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B Lymphocyte Calcium Influx

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Abstract

Dynamic changes in cytoplasmic calcium concentration dictate the immunological fate and functions of lymphocytes. During the past few years important details have been revealed about the mechanism of store-operated calcium entry in lymphocytes, including the molecular identity of calcium-release activated (CRAC) channels and the ER calcium sensor (STIM1) responsible for CRAC channel activation following calcium depletion of stores. However, details of the potential fine regulation of CRAC channel activation that may be imposed on lymphocytes following physiologic stimulation within an inflammatory environment have not been fully addressed. In this review, we discuss several underexplored aspects of store-operated (CRAC-mediated) and store-independent calcium signaling in B lymphocytes. First, we discuss the potential novel requirement for antigen-receptor linked pathways in initiating CRAC channel activation. Second, we will discuss results suggesting that coupling between stores and CRAC channels may be regulated, allowing for graded activation in response to partial depletion of ER stores. Third, we will discuss mechanisms that sustain the duration of calcium entry via CRAC channels. Finally, we discuss distinct calcium permeant non-selective cation channels (NSCCs) that are activated by innate stimuli in B cells, potential means by which these innate calcium signaling pathways and CRAC channels crossregulate one another and the mechanistic basis and physiologic consequences of innate calcium signaling.

Keywords

Calcium; CRAC channels; TRP channels; innate signaling

Overview

Calcium (Ca^{2+}) is a multifunctional second messenger that regulates varied and diverse aspects of lymphocyte differentiation and function (1). The mechanisms of antigen-receptor induced calcium signaling have been widely studied. Following the recent identification of Orai and STIM, the molecular components of store-operated calcium entry, efforts to understand the mechanism by which calcium release from endoplasmic reticulum (ER) stores regulates CRAC channel activation (i.e. calcium entry) following antigen receptor engagement have accelerated. A number of excellent reviews have detailed these mechanisms (2–5); consequently, this review focuses on three under-explored aspects of CRAC channel activation in lymphocytes as well as innate mechanisms of calcium entry that occur independently of CRAC channels. First, several studies challenge the dogma that depletion of Ca^{2+} from ER stores is both necessary and sufficient for CRAC channel activation (6,7). Accordingly, we will discuss the potential role of antigen-receptor linked kinases in CRAC channel activation. Second, while store-depletion-induced CRAC channel

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activation has generally been considered an “all or none” event, most studies are carried out under conditions of maximal calcium depletion of ER stores. As it is not yet clear if these responses fully reflect those induced by physiological stimuli *in vivo*, we will discuss results suggesting that coupling between stores and CRAC channels is regulated, allowing for graded CRAC channel activation in response to partial depletion of ER stores. Third, we will consider the fact that the duration of calcium signaling required for transcription factor activation likely exceeds the duration of proximal BCR induced signals that mobilize calcium. Therefore we will discuss mechanisms that sustain the duration of calcium entry via CRAC channels. Finally, it is becoming increasingly evident that CRAC channels are not solely responsible for calcium signaling in lymphocytes. Specifically, distinct calcium permeant non-selective cation channels are activated by innate stimuli in B cells. As these channels have been shown to have a significant functional impact on B cells, we will assess the mechanistic basis and physiologic consequences of such signaling.

Regulation of cytoplasmic calcium levels lymphocytes

Receptor-induced alterations in intracellular calcium levels regulate most aspects of lymphocyte differentiation and function (1). For example, Ca^{2+} plays a critical role in the regulation of lymphocyte cell shape and polarity (8,9), localization of antigen receptors with coreceptors and proximal signal transduction effectors (10,11), transcription factor activation/translocation (12–15), and gene expression (1,16). Consequently, pathways that dynamically regulate free intracellular Ca^{2+} concentration levels play a primary and fundamental role in immune regulation.

Calcium levels are dynamically regulated in lymphocytes by 1) intracellular receptor/channels, including IP_3 receptors, responsible for Ca^{2+} release from internal stores, 2) plasma membrane channels, including Calcium Release Activated Calcium (CRAC) channels, responsible for Ca^{2+} entry 3) the resting membrane potential (membrane potential, $[V_m]$), set by voltage-gated and calcium-activated K^+ channels (17,18), which provides the electrical driving force for Ca^{2+} entry, 4) energy dependent transporters (19), 5) non-selective cation channels (NSCCs) (20–23), 6) the Sarco-Endoplasmic Reticulum ATPase (SERCA pump) and Plasma Membrane Ca^{2+} ATPase (PMCA pump), which return Ca^{2+} to intracellular stores and extrude it from the cell, respectively (24), and 7) buffering by mitochondria (25–28). Moreover, changes in intracellular calcium concentration further regulates calcium dynamics by potentiating or attenuating CRAC channel (17,26,29–35) and IP_3R activity (36). The integrated effect of all of these elements acting in concert ultimately shapes the calcium signals that specify the distinct functional response of B cells (13,15,37,38).

Proximal mechanisms of BCR-induced Ca^{2+} signaling in B cells

Signaling through the BCR complex is mediated by immunoglobulin-associated proteins $\text{Ig}\alpha$ and $\text{Ig}\beta$, which have ITAM (immunoreceptor tyrosine based activation motif) signaling domains within their large cytoplasmic tails (39) (Figure 1). Following BCR engagement, Src family (Lyn) kinase-mediated phosphorylation of tyrosines within the $\text{Ig}\alpha$ and $\text{Ig}\beta$ ITAMs facilitates recruitment and activation of Syk kinase (40) to the signaling complex. The subsequent Syk-mediated phosphorylation of adapter proteins, which lack catalytic or enzymatic activity, facilitates protein-protein and -lipid interactions between members of the signaling cascade (reviewed in (41,42). An important adaptor in BCR signaling is SLP-65 (or BLNK, (43)) and phosphorylation of SLP-65 tyrosines facilitates its association with Btk and $\text{PLC}\gamma 2$, thereby promoting the activation of these two signaling molecules (44). One of the primary consequences of these complex interactions is the activation of $\text{PLC}\gamma 2$, which hydrolyzes inositol (4,5)-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol (1,4,5)

triphosphate (IP₃). IP₃ binds to and activates channels (IP₃ receptors [IP₃R]) on the ER membrane (for review see (45), causing Ca²⁺ to be released from ER stores. Following IP₃R/channels activation ((46), for review see (36,47), the resulting depletion of ER Ca²⁺, (not the increase in cytoplasmic [Ca²⁺]) is a central and prerequisite event in the activation of plasma membrane “store-operated” CRAC channels and Ca²⁺ entry (2,48,49). Thus, IP₃ is the primary physiological trigger for store-operated activation of CRAC channels, a critical event in the response of B cells to antigen stimulation.

Molecular mechanism of store-operated calcium entry in lymphocytes

While CRAC channel activation and regulation have been studied extensively in lymphocytes (50–53), the gene which encodes the channel pore (Orai or CRACM, (54–56)) and the proteins that sense calcium levels within the ER (Stromal Interaction molecule (STIM) proteins (57,58)) and relay this information to CRAC channels were identified only recently. CRAC channels are composed of tetramers of Orai subunits (59) with cytoplasmic N- and C-terminal tails. Mutagenesis studies of Orai have identified several residues which dictate ion selectivity, conductance, and sensitivity to divalent cations, establishing Orai as the essential pore forming subunit of CRAC channels (60–62). Of the three known Orai isoforms, Orai1 is the biologically significant variant that encodes CRAC channels expressed in lymphocytes. The critical importance of Orai1 for lymphoid function is evident from a study of two individuals, born to consanguineous parents, who presented with profound immune deficiency due to a point mutation in the protein (54).

STIM1 is a ubiquitously expressed Type I ER transmembrane protein whose N-terminus resides within the ER lumen (57,58). This luminal domain includes calcium-binding EF hand and sterile- α motif (SAM) domains (63). Estimates of the calcium affinity of the STIM1 EF hand domain are in the range of 200–600uM, which is comparable to estimates of the free calcium concentration in the ER (64). Studies of the atomic structure of the EF hand and SAM regions have also revealed an additional “hidden” EF hand, which together with the previously identified domains form a stable intramolecular association when stores are replete with calcium (63,65), generating a folded “inactive” conformation in unstimulated cells.

Following receptor engagement, IP₃-mediated depletion of ER calcium stores triggers destabilization and unfolding of the EF hand-SAM structure of STIM1, leading to its oligomerization and subsequent aggregation (59,66–69). Microscopically visible STIM1 puncta relocate to subdomains of junctional ER, (58,70–75) and move into juxtaposition with oligomers of Orai1 at the plasma membrane (66,69,76). The kinetics of STIM1 puncta formation is relatively slow and half maximal localization in Jurkat T cells in response to store depletion with EGTA occurs over a period of about 30 seconds (71). Importantly, CRAC currents develop with kinetics that are similar to STIM1 aggregation, but with a delay of approximately 20 seconds (67), consistent with a role for STIM1 in activating CRAC channels. Indeed, it was recently shown that direct interactions between C-terminal domains in STIM1 and both the N- and C-terminus of Orai1 trigger channel activation (77,78). Due to this requirement, Ca²⁺ influx through CRAC channels in lymphocytes is not widely dispersed across the cell plasma membrane, but appears to be limited to CRAC channels juxtaposed to STIM1 puncta. The clustering of STIM1 and Orai in lymphocytes around the “immune synapse” (66) suggests a role for local calcium increases in regulating downstream antigen receptor-induced signaling events. Conversely, as CRAC channels co-cluster with the antigen receptor, signaling effectors activated by the antigen receptor may function in the direct regulation of CRAC channel activation (see Figure 1). In fact, the activity of other calcium permeant channels, including TRPC family members, are known to

be regulated by direct interactions with PLC γ isoforms via a split PH domain (79–81); however, whether similar interactions regulate CRAC channel activity is currently unknown.

The mechanisms which regulate STIM1 aggregation and repositioning and its subsequent colocalization with Orai are yet to be defined. A polybasic domain near the STIM1 C-terminal tail targets STIM1 to ER-plasma membrane junctions independently of the CAD/SOAR domain required for activation of channels (77), indicating that these two processes may be independently regulated. Targeting to the ER-plasma membrane junction may reflect an interaction of the polybasic domain with negatively charged phospholipids that preferentially localize to the antigen receptor signaling complex. Remarkably, given the incredibly precise choreography required to colocalize STIM1 and Orai 1, a clear role for the cytoskeleton in the process of antigen receptor mediated calcium signaling has not yet been identified. In fact, it appears that the cytoskeleton is not essential for STIM1/Orai1 relocation, but instead may primarily be involved in regulating the size and spacing of STIM1/Orai1 puncta (74), thereby fine tuning membrane targeting of STIM1 and Orai1.

Is depletion of ER Ca²⁺ stores all it's CRACed up to be?

Although current dogma suggests that ER calcium store depletion by physiological stimuli is necessary and sufficient for CRAC channel activation, results from several laboratories suggest that critical aspects of this mechanism require further exploration. First, while depletion of ER calcium stores is necessary for channel activation, it is becoming increasingly apparent that store depletion may not be sufficient to do so. It is yet unknown whether CRAC channel activation and calcium entry are an irrevocable and inevitable consequence of calcium store depletion. Second, the mechanisms that sustain calcium entry over a period of time that exceeds the duration of proximal BCR signaling and IP₃ generation are unresolved. Data that suggest potential mechanisms for these issues are presented below.

Distal regulation of CRAC channel activity by proximal BCR effectors

While it is well accepted that BCR-linked kinases Lyn and Syk, and the adaptor SLP-65 play an important proximal role in initiating BCR-induced calcium signaling by virtue of their role in promoting IP₃ generation (see above), several studies suggest that these effectors also regulate coupling between calcium stores and CRAC channel activation in B cells. Specifically, such conclusions can be drawn from experiments in which Syk and SLP-65 deficient DT40 B cells were stimulated with IP₃ or thapsigargin to directly deplete calcium from stores, thereby circumventing the critical proximal role for these effectors in the generation IP₃ required for BCR-induced store-mediated CRAC activation. Notably Syk and SLP-65 deficient DT40 B cells exhibit defective calcium entry in response to store depletion by these agonists. Electrophysiology studies confirm these findings by further demonstrating defective CRAC channel activation in Lyn^{-/-} Syk^{-/-} DT40 B cells, and in SLP-65 deficient cells stimulated with thapsigargin or IP₃ (7), and similar results were obtained in WT B cells treated with Lyn and Syk inhibitors (B.F. unpublished data). As CRAC channel activation was not disrupted in Lyn^{-/-} DT40 B cells, Lyn and Syk appear to have distinct roles in coupling; however, it is likely that the pathways that they utilize converge on SLP-65, since Lyn^{-/-} Syk^{-/-} DT40 B cells and SLP-65 deficient cells share a similar phenotype.

If activated Lyn and Syk are indeed required for the initiation of CRAC channel activity, it is not readily apparent how store agonists such as IP₃ or thapsigargin would ever induce calcium influx. In this regard, it is possible that the low levels of Lyn and Syk activity observed in resting B cells as a result of tonic BCR signaling (82,83) may be sufficient to initiate CRAC activation. Alternatively, Syk may be activated by calcium released from

stores in response to IP₃ or thapsigargin (84). Whether thapsigargin-induced calcium release from stores activates Syk sufficiently to support coupling between stores and CRAC channels, and whether primary mammalian B cells exhibit a similar requirement for Syk, Lyn, and SLP-65 in the initiation of CRAC channel activation remains to be determined.

The data outlined above clearly challenge the concept that store depletion alone is both necessary and sufficient for activating CRAC channels. In further support of this idea, we have recently shown that a protein associated with the actin cytoskeleton, namely WAVE2, plays a critical role in CRAC channel activation. We have determined that WAVE2 regulates TCR-mediated calcium entry, but not calcium release from stores (85). Regulation of calcium entry by WAVE2 is not dependent upon its interactions with actin, but rather is mediated by the N-terminal WHD domain of WAVE2 which interacts with a complex of proteins including Rac1, Hem1, PIR121, and Abi1/2 (B.F., unpublished data). Although the precise mechanism by which WAVE2 regulates coupling of store depletion to CRAC channel activation is the focus of ongoing efforts, these studies demonstrate WAVE2, a protein with no intrinsic enzymatic activity, regulates the coupling between calcium store depletion, CRAC channel activation, and calcium influx.

Another molecule that appears to be involved in the direct regulation of Ca²⁺ influx is Btk, a B cell kinase that binds to SLP-65 and regulates PLCγ-2 activation and IP₃ generation (86). In B cells, Btk membrane localization is negatively regulated by PKCβ, which is itself activated by PLCγ-2-mediated increases in DAG and Ca²⁺ (10). In PKCβ deficient B cells (10) and in primary B cells treated with PKC inhibitors, BCR-induced Ca²⁺ signals are dramatically enhanced due to an augmentation of CRAC currents and Ca²⁺ influx via CRAC channels (B.F., unpublished data). These data suggest that localization of Btk to the plasma membrane potentiates CRAC channel activity. It is unclear if Btk directly regulates either CRAC channel activity or the coupling between intracellular Ca²⁺ stores and CRAC channels; however, this Btk-mediated effect is distinct from direct effects reported for PKC on CRAC channel inactivation (87–89). These data together demonstrate the flexibility that exists in the extent of CRAC channel activation following depletion of ER stores and indicates that, at least in B cells, CRAC channel activity can be regulated by mechanisms that are independent of depletion of ER calcium stores.

The ER calcium threshold for CRAC channel activation

Studies assessing CRAC channel activation typically utilize reagents such as the SERCA inhibitors (thapsigargin and cyclopiazonic acid (CPA)), ionophores (ionomycin), calcium chelators (EGTA and BAPTA), or antibodies that cross link ligand receptors to trigger maximal depletion of calcium from ER stores. Using these approaches, Orai1 activation appears to be tightly coupled to and maximally activated in an “all or none” fashion by store depletion. In Jurkat T cells STIM1 activation and CRAC channel activation exhibits a similar non-linear relationship to steady state ER calcium levels following progressive depletion with the SERCA inhibitor CPA (67). Similarly, data obtained in mast cells revealed that the threshold for calcium depletion of stores and activation of I_{crac} by IP₃ was also non-linear, with maximal CRAC channel activation occurring over a very narrow IP₃ concentration range (90). However, the non-linearity of CRAC channel activation by IP₃ may reflect rapid IP₃ metabolism, as a relatively non-metabolizable IP₃ analog induced dose-dependent activation of CRAC channels (91).

This highly non-linear dependence of STIM1 redistribution on [Ca²⁺]_{ER} is indicative of a cooperative mechanism of STIM1 activation, and the practical consequence is that STIM1 redistribution and CRAC channel activation are activated in a relatively binary fashion by [Ca²⁺]_{ER}. This is somewhat surprising in that it would seem to limit the possibilities for regulation of CRAC channel activation by ER calcium; however, these results do not,

preclude the possibility that modulation of channel activation occurs at a step distal to STIM1 activation. Thus additional molecules, in addition to those described above for B cells, such as the microtubule cytoskeleton ((92,93) positioned at contact sites between STIM1 and Orai1 (94) may regulate channel activation and calcium entry and play a role in generating dynamic changes in calcium required for triggering distinct fates of cells. Moreover, recent studies have identified the domain (CAD/SOAR/OASF) within the cytoplasmic domain of STIM1 that interacts with and activates Orai (77,78,95). Data suggest that the CAD domain within STIM1 may not be fully accessible to Orai and that the STIM1 C-terminal tertiary structure may also regulate the gain of coupling between stores and the channel. Currently we have no indication how this apparent conformation might be regulated in B cells, but it is possible that BCR effectors could be involved. Moreover, given that Orai1 and STIM1 expression levels influence the amplitude of whole cell CRAC currents (96), it is possible that, under physiological conditions, the amount of STIM1 that is available for interaction with Orai could also dictate the amplitude of the calcium entry signal.

Mechanisms of sustained CRAC channel activation and calcium entry

Although we have learned much about the mechanistic basis of CRAC channel activation in lymphocytes, including steps involved in calcium-dependent activation of STIM1 and the requirement for direct interactions with Orai, important aspects of the picture are still incomplete. For example, within minutes of B cell activation, the antigen receptor internalizes (97,98), Syk, Lyn and PLC γ -2 activities decrease, and IP₃ is degraded and stores refill ((23), B.F., unpublished data). Nonetheless, elevations in calcium persist and are necessary to activate, among other things, transcription factors such as NFAT (14,15). Given the transient nature of proximal BCR signaling, including the rapid degradation of IP₃, one wonders how cytoplasmic calcium elevations are sustained. If the primary factor dictating the duration of CRAC channel activation is the calcium content of stores (see above), what is the mechanisms of sustained calcium entry via CRAC channels when calcium stores are refilled and STIM1 presumably resets. In order to fully address these questions, it will be necessary to precisely resolve the relationship between ER Ca²⁺ levels and the disassembly or dissipation of STIM oligomers, and the mechanism and consequence of STIM1 disassociation from Orai1 on channel activity and sustained calcium entry

It is possible that mechanisms in addition to or distinct from ER calcium concentration independently regulate the disengagement of STIM1 and Orai. Given recent results implicating Lyn, Syk, SLP-65, and WAVE2 in the distal regulation of CRAC channel activation, the question remains as to whether these or additional molecules interact with Orai or STIM to regulate sustained channel activity independently of the calcium content of stores. Although SLP-65 is a substrate for Syk, neither Syk kinase activity nor the Syk tandem SH2 domains that anchor Syk to the Ig α and Ig β ITAMS, are required for SLP-65/Syk regulation of distal calcium signaling (99), suggesting that Syk couples SLP-65 to calcium entry in B cells via a kinase-independent mechanism. In fact, following BCR internalization, the pY630 within the Syk kinase domain mediates an association with the SH2 domain of membrane anchored SLP-65, and this interaction appears to be required for sustained calcium signaling (6) (Figure 1). Thus, Lyn, Syk, SLP-65 and possibly WAVE2 may form a signaling complex distinct from that involved in proximal signaling that regulates sustained CRAC channel activity

Innate mechanisms of calcium entry in B lymphocytes

A critical feature of adaptive immune responses is the ability of a small subpopulation of lymphocytes with receptors specific for a particular antigen to proliferate and produce antibodies and immuno-regulatory cytokines in response to that antigen, thereby

orchestrating an antigen-specific immune response. However, B cells also can be activated in a polyclonal antigen receptor-independent manner *in vivo* and *in vitro* following stimulation with either highly mitogenic pathogen-associated molecular patterns (PAMPs) that bind to pattern recognition receptors (PRRs) or other innate nonmitogenic stimuli to elicit robust proinflammatory responses. Given that dynamic changes in intracellular calcium are necessary and sometimes sufficient to trigger a range of proinflammatory responses by B cells, we examined the mechanisms of calcium signaling initiated by innate stimuli in primary B lymphocytes.

While it is established that sustained calcium signals triggered by antigen receptor engagement result from calcium entry through CRAC channels, our laboratory has shown that, in addition to CRAC channels, several distinct calcium permeant transient receptor potential (TRP) channel family members are expressed in B cells. Of the three major mammalian TRP-like gene families identified (for reviews see (100–104)), primary B lymphocytes express mRNA for TRPC1, TRPC3, TRPC6, TRPV2, TRPV4, TRPM1, TRPM5, and TRPM7 (22). Most TRP family members are permeable to both Na⁺ and Ca²⁺ and are therefore termed non-selective cation channels (NSCCs). TRP family members are not components of CRAC channels, although recent work suggests that STIM may interact with TRP family members in some cells. This latter finding may explain previous results implicating TRPC1 (105) in BCR-induced store-operated Ca²⁺ entry and TRPC3 in TCR-dependent CRAC-mediated Ca²⁺ entry (106). The fact that certain heterologously expressed TRP fragments modulate CRAC channel activity, may be due to competition for STIM proteins that limits their availability for interaction with and full activation of CRAC channels (107–110).

Activation of TRPV4 like channels by mechanical and osmotic stimuli

Calcium signals direct diverse functions of lymphocytes; therefore, distinct mechanisms must exist to generate fate specific signals. Our efforts to understand the mechanisms of signaling by innate proinflammatory stimuli initially focused on those associated with cell motility and adhesion during lymphocyte migration from the microvasculature into inflamed tissues. We examined the response of B cells to fluid shear and osmotic forces, which elicited robust calcium signals in them (22). The primary channel responsible for mechanically-induced calcium signals was found to have biophysical and pharmacological properties similar to TRPV4 (111). Our efforts to understand how mechanical stimuli activate TRPV4-like channels in B cells revealed unexpectedly diverse and complex mechanisms of calcium signaling involving non-selective cation channels, each of which is activated by a different arachidonic acid (AA) derived product (Figure 2). AA is the major polyunsaturated fatty acid component of the plasma membrane and is a precursor of numerous inflammatory mediators (eicosanoids) including prostaglandins, leukotrienes, oxilipids generated by cytochrome P450 (CYP450) enzymes, and lipoxygenase. In primary B cells, three distinct metabolites of AA (the CYP450 epoxygenase product 5,6-epoxyeicosatrienoic acid (5,6-EET), the cytochrome P450 hydroxylase product 20-HETE, and the 5-lipoxygenase product 5-HPETE) induce Ca²⁺ entry (23) via distinct calcium permeant store-independent NSCCs (21).

An intriguing feature of hypotonicity-induced calcium signaling in B cells is its reliance on phospholipase C (PLC γ -2) mediated hydrolysis of PIP₂. While hypotonic stress induces significant PLC γ -2-mediated IP₃ accumulation and calcium release from stores (23), these stores do regulate hypotonicity-induced NSCC activation, as neither thapsigargin nor IP₃-mediated calcium store depletion activates NSCCs in B cells (22). By contrast, the alternative product of PLC γ -2-mediated PIP₂ hydrolysis, namely DAG, is the upstream mediator of hypotonicity-induced NSCC activation in B cells. Specifically we found that mechanical and osmotic stimulation triggers DAG lipase-mediated cleavage of DAG and

release of AA (23). Notably, although hypotonicity generates AA, calcium entry occurs is mediated by its product 5,6-EET, which directly activates TRPV4-like channels.

While BCR engagement also triggers AA production in mature murine B cells, only mechanical stimuli activates TRPV4-like channels. At first glance, these observations are seemingly paradoxical; if BCR engagement generates DAG, then why aren't NSCCs activated in BCR-stimulated B cells? Although the mechanistic basis for this apparent anomaly is unresolved, it is possible that crucial DAG metabolites such as AA that are generated in response to BCR engagement do not reach stimulatory levels in proximity to these channels. Given that hypotonicity elicits significantly higher steady state IP₃ levels than does BCR engagement (23), it is possible that lower DAG levels (equimolar to IP₃) generated by the BCR represent insufficient substrate to produce stimulatory levels of AA and 5,6-EET for NSCC activation.

Finally, it is also somewhat unexpected that hypotonicity promotes IP₃-mediated calcium release from ER stores, yet store-operated CRAC channels contribute relatively little, if at all, to sustained hypotonicity-induced calcium signals (23). Specifically, the NSCC inhibitor ruthenium red fully suppresses hypotonicity-induced and 5,6-EET-induced calcium signaling to the same extent, yet it has no effect on BCR induced calcium entry. These results indicate that hypotonicity-induced calcium entry is predominantly mediated by TRPV4 like NSCCs. Given that hypotonicity elicits IP₃-mediated store release but does not trigger measurable CRAC channel mediated calcium entry, these results suggest that hypotonicity-activated mechanisms suppress CRAC channel activation and/or calcium entry. To do this, hypotonicity may inhibit coupling between store depletion and CRAC channel activation. It is possible that requisite signals, such as Syk or Lyn activation, that are necessary for post-store coupling (see above) are either not induced or are directly inhibited by hypotonicity-induced events. It is also possible that DAG-dependent PKC activation may potentiate CRAC channel inactivation (87). Alternatively, negative regulation of CRAC channels by hypotonicity may result from (DAG- and calcium-induced) PKC β -mediated Btk phosphorylation, blocking its localization to the BCR signaling complex, and thereby attenuating sustained calcium entry via CRAC channels ((10,86), B.F. unpublished data).

Finally, it is possible that hypotonicity-induced suppression of CRAC channel function is an indirect consequence of NSCC mediated plasma membrane depolarization (21). Work from a number of laboratories demonstrates that a hyperpolarized plasma membrane is required for maximal Ca²⁺ permeation of CRAC channels (17,33,112,113). Because NSCCs are Na⁺ as well as Ca²⁺ permeant and because they have a relatively large conductance (compared with CRAC channels), cation (calcium and sodium) entry via TRP channels depolarizes the plasma membrane and thereby dissipates the electrical driving force for calcium entry through CRAC channels (21). Calcium entry through CRAC channels exhibits a steep dependence on the plasma membrane electrical potential and even modest changes in V_m can fully attenuate calcium entry through open channels (112).

Activation of TRPC3 by pathogen derived CpG DNA

Our recent efforts to understand mechanisms of calcium signaling in B cells have focused upon BCR-independent pathogen signaling pathways. While it is well established that a range of pathogen-associated molecular patterns (PAMPs) are polyclonal B cell mitogens, if and how these molecules mobilize calcium and the role of calcium in these inflammatory responses had not been defined. We focused on unmethylated CpG DNA, the ligand of Toll-like receptor-9 (TLR9), because we found that it triggered a robust calcium signal in primary B cells. We went on to show that CpG-induced calcium influx occurs via store-independent calcium permeant TRPC3 NSCC channels. Notably, we found that TRPC3 activation by CpG does not involve TLR9, but rather utilizes the scavenger receptor B1 (SR-B1),

providing the first evidence that CpG stimulation of B cells occurs through multiple receptor systems (114) and that these functionally distinct receptors also mobilize calcium via a distinct mechanism than the BCR (Figure 3). Scavenger receptors are a diverse family of surface molecules that recognize a broad range of endogenous and pathogen-derived molecules (115,116). They are emerging as an important class of activating or co-activating receptors, which like TLR initiate and modulate immune responses via calcium dependent mechanisms.

Among the intriguing findings to emerge from our studies of SR-B1 signaling in B cells is that CpG triggers calcium release from stores yet it does not elicit detectable CRAC currents. This apparent uncoupling of store depletion from CRAC channel activation lends support to the idea promoted in this review that store depletion may be necessary but not sufficient for CRAC channel activation. A clue into this apparent block in CRAC channel activation was the finding that SR-B1 activation of TRPC3 requires PLC γ -1. This is in contrast with BCR-induced CRAC channel activation, which requires the generation of IP₃ by PLC γ -2. Interestingly TRPC3 activation by PLC γ 1 appears to be independent of its phosphorylation status or enzyme activity and rather involves direct physical interactions between PLC γ -1 and TRPC3. In fact, CpG does not induce detectable phosphorylation of either PLC γ -1 or PLC γ -2.

One can imagine several explanations for this apparent uncoupling by CpG of store depletion from CRAC channel activation. First, the inability of CpG to activate CRAC channels could reflect insufficient calcium store depletion to trigger STIM1 oligomerization and relocation to junctional ER domains (67,74). Indeed, recent studies suggest a K_d for Ca²⁺ regulation of STIM1 activation in the 200–600 μ M range (64). Although we found that BCR engagement and CpG both induce Ca²⁺ release from stores, the amplitude of CpG induced transients were sometimes smaller. Moreover, as our assessment of Ca²⁺ release was measured by changes in cytoplasmic calcium levels, any differential regulation of calcium store depletion by CpG versus BCR stimulation could also be masked by concurrent regulation of SERCA and PMCA pump activity or cellular buffering. One could also imagine that calcium is released by distinct mechanisms or from stores not in proximity to CRAC channels given the atypical requirement for PLC γ 1 in CpG responses and our inability to detect phosphorylation of either PLC γ isoforms. Due to the requirement for STIM1 migration into physical proximity with Orai1 in the plasma membrane, location could be a critical factor in the ability of stores to activate these channels. Accordingly, if IP₃ is produced by PLC γ 1 or a PLC isoform other than PLC γ -2, it may accumulate in a spatially restricted manner such that CpG activates functionally (or geographically) distinct calcium stores that are not in proximity with CRAC channels (90,117). Given recent work that indicates STIM1 and Orai1 can cluster in a highly polarized manner at the distal pole of migrating T cells, it seems likely that the targeting/localization of key molecules is critical to the cells response (76). It is also possible that TRPC3 activation indirectly regulates calcium permeation of CRAC channels.

As we demonstrated for mechanically activated TRPV4-like channels, sodium and calcium entry via TRPC3 may also depolarize the plasma membrane and dissipate the electrical driving force for calcium entry via CRAC channels.

Finally, the inability of CpG to activate CRAC channels may reflect its inability to activate distal steps involved in coupling store depletion to channel activation. As recent work suggests that Lyn, Syk, and SLP-65 regulate CRAC channel activation by mechanisms that are distinct from their proximal role in PLC γ -2 activation and IP₃ generation (6,7,99,118), the inability of SR-B1 to activate Syk (or -Btk and -PLC γ -2) may account for this block in CRAC channel activation.

In summary, we have identified multiple NSCCs in primary B cells that are activated by innate stimuli including endogenous or exogenous AA-derived inflammatory mediators and unmethylated CpG DNA. Although innate and BCR-coupled signaling pathways overlap in many aspects, they are remarkably distinct in their mechanisms of calcium entry. Calcium entry via the BCR is mediated exclusively by CRAC channels; whereas, calcium entry triggered by mechanical and osmotic stimuli and by CpG appears to be mediated exclusively by store-independent activation of NSCCs. Consequently, the ultimate fate of a B cells will reflect the intricate interplay of calcium signaling events dictated by the integrated effect of all of these pathways.

Function of NSCC channels in B cells

Ca²⁺ triggers a wide range of lymphocyte fates and functions (1,13,15,22) and our studies have identified novel mechanisms by which proinflammatory stimuli initiate calcium-dependent fates of B cells via NSCC activation. These NSCCs mediate specific responses of B cells to a wide array of stimuli including mechanical and osmotic forces, inflammatory mediators produced by B cells themselves and other leukocytes at sites of inflammation, by vascular endothelium, and by pathogens and operate independently of intracellular Ca²⁺ stores and CRAC channels linked to the BCR, yet work in concert to coordinate the immune response.

For example, we demonstrated that AA plays a central role in the mobilization of Ca²⁺ in B cells. In fact, AA derived pro-inflammatory eicosanoids have been shown to regulate cytokine production, nitric oxide, and free radicals involved in the pathogenesis of immune mediated inflammatory diseases (119,120). Eicosanoids are also produced by some prokaryotic and eukaryotic pathogens (121) and induce inflammation and host susceptibility to these pathogens (122). Eicosanoids produced by vascular endothelium increase vascular permeability and promote adhesion and transmigration of leukocytes (123). Those produced by leukocytes and the vascular endothelium have important roles in regulating capillary bed perfusion, the permeability of vascular endothelium, and expression of selectins and integrin ligands which enable macrophages, neutrophils and lymphocytes to escape from the microvasculature and lymphatics and migrate through extravascular spaces (124–127). At the same time, these mediators can activate NSCCs and promote integrin-dependent binding, which plays a critical role during B cell entry into the splenic white pulp or by allowing B cells to linger within the marginal zones adjacent to vascular sinuses (128,129). Thus, eicosanoids produced by immune cells or by pathogens at sites of inflammation may mediate “cross-talk” that promotes and coordinate the inflammatory response.

The physiological mechanism by which integrin activation is dynamically regulated and the role of eicosanoids in the activation process is poorly understood. Studies of T cells have suggested that membrane depolarization increases their binding avidity for extracellular matrix proteins. The resting membrane potential of T lymphocytes is regulated primarily by K⁺ channel activity. Depolarization induced with K⁺ channel blockers or by dissipating the K⁺ gradient activates integrins, which mediate cell adhesion to extracellular matrix proteins ICAM-1 and VCAM-1 (130). In B lymphocytes, plasma membrane depolarization induced by NSCC activation also modulates the avidity of integrins for extracellular matrix proteins (21–23,23,23). Why B cells express multiple eicosanoid sensitive NSCCs with similar functions is perplexing, although it is possible that distinct families of receptors or inflammatory mediators are each linked to different Ca²⁺ permeant channels. Thus, our findings have broad implications for understanding proximal mechanisms of integrin activation by proinflammatory mediators which regulate lymphocyte trafficking and localization. The ultimate response of a lymphocyte in vivo will; therefore, reflect the effect of these overlapping pathways on the gain or dynamic range of calcium signaling.

Interestingly, we found that the pathogen derived molecule, CpG DNA also induces calcium signals and VCAM-1 adhesion of B cells. Why it is that distinct innate stimuli utilize alternative calcium permeant NSCCs, in this case TRPC3, to activate B cells is a mystery, but in the case of SR-B1, its inability to activate Syk kinase may be critical factor in terms of how CRAC channel activity is inhibited. CpG activation of TRPC3 was found to limit the TLR9-dependent production of proinflammatory cytokines IL-6, IL-10, and IgM. Ongoing efforts are focused on trying to better define how these diverse pathways negatively regulate CRAC channel mediated signaling, and the physiological basis for this negative regulation. These results together provide a novel perspective on the complexity of calcium signaling within B cells and have far reaching implications with respect to the mechanisms regulating B cell activation, migration, and tolerance to innate stimuli including shear force and pathogen-derived molecules.

Summary and Conclusions

With the recent discovery of key molecular components of store-operated calcium signaling in lymphocytes, enormous progress has been made toward understanding the initiation of calcium signaling in lymphocytes. Despite this progress, it is becoming increasingly apparent that the regulation of intracellular calcium in response to antigen receptor-induced events is more complicated than originally thought and is subject to extensive and overlapping regulation. Moreover, evidence is rapidly mounting that complex biological stimuli, including antigen and innate stimuli found within the inflammatory milieu, cooperate to generate dynamic calcium signals in lymphocytes in vivo. As a wealth of information emerges about the molecular mechanisms of CRAC and TRP channel activation and function in lymphocytes, and as we have begun to appreciate that a range of stimuli mobilize calcium in lymphocytes via distinct and interacting pathways, our attention can begin to focus on fine details of the mechanisms of calcium regulation and how these interplay between these signals regulates the fate of immune cells in vivo.

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Figure 1. Mechanism of B cell antigen receptor (BCR) mediated calcium mobilization in lymphocytes

BCR engagement by antigen binding activates the Src kinase Lyn, which phosphorylates immunoreceptor tyrosine activation motifs (ITAMs) on the Ig α and Ig β chains of the BCR complex. Syk then binds to phosphorylated ITAMs via its tandem SH2 domains resulting in its activation and subsequent phosphorylation of both Y204 of Ig α and the adaptor protein SLP-65. SLP-65 is anchored in the plasma membrane via its N-terminal leucine zipper (LZ) motif and is further stabilized through SH2 domain mediated interactions with pY204 on Ig α , bringing it into the proximity of activated Syk. Following further phosphorylation by Syk, phosphorylated SLP-65 functions as a scaffold upon which additional proteins such as PLC γ -2 and Btk bind to form a complex that initiates calcium signaling. Activated PLC γ -2 hydrolyzes membrane PIP $_2$ into diacylglycerol (DAG) and IP $_3$, which binds to and activates IP $_3$ receptors, which are channels through which calcium leaves the ER. When ER Ca $^{2+}$ levels drop below the K $_d$ of STIM1, the intraluminal N-terminus of STIM1 undergoes a conformational change that facilitates dimerization and these dimers migrate to regions of plasma-ER membrane apposition (termed junctional ER). Direct interactions between the C-terminus of STIM1 and both the N- and C-terminus of Orai1 tetramers triggers CRAC channel activation. CRAC channel activation also appears to be modulated by many of the molecules found in the proximal signaling complex. For example, membrane localized Btk positively regulates the magnitude of calcium entry through CRAC channels following store depletion, an effect that is blocked by PKC β dependent phosphorylation of Btk. Moreover, recent studies demonstrate that although depletion of ER calcium stores is necessary for CRAC channel activation, it may not be sufficient. In particular, these studies implicate SLP-65, Syk, and Lyn in distal (post store depletion) steps of CRAC channel activation. Recent data also suggest that SLP-65 and Syk regulate sustained calcium entry even after BCR signaling diminishes and IP $_3$ levels drop, perhaps via a novel complex in which Syk remains localized to the plasma membrane via interactions between a phosphorylated C-terminal (Y630) tyrosine that interacts with the SH2 domain of SLP-65 (left side of model). It is currently unknown whether PLC γ -2 and Btk are part of this complex and how they may regulate distal steps of CRAC channel activation.

Figure 2. Mechanical regulation of calcium signaling in B lymphocytes

Shear and osmotic forces mobilize calcium in B lymphocytes via release from intracellular stores and entry across the plasma membrane. The “mechanical receptor” that triggers the calcium signaling cascade in B cells has not been identified yet subsequent steps have been defined. Mechanical and osmotic stress activate PLC γ -2 and the resulting calcium signal is critically dependent on both PLC γ -2 activity and on the expression of the adaptor SLP-65 to which PLC γ -2 anchors. In contrast to BCR-mediated signals, calcium entry in response to mechanical/osmotic stress occurs via a store-independent non-selective cation channel (NSCC) whose pharmacological and biophysical properties most closely resemble those of the transient receptor potential (TRP) channel TRPV4. TRPV4 like channels in B cells are activated by Arachidonic acid cleaved from DAG which is further metabolized by cytochrome P450 epoxygenase to form 5,6-epoxyeicosatrienoic acid (5,6-EET); a direct channel agonist. Interestingly, although PLC γ -2 is activated and high levels of IP₃ are generated by hypotonic stimulation of B cells, CRAC channels do not appear to play any role in the resulting calcium elevations. Several mechanisms could account for the apparent inability to activate CRAC channel mediated calcium entry. Calcium permeation of CRAC channels is highly dependent upon a hyperpolarized membrane potential (V_m). Calcium and sodium permeation of TRPV4-like channels in B cells produces membrane depolarization, which could attenuate Ca²⁺ permeation of activated CRAC channels (1). Alternatively, because mechanical stimuli produce much higher levels of IP₃ than BCR engagement, and therefore DAG, this may activate PKC β which inhibits Btk membrane localization (2) and this may regulate distal steps in CRAC channel activation following store depletion by IP₃ (see text for a more detailed explanation). The mechanism by which Btk potentiates CRAC channel activity is not known.

Figure 3. Scavenger receptor mediated calcium signaling in B cells

Unmethylated CpG DNA triggers a robust calcium signal in primary B cells via the Scavenger Receptor-B1. The mechanism of CpG induced calcium entry is distinct from that elicited by the BCR and by mechanical stimulation in several critical respects. First, PLC γ -1 and not PLC γ -2 is required for SR-B1 mediated calcium mobilization. Second, SR-B1 mediated calcium entry occurs via TRPC3 channels. Finally, although CpG activation SR-B1 triggers calcium release from stores, TRPC3 activation is store independent and; furthermore, PLC γ -1 appears to activate TRPC3 via a direct physical interactions between these two proteins and is not regulated by calcium release from stores. Interestingly, CpG does not activate CRAC channels in spite of its ability to trigger calcium release from ER. This suppression of CRAC channel mediated calcium entry could reflect (1) inhibition of calcium permeation of CRAC channels by TRPC3 mediated membrane depolarization, (2) insufficient IP3 production or depletion of calcium from stores to mobilize STIM1, or (3) sufficient calcium depletion and STIM1 mobilization but an inability to activate Syk, Lyn, or SLP-65 as required for positive regulation of distal coupling between stores and CRAC channels (see text).