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Small-Molecule Inhibitors of Store-Operated Calcium Entry

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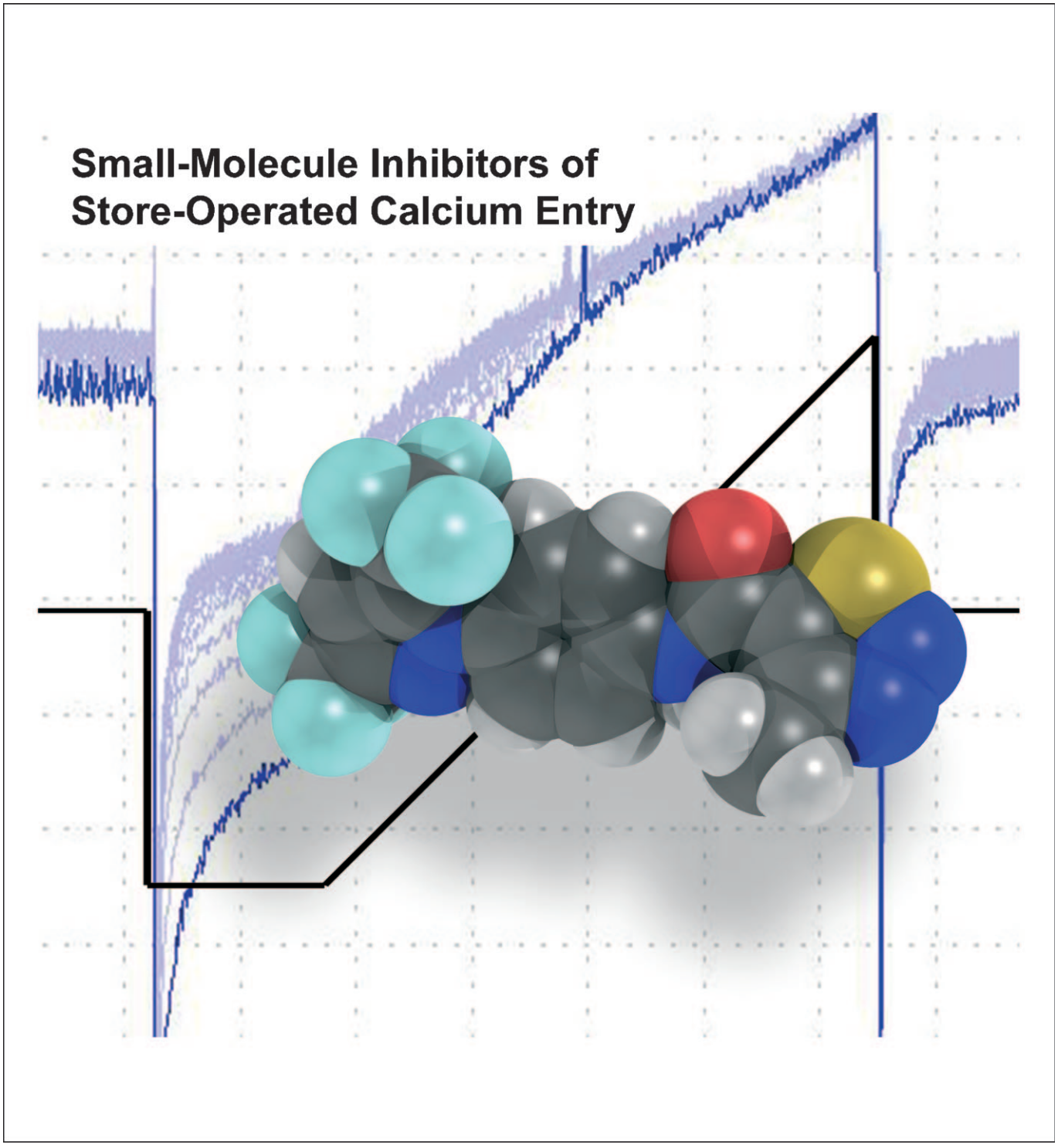
Dedicated to Dr. Robert Wilhelm



InterScience DISCOVER SOMETHING GREAT

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Controlled variation in intracellular calcium concentration is a key component of the immune response signaling pathway in lymphocytes. Store-operated calcium entry (SOCE) in these cells provides a prolonged increase in cytoplasmic Ca^{2+} concentrations and ultimately leads to the production of pro-inflammatory cytokines. Molecules that inhibit SOCE could therefore be useful immunomodulating agents for the treatment of rheumatoid arthritis, psoriasis, inflammatory bowel disease, and other conditions. Although the presence of the SOCE signaling pathway in lymphocytes and other cells involved in the immune response has been known for many years, key proteins involved in SOCE were identified only recently. The identification of these proteins may further enable the identification of agents that inhibit SOCE without affecting other cellular processes. This contribution documents representative examples of the small-molecule inhibitors of SOCE that have been reported to date. Where possible, methods that were used to characterize the mechanism of action of the inhibitors are also described.

Introduction

Calcium levels in the body are tightly controlled through an intricate network of absorption, elimination, and storage pathways.[1] In all cells, extracellular Ca^{2+} concentrations and levels of Ca^{2+} in the endoplasmic or sarcoplasmic reticulum are several orders of magnitude higher than Ca^{2+} concentrations in the cytoplasm.[2] Calcium channels in the plasma membrane can depolarize the cell by allowing the influx of Ca^{2+} from the extracellular environment. The resulting increase in levels of cytosolic Ca^{2+} influences a variety of fundamental cellular processes.

The influx of calcium ions into smooth muscle cells leads to cell contraction.[3] Reduction of calcium influx through voltage operated L-type calcium channels in these cells is the primary mechanism of action of drugs such as verapamil, diltiazem, and amlodipine that are used to treat hypertension and angina (Figure 1).

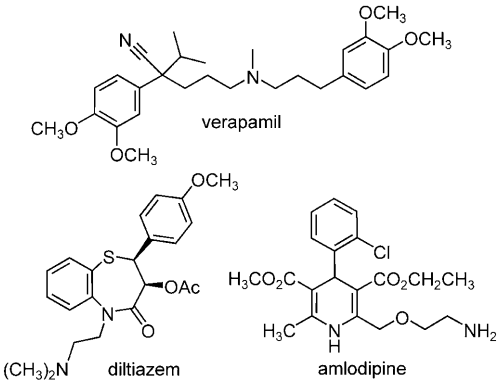

Figure 1. Structures of marketed calcium (L-type) channel antagonists.

Calcium-mediated cell-signaling pathways also play important roles in the response of B- and T-lymphocytes to extracellular signals (Figure 2).[4,5] Stimulation of cell-surface receptors leads to the activation of phospholipase C and production of inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3]. This signaling molecule mediates the release of Ca^{2+} from the endoplasmic reticulum (ER) lumen into the cytosol. The resulting depletion of luminal Ca^{2+} results in the activation of calcium-release-activated calcium (CRAC) channels in the plasma membrane and the influx of external Ca^{2+} into the cytosol. The entry of external

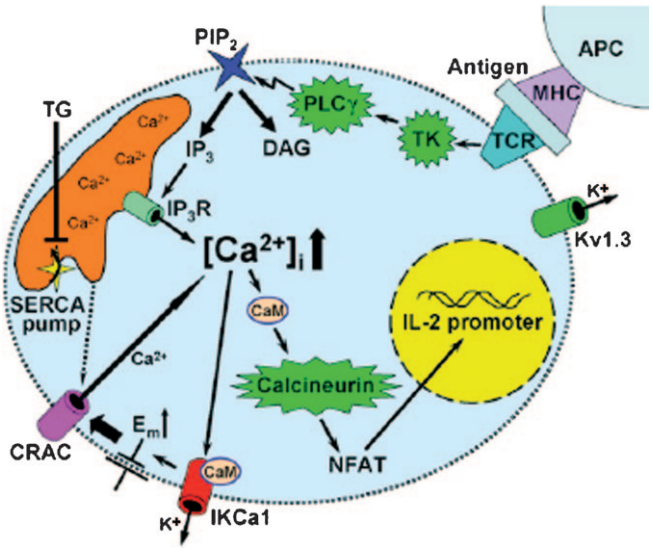

Figure 2. Store-operated calcium entry in lymphocytes (reproduced from reference [13] with permission; copyright 2007, Elsevier, Amsterdam).

Ca^{2+} following reduction of the calcium concentration in the ER is known as either capacitative calcium entry or store-operated calcium entry (SOCE), while the current generated by the opening of CRAC channels is termed I_{CRAC}.[6,7]

There are also additional, non-CRAC channels that mediate SOCE. Non-CRAC channels that contribute to SOCE are clearly distinguished both by the mechanism of channel activation as well as by the channel conductance properties.[8,9]

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The enzyme calcineurin is a critical mediator of cellular Ca^{2+} signaling, and its role in lymphocyte activation has been examined in detail. Calcineurin's phosphatase activity is regulated through the reversible binding of Ca^{2+}-calmodulin and is responsive to the prolonged oscillations in cytosolic calcium concentrations that result from SOCE. An extensively studied substrate of calcineurin is the nuclear transcription factor of activated T-cells (NFAT). Ca^{2+}-dependent NFAT dephosphorylation promotes nuclear accumulation of the transcription factor (Figure 2).[10,11] In the nucleus, NFAT helps to direct transcription of several pro-inflammatory cytokine genes such as interleukin 2 (IL-2), interleukin 4 (IL-4) and interferon γ (IFN-γ). The immunosuppressive drugs tacrolimus and cyclosporine A, together with other cellular cofactors, form complexes that bind to calcineurin and prevent the dephosphorylation of NFAT in response to elevated levels of cytosolic Ca^{2+}.[12] Interference with calcium-signaling pathways in lymphocytes is therefore recognized as a clinically useful method of reducing undesired immune responses.

Although the presence of the SOCE signaling pathway in lymphocytes and other cells has been known for 20 years, two key proteins involved in SOCE were identified only recently.[5,13–15] The CRAC channel regulatory protein stromal interaction molecule (STIM1) is a transmembrane protein located primarily in the endoplasmic reticulum, while ORAI1—also called CRACM1—is a subunit of a gated plasma membrane ion channel. There is now strong evidence that decreased calcium levels in the ER induce STIM1 to form puncta close to the plasma membrane, where this protein interacts with the ORAI subunits of the CRAC channel to permit Ca^{2+} influx into the cell.

A specific form of the inherited deficient immune response known as severe combined immunodeficiency (SCID) is characterized by a mutation (Arg91Trp) in the ORAI1 protein.[16,17] T-lymphocytes isolated from patients with this form of SCID do not show CRAC channel currents and have decreased cytokine expression, although they appear to develop normally. Two proteins closely related to ORAI1, termed ORAI2 and ORAI3, have also been identified.[6,18] These proteins are known to form calcium channels that contribute to SOCE.[19–21] The relative importance of the various ORAI paralogues in mediating SOCE may be cell-type or species dependent.[14]

The immunodeficiency of CRAC-defective SCID patients, combined with our understanding of the particular importance of SOCE to lymphocyte activation, suggests that the modulation of the ORAI1-containing CRAC channel could be an important target for the treatment of inflammatory diseases such as psoriasis, asthma, rheumatoid arthritis, and inflammatory bowel disease.[22,23] Molecules that specifically block CRAC channels may have fewer side effects than drugs that target the function of other proteins in the calcium signaling pathway (e.g. STIM1 or calmodulin), as many of these proteins are thought to contribute to a broad array of fundamental physiological processes.

Identification of SOCE inhibitors

Several methods have been used to identify molecules that interfere with SOCE.[8] The recent identification of the ORAI proteins and STIM homologues as critical components of SOCE

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Ana Minatti obtained her PhD in organic chemistry from the University of Bonn in 2005. In 2006 she was awarded an Alexander von Humboldt fellowship for her postdoctoral studies at the Massachusetts Institute of Technology. She joined the Medicinal Chemistry Department at Roche Palo Alto in 2008, where she worked toward the discovery of new immunosuppressants.

Don Button graduated from Cornell University with a BSc in 1982 and received his PhD in physiology and pharmacology from the University of California, San Diego in 1991. After a PRAT fellowship at the National Institute of Mental Health in Bethesda, Maryland, and postdoctoral research at Stanford University, he joined Roche Palo Alto in 2000. His work on drug discovery projects has focused on GPCR and ion channel targets in neuropsychiatry, inflammation, and genitourinary disease indications.

Silvia Patrick (née Cantaluppi) obtained her degree in pharmaceutical chemistry and technology at the University of Milan in 1979 and her MLS at San Jose State University in 1988. She managed library services in Milan and taught information resources at the Library School at San Jose. In 2001 she joined Roche in Palo Alto, where she has been providing targeted information services in a variety of drug discovery areas.

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now allow these experiments to be performed using recombinant expression of these proteins.[24,25]

A commonly employed approach to the identification of SOCE inhibitors uses fluorescence-based measurements of intracellular calcium concentration following depletion of intracellular stores.[8,26] The depletion of intracellular stores can be accomplished experimentally with Ins(1,4,5)P_3 or an agonist of surface receptors linked to Ins(1,4,5)P_3 formation. Alternatively, inactivation of Ca^{2+}-ATPases that transport Ca^{2+} from the cytosol into stores in the ER will lead to store depletion; thapsigargin or cyclopiazonic acid are commonly used to inactivate Ca^{2+}-ATPases in these experiments.[27]

Electrophysiological methods to characterize I_{CRAC} currents are important for studying calcium channels associated with SOCE.[9] In the whole-cell patch-clamp method, intracellular stores are depleted to activate I_{CRAC}, and current is measured during systematic variations of the cellular membrane potential. The small I_{CRAC} current, which has distinctive inward rectification and high Ca^{2+} selectivity, is directly monitored. This method is the only technique that permits direct detection of CRAC channel activity. Patch-clamp experiments therefore allow significantly more precise characterization of SOCE inhibitors than fluorescent methods, which simply report changes in cytosolic Ca^{2+} concentrations. In particular, electrophysiology experiments can exclude the possibility that inhibitor-induced membrane depolarization is responsible for a decrease in calcium influx following store depletion.

Molecules that inhibit CRAC channels are also expected to reduce both the translocation of NFAT and the level of IL-2 production in lymphocytes. It is therefore possible to identify SOCE inhibitors indirectly using established NFAT translocation[28,29] and IL-2 production[30] assays. Subsequent analysis using fluorescence detection of SOCE or electrophysiological measurements can then be performed to confirm the mechanism of action of these compounds.

It is important to consider the variety of mechanisms by which a molecule might decrease SOCE without interacting directly with the CRAC channel. Compounds that simply reduce the electrochemical driving force for Ca^{2+} entry may be mistakenly identified as direct blockers of CRAC channels in assays that rely on measurements of intracellular calcium concentrations. For example, activators of the TRPM4 channel or K^+-channel blockers could depolarize the plasma membrane and decrease the magnitude of the I_{CRAC} current.[31,32] Similarly, compounds that prevent STIM1 translocation could be mistaken for CRAC channel blockers. Careful electrophysiology studies are required to determine the precise mechanism of action of molecules that are identified as SOCE inhibitors using fluorescent probes to track changes in intracellular Ca^{2+} concentration.

Most SOCE inhibitors identified to date appear to be poorly selective for the specific block of CRAC channels. However, the identification of potent, selective inhibitors of these ion channels should be facilitated by the recent discovery of ORAI1 and STIM1 and new high-throughput ion channel inhibition assays.[33] Herein we document the inhibitors of SOCE that have been reported so far. Where possible, the methods that were used to characterize the mechanism of action of the inhibitors are also described.

Abbott Labs

In 1999, Abbott disclosed a novel class of NFAT transcription factor regulators that contain a 3,5-disubstituted pyrazole bound to a 4-amide-substituted phenyl ring (Figure 3).[30,34,35] The initial lead structures **1** and **2** were discovered in a high-throughput reporter-gene-based screen for inhibition of IL-2 synthesis in stimulated Jurkat T-lymphocytes. Testing of the compounds in a concanavalin A-induced proliferation assay using human and rat peripheral blood mononuclear cells (PBMCs) and several assays measuring cytokine inhibition in human whole blood and various human cell types demonstrated the potential of these compounds to function as novel immunosuppressive agents (Table 1).[30,35,36]

Replacement of the isoxazole heterocycle in the initial lead with a thiadiazole ring led to the very potent compound **3** (Figure 3, Table 1). As described in the following section, researchers at Astellas have reported that **3** is a potent inhibitor of thapsigargin-induced SOCE in Jurkat T-lymphocytes. Interestingly, independent studies by Abbott showed that **2** did not inhibit Ca^{2+} influx in Jurkat T-lymphocytes induced by anti-CD3. These researchers, while not identifying a specific mechanism of action, suggested that the target of compound **2** is downstream of Ca^{2+} influx.[36] Inhibition of immune response was determined to occur via a calcineurin-independent mechanism, distinguishing these compounds from classic immunosuppressive drugs such as cyclosporine A and tacrolimus.[37]

Compounds bearing fluorinated phenyl and heteroaromatic rings (e.g. **4**, Figure 3, Table 1) showed an additional boost in potency in several cytokine inhibition and cellular proliferation assays. The observation that the potency of the compounds in a concanavalin A-stimulated human PBMC proliferation assay was not reflected in IL-2 synthesis inhibition in human whole blood was attributed to the high protein binding (>99.7%) of

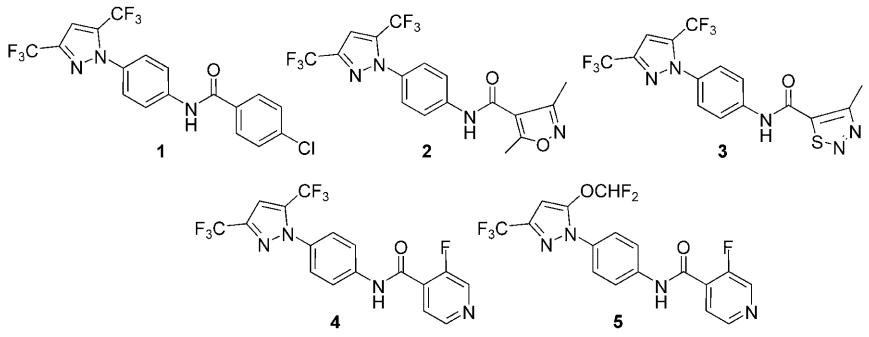

Figure 3. Abbott compounds.

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Table 1. Abbott compounds.

Compd	IC_{50} [nM]					
	PBMC proliferation human[a]	PBMC proliferation rat[a]	IL-2 whole blood[b]	IL-2 PBMC[c]	IL-4 inhibition[d]	IL-5 inhibition[d]
1	301	9603	6032	380	150	150
2	322	1266	1978	314	110	150
3	173	687	1735	102	50	80
4	42	141	496	NA	4.8	22
5	82	146	182	NA	NA	NA

[a] PBMC proliferation stimulated by concanavalin A. [b] PMA/ionomycin-induced IL-2 synthesis in human whole blood. [c] Inhibition of IL-2 production in anti-CD3/CD28-stimulated human PBMC. [d] Inhibition of IL-4 and IL-5 secretion in PMA-stimulated human T-cells.

these compounds. To decrease protein binding, the lipophilic CF_3 group at position 5 of the pyrazole was varied. In general, decreased protein binding and therefore improved potency in the whole-blood assay was achieved by introducing the more hydrophilic substituents CN, OMe, and $OCHF_2$ (i.e. **5**, Figure 3, Table 1).

The most promising compound, **5**, inhibited IL-2 production in whole blood 10-fold more potently than cyclosporine A. This compound also displayed an excellent pharmacokinetic profile in rats ($t_{1/2}$ ~6.5 h) and monkeys ($t_{1/2}$ ~8 h).[30] In two-week toxicity studies in monkeys, **5** was reported to be generally well tolerated, although some neurological side effects were observed.[38] In addition, **5** showed good efficacy in an *Ascaris*-induced nonhuman primate model of asthma as compared with cyclosporine A.[30] More recently, compound **5** was shown to prevent high-glucose-induced NFAT-c3 accumulation in cerebral arteries.[39,40]

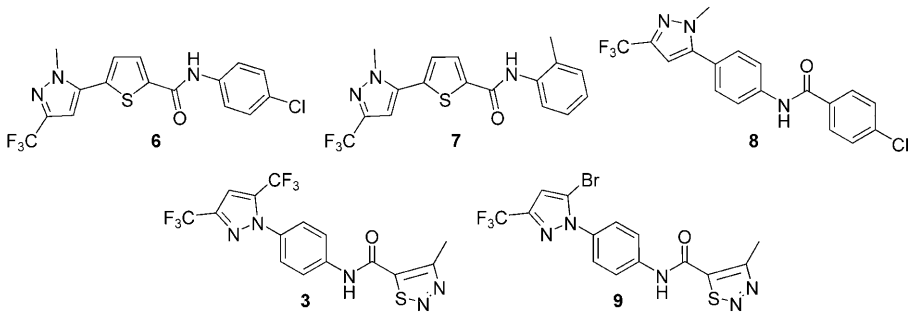

Figure 4. Astellas compounds.

Astellas Pharma

In screening assays targeted toward the discovery of novel immunosuppressive agents, researchers at Yamanouchi Pharmaceuticals (now Astellas Pharma) identified 5-pyrazol-5-yl-2-thiophene-carboxamide **6** as a moderately potent SOCE inhibitor (Figure 4).[41–46] Modification of the initial lead was guided both by the ability of the compounds to inhibit thapsigargin-induced Ca^{2+} influx in Jurkat T-lymphocytes, and selectivity versus inhibition of voltage-operated calcium channels (Table 2). These studies revealed that compounds bearing an *ortho*-substituted phenyl ring (e.g. **7**) were highly active and selective SOCE inhibitors. The authors observed a correlation between the degree of hydrophobicity of the corresponding acetamides and the inhibitory potency.

Subsequently, the role of the pyrazole moiety on the CRAC channel inhibitory activity and selectivity was studied.[42] The hydrophobic trifluoromethyl group at position 3 of the pyrazole ring proved to be essential for inhibitory activity and se-

Table 2. Astellas compounds.

Compd	IC_{50} [μM] CRAC[a]	IC_{50} [μM] VOC[b]	Selectivity[c]	IC_{50} [μM] Inhibition of IL-2[d]	ED_{50} [mg kg^{-1} p.o.] Hepatitis in mice[e]	Inhibition [%] DTH in mice[f]	ED_{50} [mg kg^{-1} p.o.] Eosinophilia in rats[g]
6	0.13	0.75	5.8	0.53	NA	NA	NA
7	0.30	10	33	0.053	12.7[h]	NA	NA
8	0.085	2.6	31	0.21	4.2	NA	NA
3	0.15	4.7	31	0.017	0.61	100[i]	2.4
9	0.092	2.6	28	0.0059	NA	8.3	NA
10	0.077	27%	>130	0.018	NA	44	NA
11	0.29	41%	>34	0.033	NA	64	1.3
12	0.4	3.5	8.8	NA	NA	NA	NA

[a] Inhibition of thapsigargin-induced Ca^{2+} influx in Jurkat T-lymphocytes. [b] Inhibition of Ca^{2+} influx in KCl-stimulated PC12-h5 cells. [c] Selectivity = $(IC_{50\,VOC})/(IC_{50\,CRAC})$. [d] Inhibition of PHA-induced IL-2 production in Jurkat T-lymphocytes. [e] Inhibition of concanavalin A-induced liver injury in mice. [f] Inhibition of TNCB-induced contact hypersensitivity in mice; percent inhibition at 30 mg kg^{-1}. [g] Inhibition of OA-induced airway eosinophilia in rats. [h] 67% inhibition. [i] ED_{50} = 1.1 mg kg^{-1} p.o.

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lectivity, as well as a small substituent at position 1, which maintained a specific torsional angle between the pyrazole and the thiophene rings. The authors also found that the NH group of the amide linkage is essential for high potency. Replacement of the central thiophene ring by other heterocycles or a phenyl ring resulted in a loss of activity.

Interestingly, compound **8**, which bears a 1,4-phenylene core and an amide linkage that is inverted relative to the original series of inhibitors, showed improved inhibitory potency and selectivity (Figure 4, Table 2). The high potency of the compounds was attributed to their extended ground-state conformation. These inhibitors also maintained a distance between the CF_3 group and the amide proton similar to that found in the original thiophene series (~9.4 Å).

A new series of inhibitors was designed based on the 1,4-phenylene core, the reversed amide and an N-bound bis(trifluoromethyl)pyrazole moiety. As observed in the original thiophene series, substituents neighboring the amide bond resulted in higher inhibitory potency and selectivity. Compound **3** (Figure 4, Table 2), now commonly known as YM58483 or BTP2, effectively and selectively blocked thapsigargin-induced Ca^{2+} influx through store-operated channels in Jurkat T-lymphocytes (IC_{50} = 150 nM).[47]. In an electrophysiology assay, Astellas showed that extracellularly applied **3** blocked Ins(1,4,5)P_3-evoked I_{CRAC} in Jurkat T-lymphocytes and in rat basophil leukemia cells (IC_{50} = 2.2 and 0.5 μM, respectively).[32] The discrepancy between these values and the ability of these compounds to suppress cytokine production at nanomolar concentrations [IC_{50}(IL-2, Jurkat) = 17 nM] was explained by the observation that **3** activates the cation channel TRPM4.[32] In these studies it was shown that **3** does not interact with K^+ channels or Cl^- channels.[32] However, He et al. later suggested that **3** may inhibit TRPC3 and TRPC5 channels.[48]

Zitt et al. determined IC_{50} values of 10 and 14 nM for **3**-mediated inhibition of thapsigargin-induced SOCE channels in Jurkat T-lymphocytes and peripheral blood lymphocytes, respectively.[26] These authors attributed the difference in the magnitude of their inhibitory constants from the value reported by Astellas to the different extracellular Ca^{2+} concentrations and pre-incubation times used in the assays. They also showed that **3** inhibits SOCE independent of the method chosen to stimulate store depletion (anti-CD_3, thapsigargin, Ins(1,4,5)P_3). Compound **3** inhibited IL-2 secretion in CD4+ cells,[26] as well as IL-5 production from human whole blood in a manner that was again similar to the known immunosuppressants prednisolone and cyclosporine A.[49,50]

Given the impressive in vitro immunosuppressive activity of compound **3**, this inhibitor served as a new lead structure for further investigations.[51] The substituent at position 5 of the pyrazole was varied, and SOCE inhibitory activity was compared with the calculated torsion angle between the pyrazole and the benzene rings. The key message was that highly active compounds show a torsion angle greater than 40°. The best results with respect to inhibitory activity and selectivity were obtained when the CF_3 moiety was replaced with small or electron-withdrawing groups such as CH_3, CN, Cl, or Br (e.g. **9**, Figure 4, Table 2). As mentioned above, identical substructures were evaluated by Abbott on a slightly different series of compounds.

Variation of the heteroaromatic right-hand portion of the inhibitors revealed that a certain degree of torsion between the amide moiety and the right-hand portion was also important for maintaining CRAC channel inhibitory activity. The introduction of a halogen atom at position 5 or a methyl group at position 6 of a pyridine-3-yl ring improved both activity and selectivity in this series (**10** and **11**, Figure 5, Table 2).[51] Various annulated pyrazoles were disclosed in a separate patent (e.g. **12** and **13**, Figure 5).[52] Compound **12** inhibited activity of the CRAC channel and had good selectivity with respect to the inhibition of a VOC channel (Table 2).[51,52] All Astellas inhibitors were potent suppressors of induced IL-2 production in Jurkat T-lymphocytes and other cytokines in different cell types.[41,42,47,51]

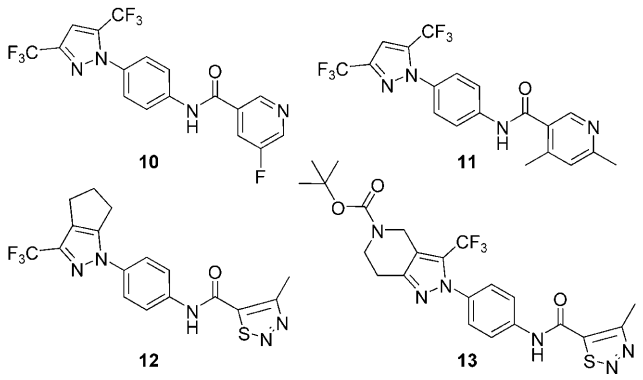

Figure 5. Astellas compounds.

In vivo studies of the orally available Astellas compounds (i.e. **3** and **8**, Table 2) revealed their ability to inhibit concanavalin A-induced hepatitis in mice, with compound **3** showing an ED_{50} value of 0.61 mg kg^{-1} p.o. in this model.[41,42] Several inhibitors also proved to be active in a contact hypersensitivity model in mice (**3**, ED_{50} = 1.1 mg kg^{-1}, Table 2).[47,51]

The effect of CRAC channel inhibition on antigen-induced eosinophilia was studied using asthmatic models in rats and guinea pigs, and compared with known corticosteroids and cyclosporine A.[49,50] Additionally, the influence of **3** in a mouse graft-versus-host disease (GVHD) model was examined. Inhibitor **3** inhibited anti-host cytotoxic T-cell activity in a dose-dependent manner. This compound also inhibited spleen T-cell proliferation (IC_{50} = 330 nM) and the sheep red blood cell induced delayed type hypersensitivity response in mice following oral administration. Little is known regarding the safety profile of **3**. It has been reported that there was no significant change in general activity in mice following oral administration up to 30 mg kg^{-1}.[53]

Finally, inhibitor **11** was identified in a patent application as a preventive or remedy for inflammatory bowel disease. This compound prevented water secretion from gastrointestinal epithelial cells and relieved diarrheal symptoms in a series of in vivo tests in rodents. In this model, **11** performed equally to

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or better than econazole, cyclosporine A, sulfasalazine, and prednisolone.[54]

Boehringer Ingelheim

In the same year that Abbott and Astellas disclosed their discovery of the pyrazole immunosuppressive agents, Boehringer Ingelheim revealed a very similar series of compounds as inhibitors of IL-2 production in T-lymphocytes (Figure 6).[55,56] Again, the 3,5-bis(trifluoromethyl)pyrazole moiety served as a common motif. Many inhibitors contained a pyridine ring attached at the 3-position of the pyrazole ring (e.g. **15**). Several examples of the invention contained amide linkages, although similar alkyl amines were also claimed.

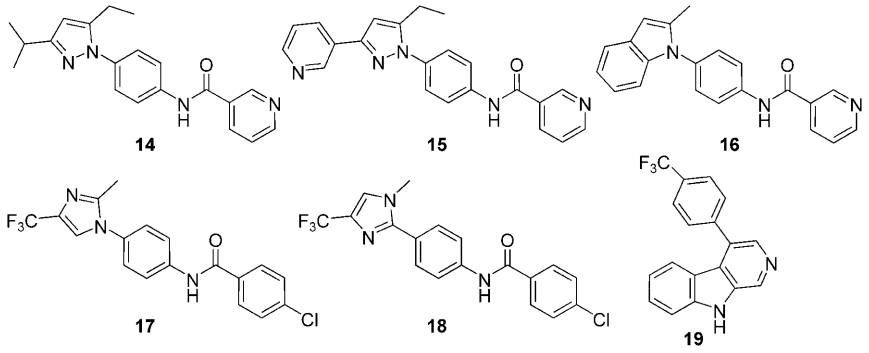

Figure 6. Boehringer Ingelheim compounds.

The compounds showed IC_{50} values less than 10 μM in an IL-2 production assay and were specifically identified as inhibitors of SOCE (Table 3). Compounds **14** and **15** showed IC_{50} values of 214 and 300 nM, respectively, for the inhibition of anti-CD3-stimulated Ca^{2+} influx in Jurkat T-lymphocytes. Additionally, **14** and **15** proved to be potent inhibitors of soluble epoxide hydrolase.[56] In vivo studies showed that **14** and **15** inhibited an allogenic transplant response in mice.[55,56]

In a subsequent series of patents, Boehringer Ingelheim researchers identified three additional compound classes as inhibitors in an IL-2 promoter assay in which the pyrazole moiety was replaced by an indole or imidazole ring.[57,58]. These inhibitors are represented by **16**, **17**, and **18**, and show IC_{50} values less than 10 μM in an IL-2 production assay (Figure 6). Notably, these applications did not refer to the ability of these compounds to inhibit SOCE.

Finally, a series of 4-substituted β-carbolines (e.g. **19**, Figure 6, Table 3) was disclosed by Boehringer Ingelheim in 2000. These compounds inhibited Ca^{2+} influx and IL-2 production in T-cells. Several inhibitors also proved to effectively suppress the graft-versus-host (GVH) reaction in mice.[59]

Synta Pharmaceuticals

As early as 2005, Synta Pharmaceuticals reported the identification of inhibitors of I_{CRAC} in human primary T-cells, Jurkat T-lymphocytes, and rat basophil leukemia (RBL) cells.[60] Importantly, it was claimed that these inhibitors showed selectivity for CRAC inhibition over modulation of $K_V1.3$, hERG, TRPM4, or TRPM7 channels. Since that time, many applications have been filed by Synta that describe a large number of structurally differentiated SOCE inhibitors.

Biphenyl compounds

The initial patents in this area from Synta disclosed the activity of a new series of I_{CRAC} inhibitors in which the pyrazole ring of the Astellas compounds had been replaced with an *ortho*-substituted phenyl ring (Figure 7).[61,62] Compounds were tested for inhibition of I_{CRAC} in RBL cells using a whole-cell patch-clamp assay. The potency of selected compounds is shown in Table 4. I_{CRAC} inhibition was observed in Jurkat T-lymphocytes and primary T-cells. The ability of compounds **20** and **22** to inhibit cytokine release was determined in whole blood following administration of the inhibitors to monkeys. After phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation, both compounds inhibited the production of IL-2 and TNF-α with a potency similar to that of cyclosporine A (Figure 7, Table 4). Studies with phytohemagglutinin-stimulated PBMCs also indicated that compounds **20**, **21**, and **22** potently inhibited cytokine production. Compound **20** also reduced degranulation in RBL cells ($IC_{50} = 0.38$ μM).

All of the I_{CRAC} inhibitors claimed in this application[61] were also reported to inhibit the activity of $K_V1.3$ ion channels, although no data were provided. In addition, some compounds were found to activate TRPM4 channels. For example, compound **23** (Figure 7) activated TRPM4 in HEK-293 cells overexpressing TRPM4 with an IC_{50} value of ~50 nM. However, I_{CRAC} inhibitor **21** had no effect on

Table 3. Boehringer Ingelheim compounds.

Compd	IC_{50} [nM]					
	CRAC[a]	SEH inhibition[b]	IL-2 reporter assay[c]	IL-2 inhibition, PBMC[d]	IL-4 inhibition, PBMC[d]	IFN-γ inhibition, PBMC[d]
14	214	464	190	NA	NA	NA
15	300	60	80	20	16	22
19	500[e]	NA	230	300	NA	NA

[a] Inhibition of anti-CD3-induced Ca^{2+} influx in Jurkat T-lymphocytes. [b] Inhibition of soluble epoxide hydrolase activity. [c] Inhibition of IL-2 promoter-driven luciferase activity in Jurkat cells. [d] Inhibition of cytokine secretion in PBMCs. [e] Inhibition of thapsigargin-induced Ca^{2+} influx in Jurkat T-lymphocytes.

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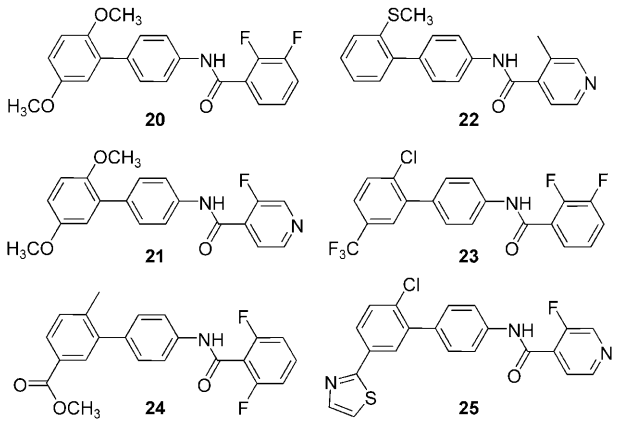

Figure 7. Early Synta compounds.

Table 4. Synta inhibitors.

Compd	IC_{50} [nM]			
	I_{CRAC}[a]	Jurkat IL-2[b]	PBMC TNF-α[c]	Other
20	100	NA	68	380[d]
21	300	NA	271	430[d]
22	200	NA	81	NA
23	NA	NA	NA	50[e]
24	20	4	NA	2520[d]
25	40	2	NA	NA
26	70	4	NA	NA
28	220	30–50	NA	NA
29	NA	< 30	NA	NA
30	NA	29	424	NA
31	NA	3	47	NA
32	NA	< 100	NA	NA
33	60	6	NA	NA
34	150	75–150	NA	NA
35	NA	5	NA	NA
36	100	11	NA	NA
37	NA	18	NA	NA

[a] Inhibition of I_{CRAC} measured in RBL cells. [b] Inhibition of IL-2 production in Jurkat T-lymphocytes. [c] Inhibition of TNF-α production from human PBMCs. [d] Inhibition of degranulation in RBL cells. [e] Inhibition of TRPM4 expressed in HEK-293 cells.

TRPM4 currents in electrophysiology studies with Jurkat T-lymphocytes.

Further investigation led to the identification of a number of I_{CRAC} inhibitors, exemplified by **24** and **25** (Figure 7), that contain esters or ester isosteres in the 5-postion of the terminal phenyl ring.[63] Both of the highlighted compounds potently decreased I_{CRAC} in RBL cells and the production of IL-2 from Jurkat T-lymphocytes (Table 4). In addition, compound **24** was an active inhibitor of degranulation in anti-IgE-stimulated RBL cells.

Central heteroaromatic ring

Substitution of the central phenyl ring of the Synta compounds provided further families of I_{CRAC} inhibitors (Figure 8).[64] The majority of the compounds exemplified in these patents featured a 2,5-substituted or a 3-substituted terminal phenyl ring and a pyridine or pyrazine central aromatic group. Pyrazine **26** inhibits RBL I_{CRAC} with an IC_{50} value of 70 nM and decreases IL-2 secretion in Jurkat T-lymphocytes at low concentrations (Table 4). Thiophene **27** has similar activity in these assays. The amide group of the thiophene inhibitors has been transposed relative to the original Synta compounds in accordance with the structure–activity relationships originally described by Astellas.

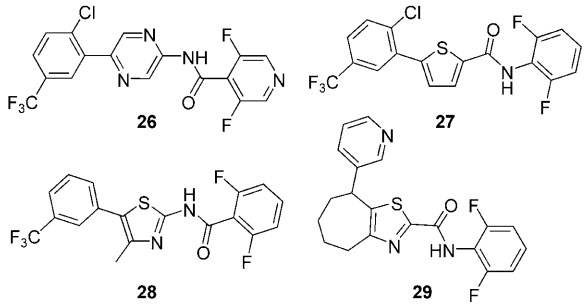

Figure 8. Synta heterocycles.

In separate applications, phenylthiazole compounds such as **28** (Figure 8, Table 4) were demonstrated to be I_{CRAC} inhibitors.[65] A large number of compounds with different substituents at the 4-position of the thiazole ring were claimed to be immunomodulators in this application. A more recent application described annulated thiazoles similar to **29** that decrease IL-2 production in Jurkat T-lymphocytes, although no electrophysiology data were provided.[66]

Heterocyclic compounds in the western portion

Small molecules containing 6,5-heterocycles were discovered by Astellas and Boehringer Ingelheim to be potent SOCE or IL-2 production inhibitors. An application from Synta describes several examples of compounds claimed to modulate I_{CRAC}, TRPM4, and $K_V1.3$ (Figure 9). The structure of inhibitor **30** is representative of the majority these compounds, and **30** was claimed to inhibit $K_V1.3$ in patch-clamp experiments.[67] Both **30** and **31** inhibited the secretion of cytokines from stimulated PBMCs (Table 4). Inhibitor **32** was specifically mentioned to be an activator of TRPM4, while closely related pyrazine **33** was reported to be a potent inhibitor of I_{CRAC} in RBL cells

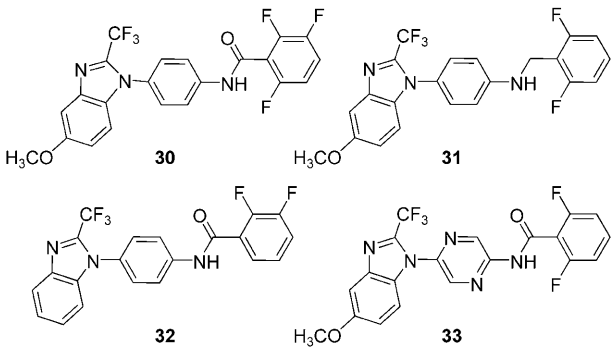

Figure 9. Synta benzimidazoles.

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(Figure 9).[68] In a delayed-type hypersensitivity test in rats, compound **33**, administered orally, was found to have efficacy similar to cyclosporine A.

Additional classes of compounds recently claimed to be I_{CRAC} inhibitors include the tetrahydropyridinyl compounds **34** and **35** (Figure 10, Table 4).[69,70] Electrophysiology data were provided for **34**, and excellent pharmacokinetic parameters were obtained for inhibitor **35** following oral administration of the corresponding dihydrochloride salt to rats. Further elaboration of this portion of the inhibitors appears to be possible. For example, **36** inhibited I_{CRAC} with an IC_{50} value of 100 nM, and compounds related to **37** were reported to inhibit cytokine production in Jurkat T-lymphocyte incubations (Figure 10, Table 4).[71]

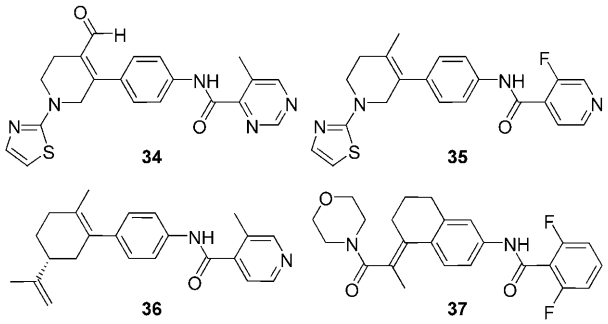

Figure 10. Recent Synta compounds.

Miscellaneous SOCE Inhibitors

SKF-96365 was one of several imidazole compounds to be clearly identified as an inhibitor of SOCE (Figure 11).[72,73] In assessments of the ability of SKF-96365 to block thapsigargin-induced calcium entry into Jurkat T-lymphocytes, the compound demonstrated an IC_{50} value for I_{CRAC} inhibition of ~12 μM. SKF-96365 is also known to be an inhibitor of voltage-gated calcium channels. In the initial publications it was suggested that this compound might decrease I_{CRAC} either by interfering with cytochrome P450 activity or by affecting the activity of calmodulin. The structurally related imidazole compounds econazole and miconazole were also determined to inhibit SOCE (Figure 11).[73] In a separate study, it was found that both econazole and an impermeable econazole derivative blocked I_{CRAC} in Jurkat T-lymphocytes when applied extracellularly, but not when added intracellularly.[74] This observation was inconsistent with the hypothesis that the imidazoles reduce SOCE indirectly by decreasing the activity of P450 enzymes.

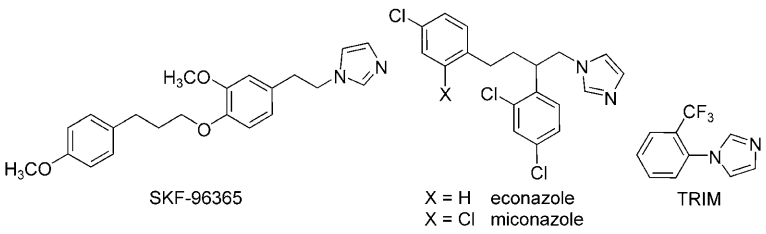

Figure 11. Imidazole derivatives.

In mouse smooth muscle, trifluoromethylphenylimidazole (TRIM), was determined to block SOCE (Figure 11, EC_{50} = 42 μM).[75] Although this compound was less potent than SKF-96365 in this system, experiments suggested that TRIM had improved selectivity versus inactivation of voltage-operated calcium channels.

Several small molecules were tested for their ability to reduce SOCE in human carcinoma cells.[76] The triazole derivative *N*-1-*n*-octyl-3,5-bis-(4-pyridyl)triazole (DPT),[77,78] sulindac sulfide, and mefenamic acid were found to inhibit SOCE following thapsigargin-induced store depletion (Figure 12). The authors suggested that SOCE might be important for cell proliferation, and the possibility that SOCE inhibitors may function as novel agents for the treatment of cancer has been highlighted recently.[79]

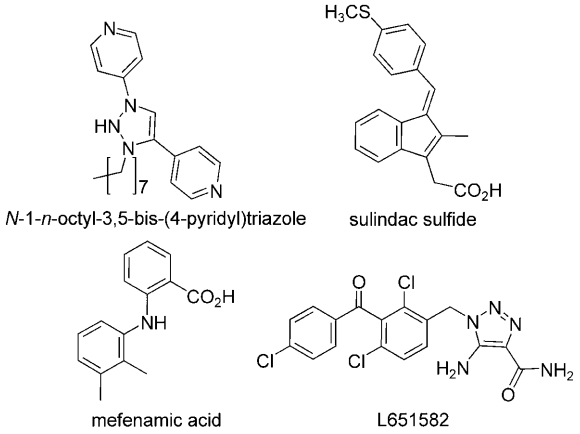

Figure 12. *N*-1-*n*-octyl-3,5-bis-(4-pyridyl)triazole, sulindac sulfide, mefenamic acid, and L651582.

Along these lines, the antiproliferative compound L651582 (Figure 12) inhibits M_5 muscarinic receptor-mediated calcium influx in Chinese hamster ovary (CHO) cells that do not depend on voltage-operated calcium channels.[80] Although no electrophysiology studies were reported, L651582 did not affect levels of Ins(1,4,5)P_3 or 3′,5′-cyclic adenosine monophosphate (cAMP), consistent with a mechanism of action involving block of SOCE.

The diphenylborinate 2-APB (Figure 13) has been widely used to characterize CRAC channel activity, although the effects of 2-APB are concentration dependent.[23,81,82] At low concentrations, some current potentiation is observed, whereas at high concentrations, application of the inhibitor causes temporary enhancement followed by I_{CRAC} block. It has been reported that this compound also blocks a variety of potassium channels and TRP channels while activating TRPV6 channels. Compounds structurally related to 2-APB have also been found to inhibit SOCE.[83] Screening of 150 phenylboronate compounds led to the identification

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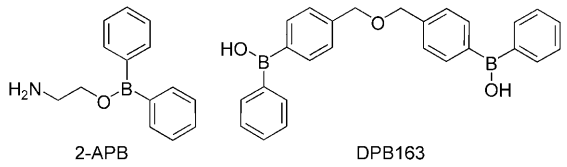

Figure 13. Borinate SOCE inhibitors.

of DPB163 (Figure 13), which reduced thapsigargin-induced SOCE in various cell lines at sub-micromolar concentrations. Receptor-operated calcium entry was also affected by these analogues.

Capsaicin (Figure 14 was reported to inhibit CRAC at high concentrations ($IC_{50} = 30$ μM).[84,85] Again, K^+ channels were also modulated at these concentrations, while activation or inhibition were described for various TRP channels. More recently, the lipophilic phenol diethylstilbestrol (Figure 14) was found to inhibit SOCE in a variety of cell types.[86] In RBL cells, the IC_{50} for inhibition of I_{CRAC} was approximately 0.6 μM. Interestingly, when diethylsilbestrol was applied intracellularly, no inhibition of I_{CRAC} was observed. Monovalent cation currents mediated by TRPM7 were not affected by this inhibitor. Furthermore, *trans*-stilbene, a structurally related alkene, did not affect I_{CRAC}.

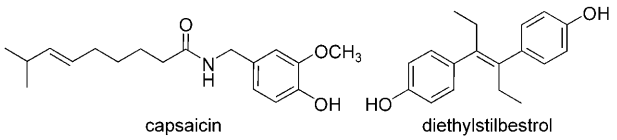

Figure 14. Phenol SOCE inhibitors.

The mechanism by which the acidic chloride channel blocker NPPB (Figure 15) blocks store-operated calcium channels was studied by scientists at AstraZeneca.[87,88] As the ability of Cl^- channel blockers to decrease I_{CRAC} was not proportional to the potency with which these compounds reduced Cl^- channel block, it was argued that the inhibition of I_{CRAC} by NPPB involves a mechanism distinct from their Cl^- channel-blocking activity. From whole-cell I_{CRAC} recordings in Jurkat T-lymphocytes, NPPB was found to be a reversible inhibitor with an IC_{50} value of 5 μM. Kinetics measurements and inhibition studies at various extracellular pH levels suggested that the neutral form of NPPB interacts directly with the CRAC channel in Jurkat T-lymphocytes. Other Cl^- channel blockers that have also been found to be weak inhibitors of I_{CRAC} in rodent cell lines include NPPA and niflumic acid (Figure 15).[89,90] Finally, the oligomycins, which affect Cl^- currents and mitochondrial ATP production, were found to inhibit I_{CRAC} (IC_{50} for oligomycin B: ~0.5 μM) in Jurkat T-lymphocytes.[91]

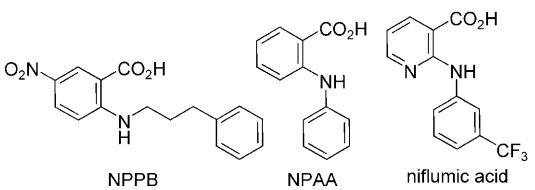

Figure 15. Cl^- channel inhibitors that decrease SOCE.

The irreversible iPLA2 inhibitor **38** (Figure 16) inhibits thapsigargin-induced calcium influx in Jurkat T-lymphocytes and other cell types, although the exact mechanism of SOCE inhibition remains undefined.[92] The identification of this inhibitor supports the potential role of iPLA2 and other cellular factors as components of the SOCE pathway.[93]

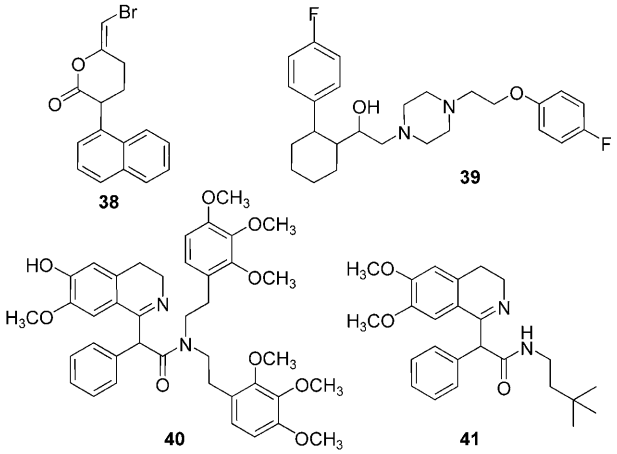

Figure 16. SOCE inhibitors.

In 2001 BASF disclosed a new class of SOCE inhibitors based on cycloalkyl-piperazinylethanol derivatives (**39**, Figure 16).[94] It was reported that these compounds were effective at low-micromolar concentrations. High selectivity for the inhibition of store-operate calcium channels compared to receptor-operated calcium channels was observed.

Boehringer Ingelheim reported a series of dihydroisoquinolines (Figure 16) that were effective blockers of thapsigargin-induced Ca^{2+} influx in RBL cells. These compounds were thought to inhibit SOCE by blocking Na^+ and K^+ channels. Compounds **40** and **41** had IC_{50} values of 3.47 and 7.57 μM, respectively.[95]

Nifedipine and nitrendipine (Figure 17), marketed dihydropyridine L-type calcium channel blockers, have been reported to block SOCE in Jurkat T-lymphocytes.[96,97] In this system, the

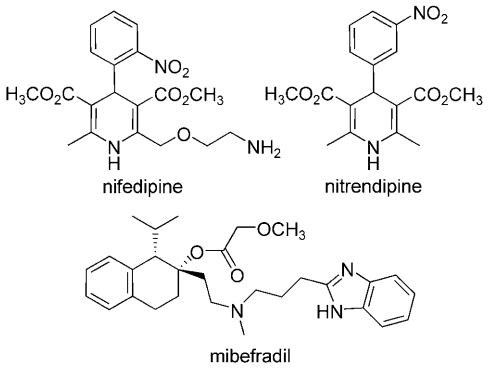

Figure 17. Voltage-operated calcium channel blockers that inhibit SOCE.

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structurally distinct L-type blockers verapamil and diltiazem (Figure 1) did not inhibit SOCE, and it was emphasized that nifedipine did not appear to inhibit calcium entry by a mechanism involving plasma membrane depolarization. Mibefradil (Figure 17), a T-type calcium channel blocker, was also found to inhibit SOCE. The effect of mibefradil was considered to be due to the specific inhibition of store-operated channels, as other T-type calcium blockers did not show an effect on calcium entry following store depletion.[98]

In experiments investigating the block of SOCE by CD95, whole-cell patch-clamp recordings revealed that C2 ceramide, C6 ceramide, and sphingosine reversibly and dose-dependently blocked I_{CRAC} in single T-cells (Figure 18).[99] C2 ceramide also blocked NFAT activity in lymphocytes, albeit at high concentrations (100 μM).

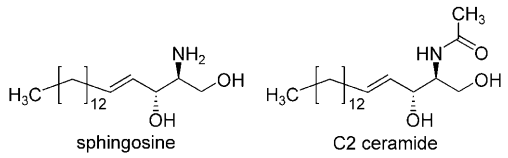

Figure 18. Sphingosine and C2 ceramide.

Several kinase inhibitors have been found to interfere with SOCE.[100] The myosin light chain kinase inhibitors wortmannin and ML-9 were found to decrease calcium influx following store depletion in human macrophages, while the kinase inhibitor methyl-2,5-dihydroxycinnamate decreased SOCE in human platelets (Figure 19).[101,102] Based on these results, the authors

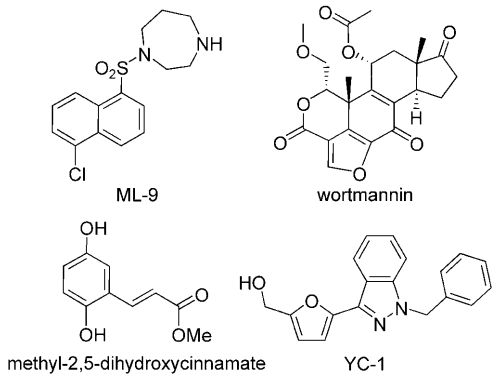

Figure 19. SOCE inhibitors.

suggested that certain kinases and phospholipases involved in microtubule formation may play a role in SOCE. Recent publications from other laboratories have suggested that the microtubule cytoskeleton may play a role in STIM1 redistribution following Ca^{2+} store depletion.[103,104] YC-1, an inhibitor of cyclopiazonic acid-activated SOCE in neutrophils, may also disrupt the integrity of the cytoskeleton necessary for calcium influx following store depletion (Figure 19).[105]

It is well known that lipophilic acids can modulate ion channel function by nonspecifically changing the physical properties of the membrane bilayer in which the channel resides.[106] More specific and higher-affinity interactions are also possible.[107] The unsaturated fatty acids arachidonic acid and oleic acid were found to inhibit SOCE following thapsigargin-induced store depletion in several cell types at micromolar concentrations.[108] Progesterone, a lipophilic steroid that is known to have a high affinity for membranes, was also found to inhibit SOCE in Jurkat T-lymphocytes.[109] Experiments with radiolabeled progesterone revealed that the extent of inhibition was proportional to the levels of membrane-bound progesterone. Interestingly, related steroids did not appear to significantly affect SOCE in this system.

The antimalarials primaquine and chloroquine (Figure 20) block I_{CRAC} induced by store depletion in rat megakaryocytes.[110] As these drugs are known to disrupt vesicle transport, it was initially postulated that vesicle transport may play a role in SOCE.[111] Primaquine was also found to reversibly inhibit SOCE in frog oocytes.[112] Mechanistic studies in oocytes suggested that membrane fusion processes were less likely than protein-signaling pathways to be involved in SOCE.

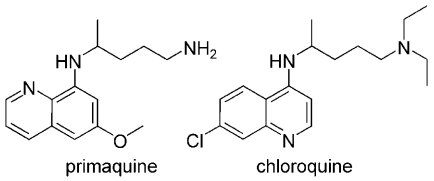

Figure 20. Antimalarial compounds that block SOCE.

Summary

In response to activation by cell-surface receptors, store-operated calcium entry (SOCE) produces a sustained influx of Ca^{2+} into the cytoplasm. While the resulting calcium-release-activated current (I_{CRAC}) was discovered more than a decade ago and has been characterized extensively, key proteins involved in this pathway were discovered only recently. The identification of these proteins is likely to accelerate efforts targeted at the discovery of inhibitors of SOCE that selectively block the calcium-release-activated calcium channel (CRAC).

Many inhibitors of SOCE were discovered using methods that measure intracellular calcium concentration following store depletion. As SOCE involves the action of several different proteins, the exact mechanism of action of many of these inhibitors remains undefined. Nevertheless, these studies have provided useful pharmacological tools for studying calcium-signaling pathways.

A number of structurally diverse agents that inhibit SOCE have also been characterized using electrophysiological methods. It is likely that these compounds are able to directly block the CRAC channel. Some of these compounds, however, are also thought to affect the function of other ion channels.

Recent patents and papers focus on series of aryl amide CRAC channel inhibitors. Characterization of these compounds

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in vitro suggests that the aryl amide inhibitors reduce cytokine production in lymphocytes and prevent degranulation of mast cells. CRAC channel inhibitors have also shown efficacy in several animal disease models.

Outlook

The identification of the ORAI and STIM proteins, along with the fairly well-understood process by which calcium functions as a second messenger in lymphocytes, will encourage further efforts in the pharmaceutical industry to identify selective CRAC channel inhibitors. The ORAI proteins, however, are widely expressed in various tissues, and the ability of even selective CRAC channel inhibitors to provide safe immunomodulators remains to be demonstrated. Although lymphocytes are particularly dependent on CRAC channel function, this pathway is thought to play a role in a number of cell types. In particular, CRAC channels may be important for signaling processes in skeletal muscle, smooth muscle, and neurons.

Small molecules, most notably thapsigargin, SKF-96365 (Figure 11), and compound **3** (Figure 3 and 4), have been useful tools for developing our understanding of SOCE. Future generations of compounds may help us gain insight into the function of the CRAC channel in various tissue types and the role that the ORAI paralogues play in CRAC. Ultimately it is hoped that one of these inhibitors will further validate the CRAC channel as an important therapeutic target.

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Keywords: calcium · inhibitors · ion channels · lymphocytes · store-operated calcium entry

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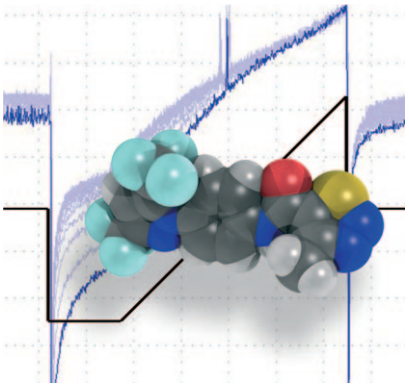
Small-Molecule Inhibitors of Store-Operated Calcium Entry



Molecules that inhibit store-operated calcium entry (SOCE) are potentially useful immunomodulating agents. The identification of proteins involved in this pathway may further enable the identification of selective inhibitors. Herein we document some examples of the small-molecule inhibitors of SOCE that have been reported to date. We also describe methods that were used to characterize the mechanism of action of these inhibitors.

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