Chapter 16
Drebrin Regulation of Calcium Signaling in Immune Cells

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Abstract Store-operated Ca^{2+} channels are plasma membrane channels that are activated by depletion of intracellular Ca^{2+} stores, resulting in an increase in intracellular Ca^{2+}; however, little is known about their regulation. Our work has shown that the immunosuppressant compound BTP2, which blocks Ca^{2+} influx into cells, interacts with the actin-reorganizing protein, drebrin. Here we review the role of drebrin in the regulation of calcium signaling, with a focus on immune cells.

Keywords Store-operated Ca^{2+} channel • BTP2 • Filopodia extension • Jurkat T cells • Acute IgE challenge • FcεRI-mediated actin organization

Abbreviations

BTP2	N-(4-(3,5-*bis*(trifluoromethyl)-1H-pyrazol-1-yl)phenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide
CRAC	Calcium-release-activated channel
Dbn1	Drebrin
FcεRI	High-affinity receptor for Fc portion of IgE
LatB	Latrunculin B
NFATc1	Nuclear factor of activated T-cell cytoplasmic 1
RBL	Rat basophil leukemia
STIM1	Stromal interaction molecule 1
TRPC	Transient receptor potential channel
WASP	Wiskott-Aldrich syndrome protein
WIP	WASP-interacting protein

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16.1 Introduction

A number of physiological processes respond to increases in intracellular Ca^{2+} levels, including the regulation of the immune response. Receptors that stimulate phospholipase C generate the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) as a result of hydrolysis of phosphoinositol 4,5-bisphosphate (PIP_2). IP_3 induces elevation of intracellular Ca^{2+} via the IP_3 receptor (IP_3R), Ca^{2+} release channels in the endoplasmic reticulum (ER) membrane. Furthermore, when Ca^{2+} stores in the ER is depleted, this stimulates a continued increase in intracellular Ca2+ by activating store-operated channels (SOCs) via store-operated Ca^{2+} entry (SOCE) (Putney 1986, 1990; Smyth et al. 2006), which allows Ca^{2+} to enter into the cell. This store-operated Ca^{2+} entry or SOCE plays critical roles in regulating the function of immune cells (Bergmeier et al. 2013). The mechanism by which this occurs continues to be under intense investigation. In this chapter, we review the evidence that this process is regulated in part by the actin cytoskeleton and that the actin-regulating protein drebrin plays an important role in this process.

16.2 Store-Operated Calcium Channels in Immune Cells

The channel Orai has been identified as the SOC in immune cells and its activity is modulated by the protein STIM1 (Soboloff et al. 2006; Peinelt et al. 2006; Zhang et al. 2005; Liou et al. 2005; Roos et al. 2005; Yeromin et al. 2006; Prakriya et al. 2006). STIM1 modifies Orai1 by translocating near to the plasma membrane, providing a link between the ER, where calcium is released, and the plasma membrane, where Orai allows Ca^{2+} into the cell (Zhang et al. 2005; Hauser and Tsien 2007; Ross et al. 2007; Wu et al. 2006; Spassova et al. 2006; Mercer et al. 2006). STIM1 is a single-spanning membrane protein with a Ca^{2+}-binding EF-hand motif that is anchored in the plasma membrane. STIM1 functions as the sensor of ER luminal Ca^{2+} levels migrates within the ER membrane to sites near the plasma membrane, interacting Ca^{2+} influx channels leading to their activation. The Orai1 protein is also multi-spanning protein localized in the plasma membrane and functions as the pore-forming subunit of the highly selective CRAC channel in the PM.

16.2.1 Role of SOCE in Immune Function

One of the best characterized Ca^{2+}-dependent transcriptional pathways leads to the activation of nuclear factor of activated T cells (NFAT), essential for transcription of many cytokine genes (Srikanth and Gwack 2013). The absence of Orai leads to a defect in Ca^{2+} influx through CRAC channels, which severely compromises activation of a wide range of immune cells (Prakriya and Lewis 2015). Indeed, mutations

in Orai1 were initially described in human immunodeficient patients that resulted in the ablation of all CRAC channel activity (Feske et al. 2006). Furthermore, the significance of Orai and SOCE in the pathogenesis of immune-related disease is underscored by its role in hypersensitivity disorders of the immune system, including mast cell activation and the generation of allergic reactions (Feske et al. 2015; Ikeya et al. 2014; Ashmole et al. 2012, 2013). In mast cells, FcεRI stimulation induces the liberation of intracellular Ca^{2+} stores and activation of SOCE, which is essential for the degranulation process, as well as the release of chemokines, and cytokines, which contribute to allergic inflammation. Mice lacking Orai exhibit defective mast cell function and allergic responses (Feske et al. 2015).

In mast cells, binding of the Fc portion of IgE to the high-affinity FcεRI triggers an increase in intracellular Ca^{2+}, an essential step for mast cell activation, degranulation, and the generation of a full mast cell response. This FcεRI-triggered increase in Ca^{2+} occurs via the previously described PLCγ pathway, leading to activation of CRAC channels and influx of extracellular Ca^{2+} (Feske 2007; Gwack et al. 2007; Ishikawa et al. 2003). Ca^{2+} increase is also critical for the degranulation of mast cells, releasing histamine and other preformed pharmacological agents from intracellular vesicles (Di Capite and Parekh 2009; Scharenberg and Kinet 1998; Turner and Kinet 1999).

16.2.2 The Actin Cytoskeleton and SOCE

Actin exists in the cell as globular G-actin and filamentous F-actin, and the polymerization of G-actin into F-actin results in the formation of microfilaments in the actin cytoskeleton (Mattila et al. 2016). The actin cytoskeleton has long been suggested to play an important role in the regulation of intracellular Ca^{2+}, although the evidence is conflicting. While actin cytoskeletal changes appear to be dispensable for ER calcium release, preventing actin depolymerization blocks SOC-mediated increase in intracellular Ca^{2+} (Hao and August 2005; Patterson et al. 1999; Smyth et al. 2007; Rosado et al. 2004; Oka et al. 2002). Cytochalasins, actin-depolymerizing agents, have also been shown to attenuate SOCE and Ca^{2+} (Rueckschloss and Isenberg 2001). Furthermore, in T cells, B cells, and mast cells, the actin-depolymerizing agent Latrunculin B is able to increase in intracellular Ca^{2+} (Hao and August 2005; Nolz et al. 2006; Rivas et al. 2004). However, not much is known about the proteins that may regulate this process. Of particular interest is the finding that mast cells from mice deficient in Wiskott-Aldrich syndrome protein (WASP), a key regulatory protein of F-actin assembly, exhibit by diminished Ca^{2+} mobilization, degranulation, and cytokine secretion (Pivniouk et al. 2003). Similarly, cells from mice deficient in other actin-regulating proteins WASP, WIP, and WAVE2 also exhibit defects in Ca^{2+} mobilization (Nolz et al. 2006; Zhang et al. 1999; Kettner et al. 2004). The actin regulators WASP, WIP, and WAVE2 have been shown to regulate SOCE and Ca^{2+} influx into mast cells and/or T cells (Nolz et al. 2006; Pivniouk et al. 2003; Kettner et al. 2004). These results strongly support the view that the actin cytoskeleton plays an important role in regulating intracellular Ca^{2+} mobilization in immune cells.

16.3 Immunosuppressant BTP Is an Inhibitor of Calcium Signaling

The class of compounds called BTPs (3,5-*bis*(trifluoromethyl)pyrazoles), exemplified by BTP2, have been found to inhibit Ca^{2+} entry into cells (Ishikawa et al. 2003; Zitt et al. 2004). Indeed, BTP2 has been shown to block T-cell receptor (TCR)-induced Ca^{2+} entry and Ca^{2+}-dependent cytokine production (Ishikawa et al. 2003; Zitt et al. 2004; Djuric et al. 2000; Trevillyan et al. 2001; Mercer 2005; Mercer et al. 2010). By inhibiting influx of Ca^{2+} into the cells, BTP2 is able to inhibit a wide variety of processes in immune cells, including inhibition of T-cell production of Th1 and Th2 cytokines (Zitt et al. 2004; Djuric et al. 2000), superoxide generation in neutrophils (Steinckwich et al. 2007), mast cell activation, degranulation, and cytokine production following IgE/FcεRI stimulation (Law et al. 2011). In vivo, BTP2 has been shown to be effective in animal models of allergic asthma, reducing Th2 cytokines and leukotrienes, inhibiting eosinophil infiltration into the lungs, and reducing bronchoconstriction and airway hyperresponsiveness (Law et al. 2011; Ohga et al. 2008).

The mechanism for the effect of BTP2 has been unclear. BTP2 has been reported to decrease Ca^{2+} influx into lymphocytes by enhancing the activity of the TRPM4 (Takezawa et al. 2006) and TRPC3 channels (Kiyonaka et al. 2009). We have shown that BTP2 is able to inhibit mast cell activation and degranulation, which is very dependent on increases in intracellular Ca^{2+} (Melicoff et al. 2009), with an IC_{50} of 23 nM (Law et al. 2011). Furthermore, we have shown that BTP2 is a potent inhibitor of mast cell degranulation in vivo at 10 mg BTP2/kg. BTP2 also inhibited IgE/FcεRI-mediated induction of mast cell production of cytokines IL-3, IL-4, IL-6, TNF-a, and GM-CSF (Law et al. 2011). Using structure-activity relationship analysis, we also showed that the trifluoromethyl group at the C3 of BTP2 is required for its activity, since deleting these trifluoromethyl groups entirely or replacing the trifluoromethyl groups of the BTP ring with less bulky methyl groups completely abrogated the activity of BTP2. By contrast, single replacement of the trifluoromethyl group at the 5- or 3-position led to different effects. While the 5-trifluorome thyl-3-methyl-pyrazole derivate had some activity, the 3-trifluoromethyl-5-methyl-pyrazole derivative of BTP2 had similar activity to the parent BTP2 compound (with an IC_{50} of 25 nM), indicating that the C3 trifluoromethyl group of BTP2 is required for its activity (Law et al. 2011).

These effects of BTP2 are similar to that which has been reported for mast cells lacking either Orai1 or STIM1 (Baba et al. 2008; Vig et al. 2008), characterized by severely impaired degranulation and histamine release, decreased leukotriene production, and cytokine production. These mice also exhibit reduced IgE-mediated allergic response in vivo (Di Capite and Parekh 2009; Baba et al. 2008; Vig et al. 2008). These findings support the conclusion that BTP2 inhibits Ca^{2+} influx into these cells and subsequent downstream functions. Interestingly, our findings on the structure-activity relationships of BTP2 derivatives were similar to that found by Kiyonaka et al. who examined derivatives of pyrazole compounds that target the

TRPC3 channel. Kiyonaka et al. found that bulky functional groups at the 3,5-positions of the pyrazole are important for the inhibition of Ca^{2+} mobilization via the TRPC3 channel (Kiyonaka et al. 2009). Based on these findings, we explored the target of BTP2 as a way to understand the mechanism of action of these compounds and the process by which SOCE and Ca^{2+} influx into cells is regulated.

16.3.1 Drebrin Is a Target of BTP

Using a combination of affinity purification and siRNA approaches, we explored binding partners for BTP2, as a means to identify BTP2-binding proteins. Using a BTP2 affinity column, gel purification, and MALDI/TOF mass-spectrometry-based protein identification, we identified drebrin as a binding partner for BTP2. Of interest is the fact that our BTP2 affinity column purification approach also actin, along with drebrin (Mercer et al. 2010). We found that BTP2 interacted directly with drebrin since drebrin expressed in bacterial cells was also able to interact with BTP2, and soluble BTP2 competed with BTP2 affinity matrix for binding to drebrin.

Further analysis of the BTP2/drebrin interaction revealed that lysines 270 and 271 within drebrin are specifically required for its ability to bind to BTP2. These lysines are found with the central actin-binding domain of drebrin, suggesting that BTP2 may inhibit specific functions of drebrin. When drebrin is overexpressed in adherent cells, it induces the formation of what we have referred to as filopodia-like extensions (FLEs), long, branched extensions and curved, thick actin bundles (Shirao et al. 1994). The formation of these FLEs in adherent cells overexpressing drebrin is significantly reduced by BTP2 treatment. Specifically, BTP2 is able to reduce the number of branch points formed per each FLE but not in the average length of these FLEs, and BTP2 treated cells had significantly reduced FLEs, and remaining FLEs were long and linear as opposed to branched. These findings suggest that BTP affects drebrin's ability to induce plasticity in the actin cytoskeleton (Mercer et al. 2010). Indeed, treatment of neuronal cells with BTP2 also affected the ability of drebrin to alter the dynamics of dendritic spines (Sonego et al. 2015). By contrast, BTP2 is unable to block the formation of these FLEs in cells that express a K270M K271M mutant that no longer interacts with BTP2 (Mercer et al. 2010).

16.4 Drebrin Regulates Mast Cell Activation by Regulating FcεRI-Mediated Increase in Intracellular Ca^{2+}

The identification of drebrin as a target of BTP2, an inhibitor of SOCE and Ca^{2+} into cells, suggested that this protein plays a role in regulating this process. This model was supported by the fact that siRNA-mediated knockdown of drebrin in Jurkat T

cells resulted in reduced Ca^{2+} influx following stimulation (Mercer et al. 2010). Furthermore, we generated drebrin-deficient mice and analyzed mast cells from these mice to determine whether drebrin indeed plays a role in the ability of these cells to signal by Ca^{2+} (Law et al. 2015). Analysis of mast-celled development revealed that drebrin deficiency leads to reduced density of skin and numbers of peritoneal mast cells, although phenotypic analysis of the skin tissue-resident mast cells did not reveal any gross or ultrastructural differences from WT mice. Interestingly, development of bone-marrow-derived mast cells (BMMCs) in vitro induced by IL-3 and stem cell factor (SCF) was not affected by the absence of drebrin, suggesting that differentiation of mast cells is independent of drebrin expression. By contrast, culture in IL-3 alone, which can also induce the development of BMMCs, resulted in normal differentiation but reduced numbers of BMMCs in the absence of drebrin (Law et al. 2015). These experiments suggest that while not critical for the development of mast cells, drebrin may play a role in their development under resource-limiting conditions such as in the presence of IL-3 alone.

Analysis of the activation of in vitro derived BMMCs revealed that drebrin is important for Ca^{2+} influx following stimulation with IgE/FcεR1, since they exhibit a significant decrease in this response. This was similar to our findings with mast cells treated with BTP2 (Law et al. 2011, 2015). Furthermore, drebrin-deficient mast cells also exhibit reduced production of cytokines in response to IgE/FcεR1, similar to those treated with BTP2. In addition, similar to mice treated with BTP2, drebrin-deficient mice significantly reduced response to acute IgE challenge in a model of passive systemic anaphylaxis, with lower levels of histamine detected in their serum compared to WT mice, as well as reduced temperature loss upon challenge with IgE and allergen (Law et al. 2015).

16.4.1 Drebrin Regulates FcεRI-Mediated Actin Organization in Mast Cells

Drebrin (Dbn1) is frequently found associated with actin in the dendritic spines of neurons and has been shown to interact with and induce changes in the actin cytoskeleton when overexpressed in cells (Shirao et al. 1994; Biou et al. 2008). Since actin has been suggested to play a role in the regulation of Ca^{2+} influx into cells (Nolz et al. 2006; Rivas et al. 2004; Oka et al. 2004; Rosado et al. 2000), we examined the actin cytoskeleton in WT and $Dbn1^{-/-}$ BMMCs. We found that $Dbn1^{-/-}$ BMMCs have higher levels of F-actin than WT cells, which was more distributed inside the cell in contrast with the localization of F-actin in WT mast cells (Law et al. 2015). Interestingly, altering the F-actin superstructure in these mast cells using Latrunculin B was able to partially rescue the ability of the FcεRI to induce degranulation in vitro in response to antigen crosslinking.

Our previous identification of drebrin as a target for the immunosuppressant BTP, a known regulator of intracellular Ca^{2+} influx into cells (Mercer et al. 2010), along with this work supporting a role for drebrin in regulating actin reorganization,

Ca^{2+} influx, and mast cell function, adds support to the idea that the actin cytoskeleton plays an important role in regulating this process. Our work suggests that drebrin may regulate the actin cytoskeleton.

As discussed previously, a role for the actin cytoskeleton in regulating calcium influx into cells has been previously suggested. Our findings with drebrin suggest that as in the case with the actin-regulating protein WIP, drebrin regulates actin changes downstream of the FcεRI. Altogether, our data supports a model where influx of Ca^{2+} is regulated by modulating actin cytoskeleton in part via drebrin, leading to mast cell activation and degranulation.

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