

Roles for Ca²⁺ Mobilization and its Regulation in Mast Cell Functions: Recent Progress

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Abstract

Ca²⁺ mobilization in response to crosslinking of IgE bound to its high affinity receptor, FcεRI, on mast cells is central to immune allergic responses. Stimulated tyrosine phosphorylation caused by this crosslinking activates store-operated Ca²⁺ entry that results in sustained Ca²⁺ oscillations dependent on Rho family GTPases and phosphoinositide synthesis. Coupling of the endoplasmic reticulum (ER) Ca²⁺ sensor, STIM1, to the Ca²⁺-selective channel, Orai1, is regulated by these elements and depends on membrane organization, both at the plasma membrane and at the ER. Mitochondria also contribute to the regulation of Ca²⁺ mobilization, and we describe recent evidence that the ER membrane protein VAP plays a significant role in the coupling between ER and mitochondria in this process. In addition to granule exocytosis, Ca²⁺ mobilization in these cells also contributes to stimulated outward trafficking of recycling endosomes and to antigen-stimulated chemotaxis, and it is pathologically regulated by protozoan parasitic invasion.

Introduction

Granule exocytosis by mast cells in response to antigen-mediated crosslinking of immunoglobulin E (IgE) receptors, FcεRI, on the plasma membrane is among the most studied Ca²⁺-dependent processes in non-excitabile cells [1, 2]. Other processes, including cytokine biogenesis and secretion, are also mediated by activation of these receptors and depend on Ca²⁺ mobilization in these cells [3]. In 2012 we reviewed the roles for Ca²⁺ mobilization in mast cell function [4]. The present article provides a summary of more recent progress on this topic from our laboratory and others.

Key to the role of Ca²⁺ mobilization in these processes is antigen-stimulated store-operated Ca²⁺ entry (SOCE), which involves coupling of the endoplasmic reticulum (ER) Ca²⁺ sensor, STIM1, with the Ca²⁺ channel protein, Orai1, in the plasma membrane (Figure 1). We previously identified a sequence containing six acidic amino acids in the C-terminus of Orai1 that are important for functional coupling [5]. More recently, we identified a corresponding basic amino acid sequence in the C-terminus of STIM1 that is essential for SOCE [6]. Supporting our conclusions, a recent NMR study provided direct evidence for a complex between this C-terminal segment from Orai1 and two anti-parallel helical segments that contain this basic sequence in STIM1 [7]. We have also characterized the roles for two different pools of PIP₂ in the regulation of stimulated STIM1-Orai1 coupling [8]. One of these pools fractionates with detergent-resistant membranes, and this pool exhibits a positive role in STIM1-Orai1 coupling. More recent data provides evidence for preferential association of Orai1 with detergent-resistant, liquid order-preferring lipids (D. Holowka, unpublished results; [9]).

TRPC channel proteins also appear to participate in Ca²⁺ mobilization in mast cells. Ma and Beaven [10] provided evidence that TRPC5 contributes to SOCE in RBL mast cells, and Cohen et al. [11] described a role for TRPC1 in the initiation of antigen-stimulated Ca²⁺ waves that emanate from the tips of cell protrusions to contribute to spatially regulated granule exocytosis. Suzuki et al. [12] also provided evidence for a role for TRPC1 in mouse bone marrow-derived mast cell degranulation. The mechanism of activation of these TRPC channels is not fully understood, but the Ambudkar laboratory has described evidence for STIM1-mediated activation that involves association of TRPC proteins with Orai proteins in other cell types [13].

Another recent study pointed to additional regulatory roles for FcεRI in signaling events with the identification an alternate-spliced form of the β subunit of FcεRI that regulates functional responses in mast cells [14]. This variant does not contain exon 3, which includes the first two

transmembrane sequences of the tetraspan Fc ϵ RI β protein. In addition to contributing to inhibition of degranulation responses in both mouse and human mast cells, knockdown of this variant, together with knockdown of full-length Fc ϵ RI β , causes greater reduction in Ca²⁺ responses stimulated by either antigen or the ER Ca²⁺ ATPase inhibitor thapsigargin than knockdown of full length Fc ϵ RI β alone. Earlier signaling events in antigen-mediated Fc ϵ RI responses are not detectably affected by knockdown of this altered splice form of Fc ϵ RI, suggesting a more downstream role for this protein in mast cell stimulation. The mechanism by which this alternatively spliced form of Fc ϵ RI β participates in these cellular responses is not yet clear, but some evidence points to regulation of microtubule assembly as a possible explanation [14].

Ca²⁺ oscillations and phosphoinositides

We previously established that Rho family GTPases Cdc42 and/or Rac1 participate in Fc ϵ RI-mediated Ca²⁺ mobilization and degranulation [15,16], but the mechanistic basis was not determined. These studies took advantage of the RBL mutant cell line B6A4C1, which is defective in Fc ϵ RI-mediated, Rho family-dependent signaling. Expression of a constitutively GTP-bound mutant, Cdc42 G12V, reconstituted normal antigen-stimulated Ca²⁺ mobilization and granule exocytosis in these cells, providing evidence that their principal defect is activation of Cdc42, which normally results from receptor stimulation. More recently, we found that these mutant B6A4C1 cells do not sustain normal antigen-stimulated Ca²⁺ oscillations, and that these oscillations can be restored by a Dock family Rho GEF or by constitutively active Cdc42 G12V, but not by Cdc42 G12V/QQ with two arginine residues in its C-terminal sequence mutated to glutamines [17]. Furthermore, we showed that these mutant cells are defective in a Ca²⁺-independent process, antigen-stimulated Fc ϵ RI endocytosis, and this can be reconstituted by Cdc42 G12V expression, similar to reconstitution of Ca²⁺ oscillations. These and other results led us to hypothesize that activated Cdc42 participates in both of these activities by facilitating PIP₂ synthesis, which is necessary for both sustained Ca²⁺ oscillations and for Fc ϵ RI endocytosis. Monitoring PIP₂ at the plasma membrane with a fluorescent protein-tagged PIP₂-specific PH domain, PLC δ -PH-EGFP, we found that oscillations in PIP₂ levels stimulated by antigen in the parent RBL 2H3 cells are absent in the mutant B6A4C1 cells, but they are restored by expression of Cdc42 G12V. These results strongly support the hypothesis that stimulated PIP₂ synthesis is important to maintain sustained Ca²⁺ oscillations that are necessary for granule exocytosis.

Other studies on phosphoinositides in IgE/Fc ϵ RI signaling yielded evidence that two different inhibitors of antigen-stimulated Ca²⁺ mobilization and degranulation, phenylarsine oxide and quercetin, are effective because they inhibit PI4-kinase and PIP5-kinase, respectively. These small molecule inhibitors of PI kinases also prevent antigen-stimulated morphological changes and Fc ϵ RI endocytosis in RBL mast cells [18], further implicating phosphoinositide synthesis in these multiple functional outcomes of IgE/Fc ϵ RI crosslinking and activation. These results also establish phenylarsine oxide and quercetin as useful pharmacologic tools for investigating roles for phosphoinositides in cellular processes.

STIM1-Orai1 coupling

We recently characterized the mechanism by which polyunsaturated fatty acids (PUFAs), including linoleic acid (C18:2 (n-6)), cause rapid inhibition of STIM1-Orai1 coupling. In this study, we developed an effective fluorimetry-based fluorescence resonance energy transfer (FRET) method for monitoring stimulated association of fluorescently tagged STIM1 and Orai1 in transfected COS7 and RBL cells, and we found that micromolar concentrations of linoleic acid

disrupt the physical coupling between these proteins in parallel with inhibition of SOCE. Saturated fatty acids of the same acyl chain length do not inhibit STIM1/Orai1 coupling, and the mechanism for this inhibition is downstream of FcεRI-mediated tyrosine phosphorylation [19]. Our current evidence suggests that PUFAs inhibit STIM1/Orai1 coupling by disrupting ER membrane organization, possibly by electrostatic interference with oligomerization of STIM1 dimers or their conformational transition that is normally activated by depletion of Ca²⁺ from ER stores.

We and others continue to characterize changes in the structural interactions occurring between STIM1 proteins and between STIM1 and Orai1 that result from Ca²⁺ depletion from ER stores and lead to STIM1-Orai1 coupling in SOCE. We observed that store depletion increases FRET between C-terminally labeled STIM1-AcGFP and STIM1-mApple, consistent with expectations of oligomerization from previous studies with N-terminally labeled STIM1 donors and acceptors [20]. However, if the calcium activation domain (CAD) of STIM1 is deleted from the cytoplasmic segment of acceptor STIM1-mApple, we observe a time-dependent *decrease* in FRET between wt STIM1-AcGFP and this STIM1-mApple ΔCAD upon stimulation of store depletion, even though these proteins oligomerize and co-localize in stimulation-dependent puncta (Korzeniowski et al., submitted for publication). A minimal model to account for these results is that full-length STIM1-AcGFP undergoes a conformational change in its C-terminal segment during activation, causing the AcGFP donor probe to move farther away from the acceptor probe, mApple, on STIM1ΔCAD, which does not undergo a conformational change. Our further experiments identified a 14 amino acid sequence just C-terminal to CAD that is necessary for the conformational transition in the donor protein: The stimulated decrease in FRET observed requires that the donor construct STIM1-AcGFP contains both the CAD sequence and the subsequent 14 amino acids (STIM1 1-462), but no additional amino acids in the distal C-terminal sequence are needed (Korzeniowski et al., submitted for publication). These results provide the first evidence in live cells for a stimulated conformational change in the C-terminal segment of STIM1, and they complement *in vitro* studies showing a more extended conformation of the C-terminal segment of STIM1 due to activating mutations [21,22].

Mitochondria

It has become increasingly apparent that mitochondria participate in buffering cytoplasmic Ca²⁺ levels, even under conditions of modest elevations in these levels due to receptor-mediated Ca²⁺ mobilization. A recent study has implicated mitochondrial buffering of cytoplasmic Ca²⁺ as a contributor to sustained Ca²⁺ oscillations stimulated by the leukotriene LTC₄ [23]. Molecular mechanisms for this role for mitochondria are just beginning to be elucidated, and the VAP family of ER-localized Type I transmembrane proteins are receiving increasing attention, in part because of a familial genetic mutation in VAP B (P58S) that is a dominant marker for the fatal neuromuscular disease known as amyotrophic lateral sclerosis (ALS; [24]). Because mast cells exhibit robust Ca²⁺ responses to antigens via IgE/FcεRI, we embarked on an effort to investigate the roles for VAP proteins and the effects of this point mutation on these responses.

Initial examination by confocal microscopy of an EGFP-VAP B construct expressed in RBL cells confirmed its distribution throughout the ER, including the nuclear envelope. Expression of mutant EGFP-VAP B P56S showed frequent localization in large ER aggregates throughout the cytoplasm, as expected from similar imaging in other cell types [24,25]. Upon store depletion of Ca²⁺ by thapsigargin, however, VAP B exhibits a tendency to cluster together with STIM1-mRFP in puncta, both for wt VAP B and the P56S mutant, suggesting that activated STIM1 exhibits some preferential association with VAP B (D. Holowka, unpublished results). In initial experiments, we employed siRNA knockdown of VAP A and VAP B to evaluate their contributions to antigen or thapsigargin-stimulated Ca²⁺ responses. We observed modest

inhibition of antigen-stimulated SOCE by VAP A + VAP B siRNA cocktails: 13 +/- 2% (SE, n=4), and similar inhibition by either VAP A or VAP B siRNA alone. In subsequent experiments, we evaluated the effect of the mutant VAP B P56S on responses to either antigen or thapsigargin. We consistently observed an elevation in basal Ca^{2+} levels in the VAP B P56S-expressing cells. In three experiments, these basal levels were $10.1 \pm 0.2\%$ greater than in control, vector only-transfected cells. We also observed that expression of the mutant VAP B protein (P56S) causes about 20% inhibition of antigen-stimulated Ca^{2+} release from ER stores (determined in the absence of Ca^{2+} influx), as well as ~20% inhibition of SOCE by antigen and somewhat less inhibition for SOCE stimulated by thapsigargin, compared to control, vector only, cells (D. Holowka, unpublished results). Over-expression of wt VAP B under these conditions causes statistically insignificant inhibition of stimulated responses. These results suggest that the VAP proteins participate in the regulation of Ca^{2+} mobilization stimulated by antigen in these cells.

To further investigate the mechanism by which VAP contributes to this process, we monitored the level of intramitochondrial Ca^{2+} under stimulating conditions using the FRET-based reporter, mitocameleon [26]. Antigen stimulation by an optimal dose of DNP-BSA resulted in a rapid increase in mitochondrial Ca^{2+} , and knockdown of VAP A + VAP B by siRNA cocktails resulted in substantial inhibition of this response. Stimulation by either antigen or thapsigargin was inhibited by these siRNAs by ~50-60% in multiple experiments (D. Holowka, unpublished results). These results indicate a significant role for VAP A and B in antigen-stimulated Ca^{2+} filling of mitochondria and in the communication between ER and mitochondrial stores.

Other roles for Ca^{2+} responses

Recycling endosomes (REs) are now appreciated as a complex organelle that contributes to multiple trafficking processes in many cell types and, in particular, has been shown to play a role in cellular events that require extensive membrane remodeling, such as migration [27] and cell division [28]. In RBL mast cells, we previously showed that antigen engagement of IgE/Fc ϵ R1 causes stimulated outward trafficking of these RE membranes to the plasma membrane in a Ca^{2+} -dependent manner [29]. To study the dynamics of this process, we constructed several different pHluorin-tagged proteins, including transferrin receptors, VAMP3, and VAMP8, which all co-localize with cholera toxin B bound to the ganglioside GM₁ in perinuclear recycling endosomes. We also constructed and characterized β -hexosaminidase-pHluorin (β -hex-pHluorin), which co-localizes with secretory granule markers. All of these undergo antigen-stimulated outward trafficking that can be monitored as an increase in fluorescence by fluorimetry or by TIRF microscopy. This trafficking, including that of β -hex-pHluorin, is inhibited by dominant negative Rab11, indicating its broad impact on the endolysosomal system (Wilson, J.D., Holowka, D., Baird, B., submitted for publication). A recent study described evidence that Rab11-dependent trafficking of recycling endosomes to the plasma membrane necessarily precedes cytotoxic granule exocytosis in T cells [30], providing a precedent for our finding of a role for Rab11 in secretory granule exocytosis in mast cells. These results offer new insights to recent reports of oscillatory PIP₂ and actin in conjunction with secretory events in mast cells [31], and they provide new tools for elucidating functional roles for recycling endosomal exocytosis in hematopoietic cell biology.

Mast cell chemotaxis toward IgE-specific antigen was first described for RBL mast cells almost 30 years ago, and we recently demonstrated that Ca^{2+} influx via Orai1 plays an important role in regulating both spontaneous motility and directional migration of mast cells toward stimulating antigen [32]. Furthermore, RBL cells expressing the Ca^{2+} sensor, GCaMP3, exhibit spontaneous Ca^{2+} transients that depend on Ca^{2+} influx, and the appearance of these transients correlates with spontaneous cell motility [26].

We discovered that infection of RBL mast cells by the obligate intracellular parasite, *Toxoplasma gondii*, inhibits antigen-stimulated Ca^{2+} mobilization and granule exocytosis, and phosphorylation of phospholipase C α by the tyrosine kinase Syk appears to be the earliest biochemical event that is disrupted in this process. Interestingly, inhibition of IgE/Fc ϵ R1 signaling is retained when tachyzoite invasion is arrested at the attachment stage by cytochalasin D treatment, suggesting that inhibition of Syk is mediated by a parasite-derived factor that is secreted during the invasion process [33]. This study provides the first direct evidence that initiation of immune subversion by *T. gondii* occurs concurrently with invasion. Infection by *T. gondii* often suppresses immune responses in host cells, and we hypothesize that this suppression results from inhibition of Ca^{2+} -dependent transcriptional activation of cytokine genes via NFAT and/or NF κ B.

In summary, Ca^{2+} mobilization is a ubiquitous mediator of mast cell functions in adaptive immune responses, and the mechanisms by which this central process is regulated by phosphoinositides and by mitochondrial Ca^{2+} buffering are being defined from a variety of experimental approaches. Questions for the future include the molecular mechanism by which the Rho family protein, Cdc42, regulates PIP $_2$ and Ca^{2+} oscillations in these cells. The mechanism by which PUFAs regulate STIM1-Orai1 coupling remains to be determined, as does the apparent role of ordered membrane structure in the activation of SOCE. Recent evidence for participation of VAP B in ER-mitochondrial Ca^{2+} coupling summarized here warrants further investigation. Potential contributions of these particular proteins (Cdc42, VAP A and B) and the processes they regulate (Ca^{2+} mobilization, RE exocytosis) to the mechanism of mast cell chemotaxis are also intriguing and compel pursuit of more detailed information. Lastly, the generality of our findings regarding regulation of stimulated mast cell Ca^{2+} mobilization and granule exocytosis by *T. gondii* infection for its suppression of immunity in other hematopoietic cells and their responses is another question that will drive future efforts in understanding the ubiquitous roles for Ca^{2+} -mediated signaling in immune responses.

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Figure Legends

Figure 1. Schematic of intracellular signaling after antigen-mediated crosslinking of IgE-FcεRI on the surface of mast cells. Binding of antigen-specific IgE to FcεRI sensitizes these receptors to antigen-stimulated signaling that is initiated by a tyrosine phosphorylation cascade. IP₃ produced by hydrolysis of PIP₂ activates SOCE that is important for granule exocytosis. Activation of protein kinase C (PKC) contributes to the exocytotic response. As described in this review, mitochondria contribute to buffering of cytoplasmic Ca²⁺ levels. Adapted from [9].

Figure 1

