

Mast cell CRAC channel as a novel therapeutic target in allergy

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Purpose of review

This review describes recent advances in our understanding of a major Ca^{2+} -entry pathway, the Ca^{2+} release-activated Ca^{2+} (CRAC) channel, that is central to mast cell activation.

Recent findings

Animals in which the genes encoding the CRAC channel have been deleted have severely compromised mast cell function and reduced allergic responses. These functional consequences reflect the ability of CRAC channels to activate a range of spatially and temporally distinct responses in mast cells, which contribute to both rapid and slow phases of an allergic response. In addition, the cells can sustain their own activation through positive feedback cycles that involve CRAC channels. Drugs that inhibit CRAC channels are proving effective in treatment of allergic responses both *in vitro* and in animal models of asthma.

Summary

CRAC channels comprise a new therapeutic target for combating allergies including asthma.

Keywords

Ca^{2+} signaling, CRAC channel, leukotrienes

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Introduction

Since their discovery by Paul Ehrlich more than 125 years ago, mast cells are now widely recognized as fundamental components of the mammalian immune system [1]. They play a key role in the initiation and then orchestration of local inflammatory responses, but it is their role in allergy and anaphylaxis that has aroused most clinical interest. Allergies arise from an overzealous immune response to generally innocuous molecules and have now reached epidemic proportions in the UK. Conservative estimates indicate that almost one in three people experiences allergy, with significant socio-economic consequences.

Allergic responses can generally be divided into two temporally distinct stages: the early and the late-phase reaction [2,3]. The early phase develops within seconds to minutes of allergen exposure and is caused by degranulation of preformed mediators from mast cells notably histamine and the chemokine tumour necrosis factor- α (TNF- α) as well as de-novo synthesis and release of prostaglandins, leukotrienes, platelet-activating factor and cytokines, which together are responsible for bronchoconstriction, mucus production, vasodilation and oedema. The late phase develops after 6–12 h and is due to recruitment of blood-borne cells including Th2 lymphocytes and eosinophils, which help sustain the inflammatory response. Mast cells play a central role in

the development of the first phase and make important contributions to the second one.

Store-operated CRAC channels activate temporally distinct responses in mast cells

Secondary exposure to allergen results in cross-linking and activation of Fc ϵ RI receptors located on the plasma membrane of tissue-resident mast cells [4].

These multimeric receptors activate phospholipase C γ through a series of tyrosine kinase-dependent phosphorylation steps, and lead to the hydrolysis of the membrane phospholipid PIP₂ to generate the second messengers InsP₃ and diacylglycerol. This causes a biphasic increase in cytosolic Ca^{2+} : an initial transient response arising from InsP₃-dependent Ca^{2+} release is followed by a sustained rise due to Ca^{2+} entry into the cell through store-operated CRAC channels in the plasma membrane. CRAC channels in mast cells participate in the development of both phases of the allergic response. Both degranulation and leukotriene production, which contribute to the rapid phase, are stimulated by Ca^{2+} entry through the channels. Likewise, CRAC channels also activate expression of genes encoding mast cell-derived chemokines and cytokines that help underlie the slow sustained phase [5].

Molecular basis of CRAC channels

Store-operated CRAC channels are opened by the emptying of internal Ca^{2+} stores, which occurs physiologically following InsP_3 -dependent Ca^{2+} release. As the Ca^{2+} content within the store falls, a signal is generated from the stores, which leads to the opening of the plasma membrane Ca^{2+} channels. For many years, the molecular basis of this ubiquitous signalling pathway remained enigmatic but recently the genes encoding Stromal Interaction Molecule (STIM) [6,7] and Orai proteins [8,9] were established as being necessary and sufficient for store-operated Ca^{2+} influx [10]. STIM proteins (of which there are two: STIM1 and STIM2) are single pass endoplasmic reticulum-resident proteins that sense the Ca^{2+} content of the stores [6,11]. Upon store depletion, STIM1 monomers come together to form oligomers, which then migrate across the endoplasmic reticulum to form punctate-like structures just below the plasma membrane [12]. At these sites, they bind to and subsequently activate the Orai family of proteins [13–15]. There are three OraIs (Orai1–3), each of which is an integral membrane protein with four transmembrane-spanning domains. Mutagenesis studies have established that Orai1 forms at least part of the CRAC channel pore and, together with substituted cysteine accessibility approaches, has identified glutamate 106 as the main Ca^{2+} -binding site that controls channel selectivity [10,13]. A functional CRAC channel is thought to be a tetramer, made up of four Orai1 subunits.

Although CRAC channels are activated by the loss of Ca^{2+} from the store, the relation between store depletion and subsequent channel activation is not linear [14,15]. A significant fraction of the store needs to be released before any CRAC channels open, giving rise to a threshold phenomenon [16]. The nonlinearity likely arises from the fact that STIM1 has high affinity for luminal Ca^{2+} and therefore a sizeable drop in store content is needed before Ca^{2+} dissociates from the sensor's EF-hand [17]. This feature of STIM1 provides a mechanistic explanation for the dissociation between Ca^{2+} release and CRAC channel activation that has been found in mast cell lines [14].

Biophysics of the CRAC channel

In addition to their unique gating mechanism, CRAC channels are also somewhat idiosyncratic in terms of their biophysical properties. The channels are exquisitely selective for Ca^{2+} and readily discriminate not only between divalent and monovalent cations, but also between different divalent cations [18]. The stringent selectivity pairs up with a tiny single channel conductance, which has been estimated to be approximately 2 fS in physiological external Ca^{2+} concentration [10]. This is approximately four orders of magnitude smaller than the conductance of most voltage-operated Ca^{2+} channels. On

the basis of the low CRAC channel conductance, it has been calculated that mast cells and T lymphocytes express approximately 10 000 functional CRAC channels in the plasma membrane. Although CRAC channels are not voltage-activated by themselves, the prevailing membrane potential determines the flux through them. Depolarization reduces the electrical driving force for Ca^{2+} entry and therefore attenuates the size of the CRAC current, whereas the converse occurs with hyperpolarization. Hence changes in the activities of other ion channels in the plasma membrane can have marked effects on Ca^{2+} flux through CRAC channels. A striking example of this is seen with TRPM4 cation channels. These Ca^{2+} -activated nonselective cation channels help determine the driving force for Ca^{2+} entry through CRAC channels in bone marrow-derived mast cells [19]. Mast cells lacking TRPM4 exhibited more pronounced degranulation, leukotriene secretion and production of $\text{TNF-}\alpha$. The IgE-mediated acute passive cutaneous anaphylactic response was also stronger [19]. By altering the membrane potential, TRPM4 channels are an effective regulator of CRAC channel-driven responses in mast cells.

In mast cells and related cell lines, CRAC channels are extensively regulated by intracellular signalling pathways. Protein kinase C, which is activated concomitantly with InsP_3 production, can inactivate the channels [20] and it has been reported that this is a consequence of phosphorylation of N-terminal serine residues 27 and 30 on Orai1 [21^{*}]. Cytoplasmic Ca^{2+} can also inactivate CRAC channels, providing a tightly controlled negative feedback pathway that might serve to prevent Ca^{2+} overload. Sphingosine is another effective inhibitor of CRAC channel activity. Hence the activity of the CRAC channels themselves in a mast cell will be determined not only by CRAC channel activity itself but also by the impact of other functional ion channels as well as a plethora of intracellular signalling pathways.

CRAC channel knockout mice: insight into mast cell function *in vivo*

The discoveries of STIM1 and Orai1 ushered in the opportunity to dissect out the role of CRAC channels in mast cell physiology through the generation of CRAC channel-deficient animals. Fetal liver-derived mast cells from mice lacking STIM1 failed to generate Ca^{2+} influx in response to FCεRI receptor stimulation and this was associated with suppression of degranulation, impaired activation of the transcription factors NFAT and NF-κB and reduced production of several cytokines including $\text{TNF-}\alpha$ and interleukins (IL) 6 and 13 [22^{••}]. In heterozygous STIM1+/- mice (used because STIM1-/- die *in utero*), the FCεRI-dependent passive cutaneous anaphylaxis reaction was substantially reduced. Similar findings were made in mast cells obtained from fetal liver

from Orai1-deficient mice [23]. Antigen-evoked degranulation and leukotriene C₄ (LTC₄) production in these mast cells were suppressed when CRAC channels were deleted, and chemokine production was reduced. Consistent with the knockout mouse studies, acute pharmacological inhibition of CRAC channels or transient knockdown of Orai1 inhibits LTC₄ production, enzyme activation as well as gene expression [24–26].

CRAC channel-independent Ca²⁺ influx in mast cells?

In both the STIM1 and Orai1 knockout mice studies, the absence of CRAC channels only partially reduced chemokine generation. Approximately 25% of antigen-dependent TNF- α production and approximately 50% of IL-6 generation remained after loss of Orai1 [23]. Similar results were observed in STIM1 $^{-/-}$ mice in response to antigen stimulation ($\sim$ 25% of TNF- α and $\sim$ 60% of IL-6 production remained) [22^{••}]. In both studies, although antigen-evoked Ca²⁺ entry was substantially reduced (by $\sim$ 70%), it was not abolished. Chemokine production could therefore be entirely dependent on CRAC channels, and the partial reduction in STIM1 and Orai1 knockout mice simply reflects incomplete loss of the CRAC channel. Consistent with this, the Ca²⁺ signal that remained in the Orai1 knockout mice was blocked by CRAC channel inhibitors [23]. However, it is nevertheless possible that other Ca²⁺ influx pathways might contribute to agonist-evoked Ca²⁺ signals and gene expression. In the mast cell line RBL-2H3, for example, it has been suggested that Ca²⁺-permeable TRPC5 channels contribute to antigen-evoked Ca²⁺ entry and subsequent degranulation [27]. Fyn kinase has also been implicated in control of FC ϵ RI-driven Ca²⁺ entry in bone marrow-derived mast cells, possibly through an association with TRPC3/6/7 [28].

Involvement of L-type Ca²⁺ channels in antigen-evoked Ca²⁺ entry has also been reported in RBL-2H3 and bone marrow-derived mast cells, in which L-type channel blockers reduced antigen-evoked cytokine production [29].

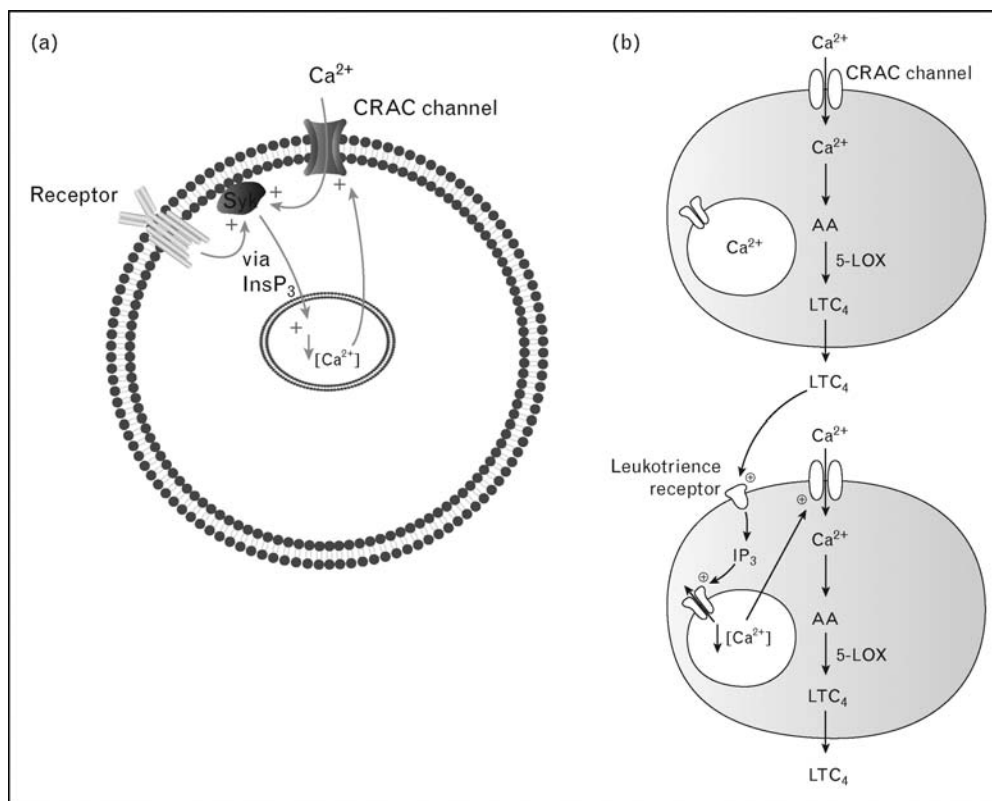
CRAC channel pharmacology

Despite the importance of CRAC channels for mast cell function and for the immune system in general, no therapeutic agents have been developed that target these channels specifically. Several agents block CRAC channels but these are plagued by numerous other actions that limit their usage experimentally and preclude clinical consideration [24]. Nevertheless, there are some popular experimental tools that are often used to probe CRAC channel function. These include low concentrations ($<1\ \mu\text{M}$) of the trivalent cation Gd³⁺ and 2-aminoethyl-diphenylborate (2-APB), the latter of which can both

potentiate and inhibit CRAC channels in a concentration-dependent manner. More recent inhibitors include a series of 3,5-trifluoromethyl pyrazole derivatives referred to as the 3,5-trifluoromethyl pyrazole derivatives (BTPs) [25], and the Synta compound [26]. BTP2 (YM-58483) inhibits CRAC channels with an IC₅₀ of approximately 2 μM when applied acutely [30]. However, when administered for several hours, its potency increases in that the IC₅₀ changes to 10 nM [31]. Nevertheless, BTP2 is not bereft of other targets, and has been found to open TRPM4 channels, for example [30]. The Synta compound blocks CRAC channels with an IC₅₀ of approximately 3 μM in mast cells. Both BTP2 and Synta compound are being used to probe the role of CRAC channels in in-vivo and in-vitro animal models of disease [24].

Positive feedback steps in mast cell activation

An intriguing property of mast cells is that they can sustain their own activation through mechanisms that involve CRAC channels. One way whereby this is accomplished is through Ca²⁺-dependent activation of the nonreceptor tyrosine kinase Syk (Fig. 1a) [26]. Upon cross-linking of FC ϵ RI receptors on the cell surface, the Syk family tyrosine kinase Lyn phosphorylates immunoreceptor tyrosine-based activation motifs on the β and γ chains of the receptor. This leads to further binding of Lyn along with recruitment of Syk via Src homology 2 domains. Syk is then phosphorylated and thereby activated by Lyn and, in turn, leads to activation of phospholipase C isoforms γ 1 and 2. Phospholipase C activation generates InsP₃, which depletes the stores and opens CRAC channels in the plasma membrane. Inhibition of CRAC channels or knockdown of Orai1 both prevented sustained Syk phosphorylation in response to antigen [26]. Once Syk has been activated by receptor stimulation, open CRAC channels maintain Syk activity, leading to sustained store depletion and thus prolonged Ca²⁺ influx. This form of positive feedback is an auto-crine mechanism that helps maintain individual mast cell activation. A second mechanism for positive feedback involves paracrine signalling and arises from a dynamic interplay between CRAC channels and LTC₄ production (Fig. 1b) [32^{••}]. Ca²⁺ microdomains near open CRAC channels lead to the activation of the MEK/ERK pathway, and this results in the synthesis and secretion of the pro-inflammatory molecule LTC₄ [33]. LTC₄ then activates cysteinyl leukotriene type I (cysLT1) receptors on adjacent mast cells. These G-protein-coupled receptors couple to phospholipase C, leading to CRAC channel activation through InsP₃-driven store depletion. Ca²⁺ entry through the open CRAC channels leads to further LTC₄ synthesis and secretion and the feed forward loop continues [32^{••}]. In this way, an intercellular Ca²⁺ wave

Figure 1 Positive feedback steps that sustain mast cell activation

(a) The tyrosine kinase Syk depletes stores, via InsP_3 generation, and thus opens CRAC channels. The subsequent Ca^{2+} entry through the channels sustains antigen-dependent Syk activity, maintaining store depletion and hence CRAC channel activity. (b) Paracrine signalling co-ordinates activity in a mast cell population. CRAC channel activation leads to a Ca^{2+} rise that stimulates Ca^{2+} -dependent phospholipase A_2 to generate arachidonic acid (AA), which is then metabolized by 5-lipoxygenase to produce LTC_4 . LTC_4 is secreted and activates cysteinyl leukotriene type 1 receptors on adjacent cells. This leads to activation of CRAC channels, Ca^{2+} entry and LTC_4 generation, resulting in a positive feedback cycle.

propagates through the mast cell population, helping co-ordinate mast cell activity.

CRAC channels and mast cell-linked disorders

Given the importance of CRAC channels in mast cell physiology and the possibility of chronic activation due to the positive feedback cycles described above, it is perhaps no great surprise that mast cells have recently been implicated in a range of common and often debilitating diseases.

Nasal polyposis

Nasal polyps arise from a chronic inflammation of the upper airways and occur in around 2% of the population. Symptoms include itchy eyes, sneezing, rhinorrhoea, nasal itch, sleep disorders, headaches and more severely the loss of sense of smell [34]. Development of polyps is associated with perennial nonallergic rhinitis, asthma, aspirin intolerance, allergic fungal rhinosinusitis and cystic fibrosis. Mast cells are abundant in nasal polyps and, as

with allergic rhinitis, are heavily implicated in polyp development [35]. Mast cells isolated from surgically removed human polyps express functional CRAC channels [32^{••}]. Ca^{2+} entry through the channels leads to the synthesis and secretion of LTC_4 , which activates cysLT1 receptors on other mast cells leading to the opening of CRAC channels [36[•]]. The positive feedback cycle between CRAC channels and LTC_4 production is therefore expressed in nasal mast cells. This is particularly germane to polyposis because polyps are poorly vascularized. Signalling molecules like LTC_4 will therefore linger for longer, sustaining mast cell activation. This feed forward loop suggests a novel tact for treating polyposis, namely to target CRAC channels and cysLT1 receptors simultaneously [36[•]]. Crucially, because of the inherent instability introduced by the positive feedback step, partial block of CRAC channels together with partial block of cysLT1 receptors should be very effective in dampening down mast cell activation. A combination approach, targeting both the channels and the receptors and using submaximal concentrations of antagonists, suppressed mast cell activity *in vitro* [36[•]]. Once CRAC

channel inhibitors become available for clinical use, it will be interesting to test this concept *in vivo*.

Allergic asthma

Features of asthma include inflammation of the airways, bronchial hyper-responsiveness and airway obstruction. Bronchoconstriction arises from contraction of underlying smooth muscle and this is exacerbated by bronchial wall thickening due to hypertrophy of the airway smooth muscle. Store-operated Ca^{2+} entry is present in airway smooth muscle and contributes both to the contractile response and to smooth muscle cell proliferation [37]. STIM1 and Orai1 mRNA are found in human airway smooth muscle and knockdown of either reduces store-operated Ca^{2+} influx [38,39]. Single channel recordings place the conductance of the store-operated channel to be 25 pS, considerably larger than that of the CRAC channel [40]. Whether this is due to the presence of a hetero-multimeric channel complex containing Orai1 together with other pore-forming components or the existence of several distinct Ca^{2+} entry pathways is currently unclear. Inhibition of store-operated Ca^{2+} channels with BTP2 was effective in reducing bronchoconstriction in an *Ascaris*-induced nonhuman primate model of asthma [41]. Moreover, orally administered BTP2 reduced eosinophil infiltration into the airways as well as airway responsiveness that was evoked following repetitive exposure to ovalbumin in sensitized guinea pigs [42]. These effects are due in part to a direct action on airway smooth muscle, preventing contraction.

Inhibition of CRAC channels in lung mast cells would reduce exocytosis of histamine-containing granules and suppress the production and secretion of LTC_4 as well as decrease synthesis and release of cytokines that recruit other cell types to the inflamed airway. Hence targeting CRAC channels in mast cells and airway smooth muscle could prove to be a very effective therapy for the management of asthma.

Conclusion

CRAC channels in mast cells constitute a novel and potentially important target for drugs aimed at combating mast cell-linked disorders including allergic rhinitis, polyposis and asthma. Moreover, because the channels are, along with leukotrienes, a central component of a positive feedback cascade that helps sustain mast cell activation, partial block of both CRAC channels and leukotriene receptors should dampen down mast cell activity. An advantage of therapeutic strategies that exploit this approach is that low doses of inhibitors need to be used, thereby avoiding the off-target effects that often arise when dosage is raised. Although CRAC channel blockers are still in the early stages of development, the discovery of the molecular basis of the channel along with effective

high-throughput screening means we can look forward to rapid progress in drug discovery programmes.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 67).

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