca^{2+} mobilization events in the salivary glands but also clearly established the sequence and spatial aspects of the $[Ca^{2+}]_i$ changes at a cellular level. Studies using fluorescent dyes to monitor $[Ca^{2+}]_i$ rapidly evolved from studies in cell populations to those in single cells. A primary observation made was that cell stimulation induced biphasic increase in $[Ca^{2+}]_i$; an initial rapid increase in $[Ca^{2+}]_i$ which was transient in nature and decreased to a lower more sustained elevation [4, 5, 9, 10, 17]. Further, the initial increase in Ca^{2+} was not altered by removing Ca^{2+} from the external medium, suggesting that it was primarily due to release from an internal Ca^{2+} store while the subsequent, sustained, increase in Ca^{2+} was completely dependent on the presence of external Ca^{2+} and could be inhibited by the addition of La^{3+} or Ca^{2+} chelators to the external medium. These results confirmed that the second phase of Ca^{2+} increase was due to Ca^{2+} influx into the cell from the external medium. These studies firmly established that while fluid secretion as well as Rb^+ and Cl^- efflux could be stimulated by intracellular Ca^{2+} release, prolonged secretion was dependent on the sustained elevation in $[Ca^{2+}]_i$ which was achieved primarily by Ca^{2+} influx. It has now been demonstrated that sustained activation of the three ion flux systems that are critical in the regulation of fluid secretion, namely the K_{Ca} channels, the $Na^+/K^+/Cl^-$ cotransporter, and TMEM16A channel are all dependent on Ca^{2+} influx as is the membrane trafficking of the water channel, AQP5 [5]. Studies in single cells also demonstrated an important feature of Ca^{2+} signaling. It was shown that low concentrations of agonists produced oscillatory changes in $[Ca^{2+}]_i$, some of which were also spatially restricted within a certain domain in the cell. The pattern of these oscillations was highly cell-type and agonist specific [6, 9, 10]. These studies also provided interesting clues to the underlying mechanisms that contribute to cytosolic $[Ca^{2+}]_i$ increase.

Mechanisms that Contribute to Cytosolic $[Ca^{2+}]_i$ Increase

The major mechanisms that contribute to regulation of $[Ca^{2+}]_i$ following agonist stimulation of cells are Ca^{2+} channels in the intracellular membranes, including the ER, and those in the plasma membrane, calcium pumps, and mitochondria. The role of mitochondria in the spreading of cytosolic calcium waves has been studied somewhat in detail in some exocrine gland cells and the function of the Ca^{2+} pumps have also been examined in isolated cells preparations [3, 4, 6, 8–11, 13]. Here we will focus the discussion on the intracellular Ca^{2+} release and Ca^{2+} entry mechanisms.

The IP_3-Sensitive Ca^{2+} Channel

One of the best characterized and most important Ca^{2+} channels in cells is the IP_3-sensitive Ca^{2+} channel in the ER which allows Ca^{2+} to be rapidly released from the ER Ca^{2+} stores into the cytosol [19, 20]. The free [Ca^{2+}] in the ER lumen is estimated to be between 70 and >300 μM, thus providing a large driving force for Ca^{2+} release from this pool. This channel is regulated in a complex way by a number of different factors and proteins and in turn IP_3R regulates a number of key signaling events in the cell. As mentioned above, two major isoforms IP_3R2 and IP_3R3 are found in salivary gland acinar cells which are concentrated in the luminal end of the cell. This localization is consistent with the observation that agonist stimulated rise in $[Ca^{2+}]_i$ initiated at the luminal pole of the cell. It has been well established that Ca^{2+} stimulates IP_3-mediated Ca^{2+} release at lower concentrations and inhibits it at higher concentrations, >300 nM. The ability of Ca^{2+} to increase Ca^{2+} release is completely dependent on IP_3. As the IP_3 concentration increases, the receptor is more sensitive to lower [Ca^{2+}]. This feed-forward and feedback regulation by Ca^{2+} ensures an open state of the channel when $[Ca^{2+}]_i$ is low whereas when cytosolic Ca^{2+} levels are high, release from the ER Ca^{2+} store is restricted. Such regulation provides a process by which the ER Ca^{2+} stores can be protected and $[Ca^{2+}]_i$ can be tightly regulated within the range required for the physiological function of the cell. Such regulation of IP_3R can also account for $[Ca^{2+}]_i$ oscillations, although not in all cell types.

Ca^{2+} Influx

Although evidence for the involvement of Ca^{2+} influx in salivary fluid secretion was provided almost 30 years ago [18] the molecular mechanism(s) involved in regulating this process proved to be a major challenge in the field of salivary gland physiology as well as calcium signaling in non-excitable cells. Interestingly, some of the earliest studies of Ca^{2+} influx were performed with salivary gland cells. These studies showed that Ca^{2+} influx into cells was increased several fold following stimulation of the cells with an agonist and inactivated when the stimulus was removed or an antagonist was added giving rise to the suggestion that Ca^{2+} influx was activated either directly as a result of agonist binding to the receptor, i.e. via receptor-operated Ca^{2+} entry (ROC) or by an intracellular second messenger via a second messenger operated entry (SMOC). An important study demonstrated that when cells were treated with the SERCA inhibitor, thapsigargin, there was slow depletion of internal Ca^{2+} stores and activation of Ca^{2+} influx which provided conclusive evidence that internal Ca^{2+} store depletion per se was the signal for activation of Ca^{2+} influx while refilling induced inactivation. This Ca^{2+} influx was termed capacitative Ca^{2+} entry (CCE) and currently is also referred to as store-operated Ca^{2+} entry (SOCE) [15]. SOCE is the primary mode of Ca^{2+} influx in salivary gland cells following stimulation with muscarinic and α_1-adrenergic agonists [4, 5, 14].

The first model proposed by Putney for the regulation of SOCE suggested that the internal Ca^{2+} store is physically linked to the plasma membrane such that Ca^{2+} entering the cell directly enters the ER Ca^{2+} store from where it is released via the IP_3-sensitive channel [15]. According to this model the resting level of Ca^{2+} in the ER exerted an inhibitory effect on the Ca^{2+} influx pathway and when the Ca^{2+} level decreased, following IP_3-induced Ca^{2+} release, the inhibitory effect was removed and Ca^{2+} influx activated. However, using Mn^{2+} as a Ca^{2+} surrogate ion researchers clarified that the route of Ca^{2+} entry into ER was via the cytosol; i.e. Ca^{2+} entered the cell from where it was pumped into ER by the SERCA activity [15]. This Ca^{2+} entry pathway was shown to be involved in regulating key cellular functions; including exocrine secretion, platelet aggregation, endothelial cell permeability and migration, cell proliferation, T-lymphocyte activation, and mast cell degranulation. Thus identifying the mechanisms and components of this pathway has been a major focus of interest in the field of Ca^{2+} signaling.

A major problem in resolving this important mechanism was the lack of knowledge regarding the molecular components of the SOCE channel. Several models have been proposed to explain the mechanism(s) by which plasma membrane channels mediating SOCE are regulated; i.e. how the status of the ER-$[Ca^{2+}]$ is sensed and transmitted to the plasma membrane to regulate SOCE [15, 16, 21]. Two major hypotheses which have been proposed are the conformational coupling hypothesis and the diffusible messenger hypothesis. According to the former the depletion of Ca^{2+} in the ER lumen is detected by IP_3R which undergoes a conformational change that is transmitted to the plasma membrane channels. However, later studies excluded a direct role for IP_3R in gating the SOCE channel (further discussed below). The second model proposed the involvement of a diffusible metabolite that was released from the ER together with Ca^{2+}. However, this model has not been supported with consistent and conclusive data and several studies have ruled out the possibility of such a diffusible factor. Other possible models include recruitment of channels to the plasma membrane by trafficking. While the exact mechanism involved in SOCE was not established until very recently, it was evident from early studies that the physical proximity of the ER and plasma membrane was critical in the activation of SOCE and that the activation was a relatively rapid process.

Resolving Receptor-Mediated Ca^{2+} Signaling Mechanisms in Salivary Gland Acini

Receptors

Identification of the specific muscarinic acetylcholine receptor (mAChR) subtypes mediating stimulation of salivary secretion was important not only for clarifying the components of salivary gland Ca^{2+} signaling but also of considerable clinical interest. It was reported earlier that the M(1) and M(3) subtypes are the major muscarinic acetylcholine receptors although their physiological relevance was not resolved until later by studies from Mikoshiba and Wess laboratories [22, 23]. These studies confirmed the earlier findings by demonstrating that carbachol-induced $[Ca^{2+}]_i$ increase was markedly impaired in submandibular gland cells from mice lacking M(3) receptors and completely absent in those lacking both M(1) and M(3) receptors. This demonstrated that M(3) and M(1) play major and minor roles, respectively, in the cholinergically induced $[Ca^{2+}]_i$ increase. Two-dimensional Ca^{2+}-imaging analysis revealed a patchy distribution of M(1) in submandibular gland acini while there was ubiquitous distribution of M(3). In vivo administration of a high dose of pilocarpine (10 mg kg^{-1}, s.c.) to M(3) knockout mice (M(3)−/− mice) caused salivation comparable to that in wild-type mice, while no salivation was induced in M(1)/M(3)−/− mice, indicating that salivation in M(3)−/− mice is caused by an M(1)-mediated $[Ca^{2+}]_i$ increase. In contrast, a lower dose of pilocarpine (1 mg kg^{-1}, s.c.) failed to induce salivation in M(3)−/− mice, but induced abundant salivation in wild-type mice, indicating that M(3)-mediated salivation has a lower threshold than M(1)-mediated salivation. In addition, M(3)−/−, but not M(1)−/−, mice, had difficulty in eating dry food, as shown by frequent drinking during feeding, suggesting that salivation during eating is mediated by M(3) and that M(1) plays no practical role in it. These results provide conclusive evidence the M(3) subtype is essential for parasympathetic control of salivation.

Protease-activated receptor-2 (PAR-2) is expressed in the salivary glands and when stimulated with an agonist, SLIGRL-OH, induced salivary flow in mice lacking M(3) and M(1) muscarinic receptors. In PAR-2−/− mice SLIGRL-OH-stimulated secretion was abolished [24]. Furthermore, compared with the secretion in WT mice, PAR-2-mediated salivary secretion and $[Ca^{2+}]_i$ response were enhanced in mice lacking M(3) or both M(1) and M(3) mAChRs, in which mAChR-stimulated secretion and $[Ca^{2+}]_i$ response in acinar cells were severely impaired. Although the mechanism underlying the enhanced PAR-2-

mediated salivary secretion in M(3)-deficient mice is not clear, the result suggests the presence of some compensatory mechanism involving PAR-2 in the salivary glands deficient in cholinergic activation.

GPCR-Signal Transduction Mechanisms

While functional studies of either salivary or pancreatic secretion have not been carried out with mice lacking $G_{\alpha q/11}$ or PLCβ, these proteins are associated with muscarinic receptor-mediated Ca^{2+} signaling. They are co-localized with receptors and key Ca^{2+} signaling proteins and this association has also been biochemically verified by co-immunoprecipitation. Spinophilin (SPL) and neurabin (NRB) are structurally similar scaffolding proteins. SPL binds regulators of G protein signaling (RGS) proteins and regulate the intensity of Ca^{2+} signaling by GPCRs. Studies with SPL(−/−) and NRB(−/−) mice show that SPL and NRB reciprocally regulate Ca^{2+} signaling by GPCRs. Deletion of SPL in mice enhanced binding of RGS2 to NRB and Ca^{2+} signaling by αAR, whereas deletion of NRB enhanced binding of RGS2 to SPL and reduced Ca^{2+} signaling by αAR. This was due to reciprocal modulation by SPL and NRB of the potency of RGS2 to inhibit Ca^{2+} signaling by αAR. These findings suggest a novel mechanism of regulation of GPCR-mediated Ca^{2+} signaling in which SPL/NRB form a functional pair of opposing regulators that modulate Ca^{2+} signaling intensity by GPCRs [25, 26].

RGS proteins accelerate the GTPase activity of Gα subunits to determine the duration of the stimulated state and control G protein-coupled receptor-mediated cell signaling. RGS2 is an RGS protein that shows preference toward Gα(q). In cells derived from RGS2(−/−) mice the kinetics of IP_3 production was modified without an effect on the peak level of IP(3) [27]. The cells were also adapted to deletion of RGS2 by reducing Ca^{2+} signaling excitability. Reduced excitability was achieved by adaptation of all transporters to reduce Ca^{2+} influx into the cytosol. These findings highlight the central role of RGS proteins in Ca^{2+} signaling and reveal a prominent plasticity and adaptability of the Ca^{2+} signaling apparatus. It is highly likely that such modulation of Ca^{2+} signaling will significantly impact fluid secretion.

Functional Significance of $InsP_3R$ Family

$InsP_3R2$ and $InsP_3$-3 are the main receptors found in acinar cells leading to the question as to whether this is reflective of redundancy or do particular sub-types make specific contribution to the function of the gland. Genetic knockout of individual or a combination of $InsP_3R$ genes provided significant insight into these issues [28]. Futatsugi and colleagues reported that targeted ablation of either the type-2 or type-3 $InsP_3R$ individually, had no significant effects on muscarinic-receptor stimulated secretion and the animals had no overt phenotype. Consistent with these observations, the peak secretagogue-stimulated increases in $[Ca^{2+}]_i$ were not altered by the deletion of $InsP_3R3$ and only modestly impacted by the loss of the $InsP_3R2$. In $InsP_3R2$−/− acini, marked changes were only seen at low secretagogue or $InsP_3$ concentrations. In both cases the spatial aspects of the signals were largely unaffected. Taken together these data indicate that the complement of $InsP_3R2$ or $InsP_3R3$ in isolation, or perhaps in combination with relatively low levels of $InsP_3R1$, is sufficient to maintain signaling and preserve stimulated exocytosis. However, analysis of the compound $InsP_3R2$/$InsP_3R3$−/− animal revealed a much more striking phenotype. Although animals were born normally, they failed to survive past weaning, largely due to a failure to ingest and subsequently assimilate food. Even when fed wet mashed food to overcome the salivary deficit, the animals failed to thrive as a result of diminished pancreatic secretory function. The immediate cause of this was an almost complete absence of any measurable secretagogue-induced Ca^{2+} signal-even at supramaximal concentrations of agonist. The conclusion from these data is that while the residual expression of $InsP_3R1$ is not sufficient

to mount a Ca^{2+} signal, either the $InsP_3R2$ or $InsP_3R3$ in isolation. In addition, Ca^{2+} release via $InsP_3R$ was markedly influenced by the levels of cellular ATP, potentially linking the extent of Ca^{2+} release to the metabolic status of the cell. In acini, ATP (~Kd 40 μM) enhanced Ca^{2+} release at low levels of stimulation but did not influence release at high $InsP_3$ levels. In contrast, in $InsP_3R2-/-$ mice presumably dominated by $InsP_3R3$, ATP modulated release at all $InsP_3$ levels although the Kd for this effect was 10 fold higher (~450 μM). Interestingly, the properties of the wild-type animal were essentially identical to those shown for the $InsP_3R2$ in isolation, while the $InsP_3R2-/-$ mice mirrored those of the $InsP_3R3$. These data indicate that while $InsP_3R2$ and $InsP_3R3$ are interchangeable for Ca^{2+} release, the individual $InsP_3Rs$ are not redundant. Further, when expressed together the properties of $InsP_3R2$ dominate over $InsP_3R3$. These results reveal IP_3R2 and IP_3R3 as key molecules in exocrine physiology underlying energy metabolism and animal growth. More importantly, the data show that there is minimal Ca^{2+} signaling including Ca^{2+} entry that occurs in the absence of IP_3Rs, i.e. supported by PIP_2 hydrolysis alone.

Ca^{2+} Entry

As discussed above, agonist-induced Ca^{2+} entry is required for sustained fluid secretion. Members of the transient receptor potential (TRP) channels have been proposed as components of the SOCE-channel [29–31]. Studies reported by Ambudkar and co-workers have provided evidence for the involvement of TRPC1 in SOCE in the human salivary gland cell line, HSG [4, 32–35]. These investigators provided further confirmation by carrying out studies using mice lacking TRPC1. Neurotransmitter-regulated salivary gland fluid secretion in TRPC1−/− mice was severely decreased (by 70%) [14]. Further, SOCE stimulated either by agonist- or thapsigargin (Tg) was significantly reduced in salivary gland acinar cells isolated from TRPC1−/− mice. Deletion of TRPC1 also eliminated sustained Ca^{2+}-dependent potassium channel activity, which depends on Ca^{2+} entry and is required for fluid secretion. Expression of key proteins involved in fluid secretion and Ca^{2+} signaling was not altered. In addition, inhibitors of SOCE, 1 μM $Gd^{3}+$ as well as 20 μM 2APB completely blocked SOCE in agonist and Tg-stimulated submandibular gland acini which demonstrated that SOCE is the primary Ca^{2+} entry pathway into these cells (this agrees with the lack of Ca^{2+} influx in acini from IP_3R2 + $IP_3R3-/-$ mice discussed above). Together, these data demonstrate that reduced SOCE accounts for the severe loss of salivary gland fluid secretion in TRPC1−/− mice, thus providing the first evidence that SOCE supports fluid secretion. This important study finally established TRPC1 as a critical channel component of SOCE in salivary gland acinar cells which is essential for neurotransmitter-regulation of fluid secretion.

Another TRP channel protein, TRPC3, was shown to function as SOCE channel in vivo to mediate a significant portion of the receptor-stimulated Ca^{2+} influx in exocrine pancreatic cells. TRPC3-mediated Ca^{2+} influx in these cells affected the frequency of Ca^{2+} oscillations and moreover excessive Ca^{2+} influx by TRPC3 during supramaximal receptor stimulation was toxic to acinar cells and responsible in part to the cell stress and damage in pancreatitis. Such pancreatic damage was abrogated in TRPC3−/− mice. Therefore, it was suggested that inhibition of acinar cell TRPC3 and/or other Ca^{2+} influx channels may be considered as a strategy to control and reduce the severity of pancreatitis [36].

Other Components of SOCE

Recently two novel proteins, STIM1 and the Orai proteins, were identified as critical components of SOCE [16, 37]. STIM1 is a multidomain, Ca^{2+} binding protein that functions as the ER Ca^{2+} sensor. At the resting state STIM1 is diffusely localized in the ER with its luminal EF hand domain bound to Ca^{2+}. Depletion of ER Ca^{2+} results in dissociation of Ca^{2+} from the STIM1-EF hand domain as well as aggregation, translocation, and clustering

of STIM1 in close proximity to the plasma membrane where it interacts with the channels involved in SOCE. Orai1 is a plasma membrane protein, with four transmembrane domains, that has now been established as the main poreforming component of store-operated CRAC (calcium release activated Ca^{2+}) channels that are found in T lymphocytes and other hematopoietic cells. Orai1 is gated by STIM1 in response to store depletion and co-expression of Orai1 and STIM1 generated CRAC channel function [38–40]. As predicted by the contribution of TRPC1 to SOCE, STIM1 was shown to bind and activate TRPC channels, including TRPC1 and TRPC3 [34, 35, 41–43]. Co-expression of STIM1 and TRPC1 resulted in generation of non-selective cation channels that are distinct from CRAC channels in their characteristics. These channels have been referred to as store-operated Ca^{2+} (SOC) channels. STIM1 gates Orai1 and TRPC1 by different mechanisms [43, 44]. While the STIM1-SOAR domain in the C-terminus of STIM1 interacts with the C terminus of Orai1 to gate the CRAC channel, the C-terminal polybasic (KK) motif of STIM1 activates TRPC1 by an electrostatic gating mechanism which results in SOC channel activation [39, 45, 46]. In aggregate, these data finally validated the conformational coupling model for the regulation of SOCE and ruled out other previously proposed models.

More intriguingly, it was shown that TRPC1 function is completely dependent on Orai1 [31, 46, 47]. When endogenous Orai1 expression is suppressed, TRPC1 + STIM1 function is eliminated. How exactly Orai1 regulates TRPC1 is a critical and as yet unresolved question. While Orai1 knock-out mice appear to have severe growth problems, STIM1 knockout mice survive [37]. Salivary gland function in either mouse model has not yet been tested. However in a recent study, TRPC1, STIM1 or Orai1 were knocked down in isolated exocrine cell preparations and this lead to reduced SOCE and frequency of Ca^{2+} oscillations [48]. Thus, although the exact physiological role of Orai1 and STIM1 in exocrine gland function needs to be confirmed by using relevant mouse models, these recent data provide further demonstration for the requirement of all three proteins in Ca^{2+} signaling in exocrine acinar cells.

Spatial Aspects of Ca^{2+} Signaling Mechanisms

The highly polarized nature of epithelial secretory cells in exocrine glands necessitates targeting of functionally relevant proteins to specific locations in the cells. Further, key signaling proteins are assembled into complexes which confines the Ca^{2+} signaling apparatus, and $[Ca^{2+}]_i$ signals, to specific cellular microdomains. Such high degree of compartmentalization in the cellular localization has been shown for several Ca^{2+} signaling molecules, including G protein coupled receptors and their associated proteins, Ca^{2+} influx channels at the plasma membrane and Ca^{2+} release channels in the endoplasmic reticulum [8, 49, 50]. Although the physiological significance of polarized Ca^{2+} signaling can be predicted and has been experimentally validated to some extent, little is known about the mechanism of targeting, assembly, and retention of Ca^{2+} signaling complexes. For example, the consequence of polarized enrichment of Ca^{2+} signaling complexes at the apical pole facilitates the generation of an apical Ca^{2+} signal at low physiological agonist concentrations and limits propagation of the signal to the basal pole [6, 7, 9, 10, 12]. Close examination of the initiation and propagation pattern of repetitive Ca^{2+} waves evoked by the same agonist reveal that consecutive Ca^{2+} waves originate from the exact same initiation site and propagate along the same pattern. This indicates that the initiation site of the Ca^{2+} signal is likely defined by stably localized Ca^{2+} signaling proteins at the initiation sites. Consistent with this prediction, immunolocalization revealed concentration of Ca^{2+} signaling proteins at the apical pole of polarized cells. The Ca^{2+} signaling complexes at the apical pole appear to be much more sensitive to agonist stimulation than the complexes at the basal pole. The polarized localization of proteins and organelles results in generation of Ca^{2+} microdomains, primarily by limiting the diffusion of Ca^{2+} in the cell and segregating locally generated Ca^{2+}

signals. An example of this is found in pancreatic acinar cells where mitochondria cause a fire wall effect and restrict $[Ca^{2+}]_i$ elevations to the apical region [9, 10, 13]. A particularly interesting microdomain was discovered recently by measurement of $[Ca^{2+}]_i$ using TIRF microscopy [51]. These measurements revealed a very high and sustained Ca^{2+} microdomain underneath the plasma membrane (PM) when SOCE is activated. The exact components required to sustain this microdomain as well as the functional relevance of this Ca^{2+} signal is not yet known. It is suggested that such localized sustained $[Ca^{2+}]_i$ elevation is likely utilized for regulation of specific functions such as ion channel activation, activation of Ca^{2+} -dependent signaling; e.g. calcineurin, NFAT and NFκB. The frequency and amplitude of the $[Ca^{2+}]_i$ signal determine the type of pathway activated as each is regulated by a specific type of Ca^{2+} sensor.

Interestingly, TRPC1 is primarily localized in the basal and lateral regions of acinar cells while TRPC3 is also seen in the apical region of the cell [14, 48]. Orai1 appears to be localized in the lateral membrane towards the luminal region and possibly at very low levels in the basal membrane [48, 49]. Following cell stimulation, STIM1 moves to the lateral and basal region of the cells where it co-localizes with Orai1 and TRPC channels which are localized there. The localization of these key components of SOCE suggests that they can contribute to the apical and basal $[Ca^{2+}]_i$ signals. It is important to note that ion flux components as well as AQP water channels display polarized localization in acinar cells, consistent with the vectorial transport of ions and water from the basolateral region of the cell to the apical pole. Ca^{2+} regulated K_{Ca} channels as well as NKCC1 are localized in the basolateral region of acinar cells while TMEM16A and AQP5, which are also dependent on $[Ca^{2+}]_i$ increase, are localized in the luminal membrane [4, 5]. Thus, it can be speculated that these different ion channels could be regulated by local changes in $[Ca^{2+}]_i$ which are generated by the activity of Ca^{2+} channels residing in those specific cellular domains. The exact local and global $[Ca^{2+}]_i$ increases contributed by Orai1 channels, localized in the lateral and luminal region of the cells, as well as TRPC1 which are localized in the basolateral regions in response to stimulation of cells by agonists needs to be determined. However, it is reasonable to hypothesize that amplitude and spatiotemporal signature of the $[Ca^{2+}]_i$ increase will determine which ion channels involved in fluid secretion are regulated. Future studies should be directed to addressing some of these very physiologically relevant issues.

Conclusions and Questions

In summary, in the past four decades since the identification of Ca^{2+} as a critical factor in salivary gland function, several key components that are involved in generating and regulating cellular $[Ca^{2+}]$ signals have now been identified (Fig. 1). If we consider the muscarinic receptor signaling system, one of the main G-protein couples receptor signaling pathways that regulates salivary gland function, we now know that M(1) and M(3) receptors are functionally relevant. Downstream from these receptors, $G_{\alpha q/11}$, PLCβ, and RGS proteins are involved in signal transduction and tuning the level of stimulation. Activation of the receptor and downstream signal transduction components leads to PIP_2 hydrolysis and generation of IP_3. While the role of DAG, other than in activation of PKC, is not yet known, IP_3 binds to IP_3R initiating intracellular Ca^{2+} release. IP_3R2 and IP_3R3 have been shown by Mikoshiba and colleagues to have critical and non-redundant functions in determining the magnitude and pattern of $[Ca^{2+}]_i$ signals. This important study also showed that non-IP_3R-dependent mechanisms do not have any significant contribution to agonist-stimulated $[Ca^{2+}]_i$ signals. Interestingly, localization of these receptors in the cell is consistent with the spatial aspects of receptor-mediated $[Ca^{2+}]_i$ increases and modulation of local cellular functions. Importantly, key aspects of the elusive Ca^{2+} influx pathway have now been elucidated. Studies from the Ambudkar lab have shown that TRPC1 is a major contributor to

SOCE in salivary gland acinar cells and thus in the regulation of salivary fluid secretion. Muallem and co-workers have reported the relevance of TRPC1 in pancreatic acini and also demonstrated that TRPC3 contributes to SOCE in pancreatic acinar cells. Further, TRPC channels are regulated by STIM1 and Orai1. Important insights into the organization of the Ca^{2+} signaling mechanisms have now been provided. Studies from several leading researchers demonstrate that Ca^{2+} signaling proteins as well as Ca^{2+} signals and cellular functions are compartmentalized in cells. Concentrated localization of IP_3Rs in the luminal region of the cells is consistent with the $[Ca^{2+}]_i$ signals detected at that location as well as the main secretory functions that are triggered at the luminal membrane by Ca^{2+} elevations. Furthermore, consistent with the predicted site of Ca^{2+} influx, key components of SOCE; TRPC1, Orai1, and STIM1, are localized in the lateral and basal membrane regions in stimulated cells. Although the physiological relevance of Orai1 and STIM1 in the exocrine glands have yet to be described, the occurrence of these two proteins in areas where TRPC1 is located points out to the close functional interactions between these proteins. Together, these studies elucidate the key components of Ca^{2+} signaling in exocrine gland cells and provide insight into the spatiotemporal aspects of their contribution to salivary gland function. Further studies should address how exactly the Ca^{2+} signals are sensed and detected. What Ca^{2+} sensors are involved and how the Ca^{2+} signal is translated to regulate specific cellular functions? The mechanisms involved in the targeting and assembly of signaling complexes to various cellular locations is another area that needs to be examined in great detail. Of course, finally the effect of pathology or disease on Ca^{2+} -signaling or alternatively the contribution of Ca^{2+} signaling, or its deregulation, to exocrine gland dysfunction is a crucial area that will need to be addressed. Such studies will not only provide us with a comprehensive knowledge of the physiology of these important glands but also allow greater understanding of pathology and disease and lead to identification of new clinical targets and development of new therapeutic strategies.

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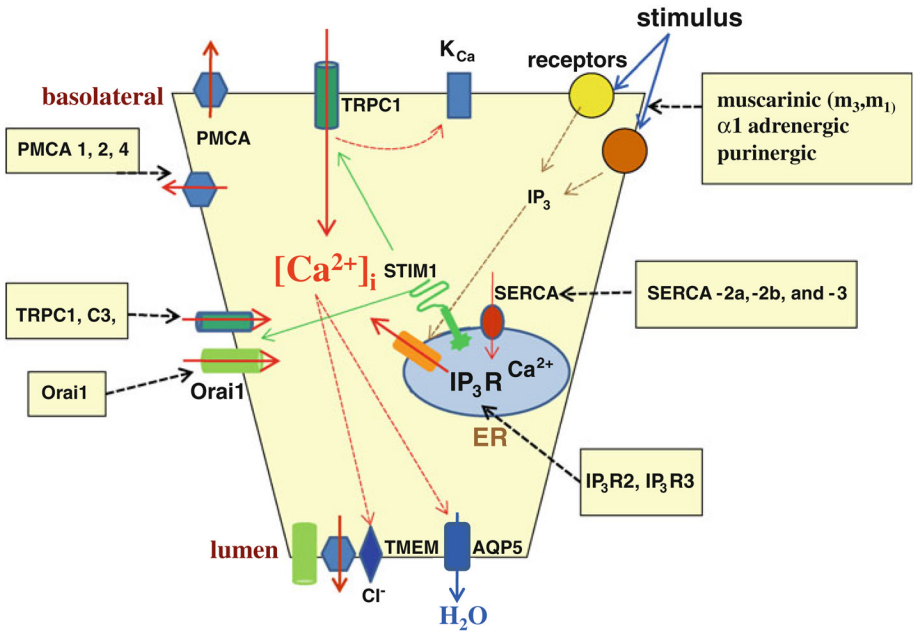

Fig. 1.

Ca^{2+} signaling mechanisms regulating salivary gland fluid secretion. The figure shows Ca^{2+} mobilizing events in acinar cells that are initiated by a stimulus and lead to fluid secretion. All molecular components, and the cellular domains, are labeled. *Dashed arrows* indicate mechanisms regulated by $[Ca^{2+}]_i$ increase and those inducing $[Ca^{2+}]_i$ increase. *Boxes* list the signaling proteins identified in these cells