REGULATION OF CALCIUM ENTRY PATHWAY IN JURKAT T CELLS

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

PETRONILLA A. FRUASAHA
B.S., Central State University, 2005

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Petronilla A. Fruasaha ENTITLED Regulation of Calcium Entry Pathway in Jurkat T Cells BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Calcium release-activated calcium channels (CRAC) control influx of calcium in human T lymphocytes. Hour-long calcium elevations are necessary for efficient gene expression during T cell activation and proliferation. We report here that, the time course for store-operated Ca$^{2+}$ entry is short-lived (3-4 min) and therefore, cannot account for the prolonged Ca$^{2+}$ elevations necessary for NFAT translocation into nucleus. Previous findings strongly suggest that T cell activation is accompanied by cytosolic alkalinization. Here, we show that pH changes in Jurkat T cells following activation with mitogenic lectin, phytohemagglutinin (PHA), depends on the length of time of exposure and the concentration (potency) of the mitogen. For full understanding of ion fluxes involved in this process, it is important to distinguish CRAC channel subtype functions in these cells during activation as well as elucidate the pH mediated changes in Ca$^{2+}$. In some experiments we show low pH with high concentrations of PHA. We also investigated the dependence of 2-APB action on intracellular pH and show that it mediates its effect by acidification but consistently inhibits CRAC with NH$_4^+$-induced alkalinization. Finally, we showed that in agreement with a previous study, PIP3 specifically, can elicit calcium elevations in Jurkat T cells.
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DEDICATION

Dedicated to my late grandmother, Mama Susanna Guese Ndefru whose wonderful life greatly inspires me. Her beautiful and incredible warm memories light up my days.
I. INTRODUCTION AND LITERATURE REVIEW

Calcium Release-Activated Calcium (CRAC) channels: an overview

Calcium release-activated calcium (CRAC) channels have been widely known to regulate intracellular calcium (Ca\(^{2+}\)) in many cells (Putney et al., 1990; Zweifach and Lewis, 1993). These channels are known to get activated when the endoplasmic reticulum (ER), a Ca\(^{2+}\) store, is depleted of Ca\(^{2+}\) (Putney et al., 1990; Zweifach and Lewis, 1993). Its current (I\textsubscript{CRAC}) has been shown to be inwardly rectifying and highly selective for Ca\(^{2+}\) (Kozak et al., 2002; Feske et al., 2005). CRAC has been recently found to contain three proteins, Orai1 (CRACM1), Orai2 (CRACM2) and Orai3 (CRACM3). These three proteins are found in the CRAC channels’ pore-forming unit with Orai1 being the dominant one in store-operated calcium entry (SOCE). (Vig et al., 2006a; Soboloff et al., 2006b; Vig et al., 2006b; Gwack et al. 2007). HEK 293 cells, human T cells and fibroblast cells have been shown to have a dominant Orai1 (Gwack et al., 2007). Clearly, these proteins have different functions and mechanisms of action or regulation that is important to elucidate. All Orai proteins are made of four transmembrane segments (Fig. 1b) and a putative coiled-coil domain at their C-terminus. Orai1 differs from the others because of regions which are proline (2)- and arginine (1)-rich in its N-terminus. Orai3 on the other hand differs from the others by having a much longer second extracellular loop compared to Orai1 and Orai2 (Cahalan et al. 2007).

The interaction of stromal interacting molecule (STIM) with Orai proteins elicits CRAC activation (Roos et al., 2005; Zhang et al., 2005). The two STIM proteins (STIM1
and STIM2) found in the membrane of the ER are single-pass transmembrane proteins (Fig. 1a). STIM has an EF hand which serves as a Ca$^{2+}$ sensor (Zhang et al., 2005, Liou et al., 2005). The EF hand on STIM binds Ca$^{2+}$ constitutively. Emptying of the endoplasmic reticulum (ER) stores triggers Ca$^{2+}$ dissociation from the EF hand leading to a conformational change in STIM. This facilitates its apposition (Fig. 1c) to the plasma membrane close to the Orai protein (Zhang et al., 2005). It has recently been shown that oligomerization of STIM1, mediated by store depletion, is necessary for its accumulation at the ER-plasma membrane junctions or puncta (Luik et al., 2008). When Ca$^{2+}$ stores are full, STIM1 proteins are found dispersed on the ER membrane (Fig. 1c).

![Figure 1: A schematic representation of Orai1 and STIM1 protein structures and a proposed interaction mechanism (Gwack et al., 2007).](image-url)
STIM and Orai interaction causes CRAC activation and influx of Ca\(^{2+}\) into the cytoplasm of the cell. The STIM2 protein is 61\% homologous to STIM1, their differences are found in the C-terminal region after the ERM/coiled –coil domain (Zheng et al., 2008). STIM1 contains a glutamine- and a serine/threonine-rich region, besides the serine/proline-and lysine-rich region also found in STIM2. It has been shown that STIM2 inhibits STIM1-mediated SOCE (Soboloff et al., 2006a) but in this study we are only interested in STIM1.

Pharmacological Properties of CRAC Channels

2-aminoethoxydiphenyl borate (2-APB) is a compound that can differentiate between Orai proteins. 2-APB has been formerly shown to bind inositol 1, 4, 5-trisphosphate receptor (InsP\(_3\)R) (Ma, Venkatachalam et al., 2001) antagonistically. In this study we bypassed the release of Ca\(^{2+}\) from the ER store mediated through inositol trisphosphate (IP\(_3\)) binding to its receptor, InsP\(_3\)R.

Table 1: Properties of the Mammalian CRACM Proteins from (Lis et al., 2007)

<table>
<thead>
<tr>
<th></th>
<th>CRACM1</th>
<th>CRACM2</th>
<th>CRACM3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Store-operated</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Activation time (t(_{1/2}))</td>
<td>35 ± 7 s</td>
<td>21 ± 3 s</td>
<td>63 ± 7 s</td>
</tr>
<tr>
<td>Ca(^{2+}) -dependent inactivation (fast)</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Strong</td>
</tr>
<tr>
<td>Ca(^{2+}) -dependent inactivation (slow)</td>
<td>Strong</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Ca(^{2+}) -dependent reactivation</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Selectivity</td>
<td>Ca(^{2+}) &gt;&gt; Na(^{+}), Ba(^{2+})</td>
<td>Ca(^{2+}) &gt;&gt; Na(^{+}), Ba(^{2+})</td>
<td>Ca(^{2+}) &gt;&gt; Na(^{+}), Ba(^{2+})</td>
</tr>
<tr>
<td>Monovalent permeation in DVF solutions</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Strong</td>
</tr>
<tr>
<td>2-APB effect at 50 μM</td>
<td>Block</td>
<td>Reduction</td>
<td>Potentiation</td>
</tr>
</tbody>
</table>
The action of 2-APB on CRAC is dose-dependent. Low [2-APB] (≤5 μM) activates all Orai proteins while high [2-APB] (≥ 10 μM) blocks Orai1 current (Peinelt et al., 2006; Prakriya et al., 2001), reduces Orai2 and potentiates Orai3 (Lis et al., 2007; Zhang, Kozak et al., 2008) as shown in Table 1.

More recent studies have shown that 2-APB’s mechanism of action is through the reversal or prevention of STIM1 localization in puncta near the plasma membrane (DeHaven et al., 2008). They show that 50 μM 2-APB disperses STIM1. This dispersion is similar to that seen with filled Ca\(^{2+}\) stores before thapsigargin (TG) mediated depletion as opposed to after TG store depletion (TG mechanism of action is discussed later). On the other hand, they suggest that potentiation occurs in a STIM1-relocalization-independent manner. A contrary previous finding shows that STIM1 is not indispensable in the activation of CRAC by means of store depletion (Zhang, Kozak et al., 2008). The C-terminus of STIM1 (C-STIM1) has been shown to be the effector that activates CRAC current. Therefore, activation of CRAC could be achieved independently of STIM1.

**Calcium Signaling**

Ca\(^{2+}\) is a ubiquitous second messenger in many cellular pathways. In T cells, high sustained [Ca\(^{2+}\)] has been reported to be necessary for their full activation (Rasmussen and Barrett, 1984; Timmerman et al., 1996, Lewis, 2001) following inositol 1, 4, 5 triphosphate (IP\(_3\)) generation (Berridge and Irvine, 1984; Berridge et al., 1993) and subsequent store depletion (Putney, 1990). Timmerman et al., 1996 further states that
high sustained Ca\(^{2+}\) but not transient spikes, are required to retain nuclear factor of activated T cells (NFAT) transcription factors in the nucleus (discussed below). Cardenas and Heitman, (1995) suggest that this is achieved by a long-lasting Ca\(^{2+}\) plateau following a sharp transient Ca\(^{2+}\) influx. In addition to sustained, elevated Ca\(^{2+}\), long-lasting Ca\(^{2+}\) oscillations are also required for T cell gene expression (Negulescu et al., 1994). In the process of gene expression, a variation in Ca\(^{2+}\) signal amplitude promotes activation of different genes (Dolmetsch et al., 1997). Previous findings (reviewed in Rasmussen and Barrett, 1984) state that elevated [Ca\(^{2+}\)] influx is short-lived since the nonionic mitochondrial matrix takes it up. This however is not SOC. We show here that, SOCE of CRAC in Jurkat T cells as well as in our HEK293 expression system is also short-lived. This finding is quite significant because it suggests that SOCE cannot account for all Ca\(^{2+}\) necessary for full activation. Jurkat T cells (Schneider et al., 1977) have been used to model T lymphocytes for over 30 years. Therefore it is important to describe in detail all Ca sources in this system.

According to Rasmussen and Barrett, 1984, there are three possible explanations for this short time course of Ca\(^{2+}\) rise. 1) Released Ca\(^{2+}\) is taken up into the nonionic Ca\(^{2+}\) pool in the mitochondrial matrix. 2) The plasma membrane Ca\(^{2+}\) ATPase (PMCA) pump is activated by calcium-calmodulin (Ca\(^{2+}\)-CaM) when [Ca\(^{2+}\)] increases which pumps Ca\(^{2+}\) out of the cell. 3) Activated cells that have emptied Ca\(^{2+}\) from their ER pool can no longer replenish it as long as they remain activated.

In T lymphocytes (Fig. 2), antigen recognition by immunoreceptors (TCR-CD3) subsequently activates phospholipase C gamma (PLC\(_{\gamma}\)) by means of a transient and rapid phosphorylation of serine and tyrosine residues (Park et al., 1991). PLC\(_{\gamma}\) then hydrolyses
phosphatidylinositol 4, 5 diphosphate (PIP$_2$) to IP$_3$ and diacylglycerol (DAG) (Imboden and Stobo, 1985). When IP$_3$ binds to its receptors on the ER membrane, Ca$^{2+}$ is released from the ER stores into the cytoplasm where the Ca$^{2+}$ sensor protein, calmodulin (Wang, 1979; Weinstein and Mehler, 1994) detects the rise in Ca$^{2+}$ when at least three of its four Ca$^{2+}$-binding sites are occupied (reviewed in Rasmussen and Barrett, 1984).

Calcineurin, a serine/threonine phosphatase (Klee et al., 1979; Stewart et al., 1982) is activated when Ca-calmodulin binds to its binding site on the carboxy terminus of calcineurin leading to a 10-20 fold increase in enzyme’s phosphatase activity (Sagoo et al., 1996). Prior to that, enzyme activity is increased 2-3 fold when calcineurin A (60 kDa phosphatase catalytic subunit) binds calcineurin B (25 kDa high-affinity calcium binding subunit) (Feng and Stemmer, 1999). The autoinhibitory domain in the carboxy terminal is displaced as a result of increased enzyme activity leading NFAT to bind to its catalytic site on calcineurin which then undergoes dephosphorylation (Sagoo et al., 1996).

Nuclear factor of activated T cells (NFAT) (Shaw et al., 1988) is a substrate for calcineurin. NFATc1 (formerly called NFATp) and NFATc2 isoforms in T cells play a role in the completion of T cell differentiation but not activation of naïve T cells (Peng et al., 2001). NFAT is normally localized in the cytoplasm of the cell and are retained by the negatively charged phosphate groups. Dephosphorylation by calcineurin (reviewed by Rao et al., 1997) therefore makes NFAT more lipophilic, allowing it to permeate the membrane to be translocated to the nucleus in a complex with calcineurin (Shibasaki et al., 1996). Phosphorylation exposes the nuclear export sequence (NES) which binds exportin-crm1 keeping NFAT in the cytoplasm of the cell.
Calcineurin suppresses crm1-dependent export (Zhu and Mckeon 1999) and dephosphorylates NFAT leading to its conformational change which, masks NES and exposes the nuclear localization sequence (NLS) (Okamura et al., 2000). The NLS binds importin causing the translocation of NFAT to the cytoplasm of the cell. The inhibitors of calcineurin, cyclosporin A (CsA) and FK506 (Mattila et al., 1990; Lui et al., 1991) can block the Ca\(^{2+}\) - activated signaling pathway (Schreiber and Crabtree 1992).

In the nucleus, NFAT can then mediate gene transcription of cytokines like interleukin 2 (IL-2) necessary for lymphocyte proliferation and differentiation (Shaw et al., 1988) as well as IL-4, INF-\(\gamma\), GMCSF, II-3, IL-13, TNF-\(\alpha\) (reviewed in Rao et al., 1997), and cell surface receptors CD95L, CD40L CD69 (Hodge et al., 1996). At the
NFAT/AP-1 sites in the IL-2 promoter, NFAT cooperates and heterodimerizes with the
AP-1 transcription factor (Chen et al., 1998) which, is made of dimers of Fos/Jun family
of proteins (Jain et al., 1992; Northrop et al., 1993; Rao, 1994). NFAT also cooperates
with another transcription factor, NFκB to mediate transcription (Crabtree et al., 1989).

Crabtree also discusses the time course (Table 2) for transcription factors obtained
from previous findings. Although NFAT appears in 20 min (Table 2) and its levels
increase steadily for 2 h (Shaw et al., 1988), it has been shown that following
immunoreceptor activation, it takes up to 2 h before NFAT commitment (Discussed in
Crabtree, 1989).

<table>
<thead>
<tr>
<th>Name</th>
<th>Time</th>
<th>Location</th>
<th>Ratio (A/NA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immediate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFAT-1</td>
<td>20 min</td>
<td>Nucleus</td>
<td>~50</td>
</tr>
<tr>
<td>NFκB*</td>
<td>30 min</td>
<td>Nucleus</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Early</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2*</td>
<td>45 min</td>
<td>Secreted</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>IL-2 receptor*</td>
<td>2 h</td>
<td>Secreted</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

*cloned genes. ^Events are independent of protein synthesis. ^Events require protein synthesis. A/NA
refers to the relative levels in activated versus nonactivated cells at peak induction.

Here we show that the time course for SOCE in Jurkat T cells is insufficient to fulfill this
obligation. A previous suggestion states that **CRAC mediated Ca influx upon TCR
engagement last >1 h** (Donnadieu et al., 1992). IP₃ production is markedly decreased in
10 min (Guse et al., 1993).
In order to mimic IP₃ mediated store depletion, the IP₃ pathway was bypassed in our lab with the use of sarcoplasmic endoplasmic reticulum calcium ATPase pump (SERCA) blockers like thapsigargin isolated from a plant called *thapsia garganica* (Thastrup et al., 1989) and the mycotoxin, Cyclopiazonic acid (CPA) (Siedler et al., 1989) to achieve store depletion. In the absence of 2 mM Ca²⁺ solution, 10 µM CPA in a solution containing 2 mM EGTA is added. See similar experiment performed in our lab (Fig. 3).

CPA and TG bind and block the SERCA found on the ER membrane. Blocking of the SERCA pump prevents Ca²⁺ from being pumped from the cytoplasm into the ER store. CPA blocks the SERCA reversibly while TG blocks it irreversibly.

The ER Ca²⁺ already present before the SERCA block leaks through the leak channels (Camello et al., 2002) depleting the ER store completely of Ca²⁺ after adding CPA. This results in a transient Ca²⁺ release (Mason et al., 1991).
Ca$^{2+}$ is also stored in the mitochondria and nuclei of the cell (Lee et al., 1988). Indo-1/AM-measurement has also been reported in the microsomes after loading showing the presence of Ca$^{2+}$ in them (Blatter and Wier, 1990). Liver mitochondria contain esterases like carbonic anhydrase (Dodgson et al., 1980) that can also hydrolyze fura2-AM (Gunter et al., 1988). Compartmentalization of fura2 could be minimized by reducing temperature and fura2 concentration. For this reason, we used fura-2 AM at a concentration of 1 µM. The loading and observations were then performed at room temperature for homogeneity (Malgaroli et al., 1987; Poenie et al., 1986).

**pH Measurement**

pH studies were performed as stated in the Materials and Methods. Under normal physiological conditions, pHi changes are generated by Na$^+$/H$^+$ exchangers. pHi changes mediated by Na$^+$/H$^+$ exchange are attenuated in the presence of 5(N-ethyl-N-isopropyl)amiloride (EIPA), an inhibitor of Na$^+$/H$^+$ exchange (Cragoe et al., 1967; Vigne et al., 1983) or in low [Na$^+$] medium (Gukovskaya et al., 1990). The rise in Na$^+$/H$^+$ activity leads to [Na$^+$] and pH increase (Paris and Pouysségur 1983 and 1984; Rosoff et al., 1984, Boron et al., 1984). In the human T-cell leukemia line, Jurkat, the pHi signal is suggested to be mediated by a [Ca$^{2+}$] increase (Rosoff and Cantley 1985). An increase in [Na$^+$], and/or pH, stimulates effector molecules like PLC (Gukovskaya et al., 1990). These findings together show a tight relationship of Ca$^{2+}$ with pHi changes which we looked at more closely.
In order to experimentally change the internal pH we used a weak base and a weak acid, ammonium chloride (NH₄Cl) and sodium propionate, respectively. It has previously been shown that NH₄⁺ stimulates Ca²⁺ influx and also mobilizes intracellular Ca²⁺ in platelets (Ghigo et al., 1988). However, others have shown that NH₄⁺ and other chemicals like monensin and phorbol ester, 12-O-tetradecanoyl phorbol-13-acetate (TPA or PMA) elicit [Ca²⁺]ᵢ rise by bypassing the Na⁺/H⁺ exchanger upon artificial cytosolic alkalinization (Grinstein and Cohen, 1985; Gukovskaya and Zinchenko, 1986). This is accomplished by elevating [Na]ᵢ and/or pHᵢ (Grinstein and Cohen, 1985; Klip et al., 1984). In addition, in single mouse thymocytes, NH₄⁺ has been shown to give fluorescent transients in pHᵢ measurements which, decrease with successive additions (Rogers et al., 1983).

**Jurkat T cell activation**

Activation of Jurkat T cells was performed using phytohemagglutinin (PHA) (Whitney and Sutherland, 1972). PHA is a mitogenic plant lectin (Nowell, 1960) that binds to the glycosylated groups of immune cells promoting immune reactions. Its potency allows detection and observation of early changes in lymphocyte activation (Whitney and Sutherland, 1972). Several experiments have been performed showing PHA-mediated cytoplasmic Ca²⁺ increase in various cells (Ng et al, 1990). It has been long established that rapid Ca²⁺ influx is enhanced within 38 min (Allwood et al. 1971) and one hour (Whitney and Sutherland, 1972) after PHA activation. At the time, increased Ca²⁺ influx was attributed to increased adenylyl cyclase activity leading to a
subsequent increase in the cyclic 3’5’-adenosine monophosphate (cAMP) intracellular concentration (Smith et al., 1971; Parker et al., 1971). Smith showed that cAMP levels decreased to normal after 6 hours and then below the control after 12 hours.

Others have used quin2 with fluorescent microscopy in mouse thymocytes (Tsien, 1980) but not in Jurkats. Here, we observe PHA-mediated cytoplasmic Ca\(^{2+}\) increase in Jurkat T cells through CRAC using fura-2 as our Ca\(^{2+}\) indicator. We also measured the corresponding pH changes resulting from activation.

**Phosphatidylinositol 3,4,5-triphosphate (PIP\(_3\))**

PIP\(_3\) is a plasma membrane-bound phospholipid formed by the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) to PIP\(_3\) by the enzyme, phosphoinositide 3 kinase (PI3K). PI3K, identified by (O’Shea, 1986), phosphorylates phospholipids. We show here that addition of (di-C\(_8\) PIP\(_3\)) to Jurkat cells causes an increase in cytoplasmic Ca\(^{2+}\) in a waveform manner. We used di-C\(_8\) PIP\(_3\) because it mimics the biochemical functions PIP\(_3\) such as membrane binding and Ca\(^{2+}\) transport (Hsu et al., 2000). As previously seen, PIP\(_3\) mediates Ca\(^{2+}\) oscillations (waves) in Jurkat T cells (Negulescu et al., 1994; Dolmetsch et al., 1997) manifested in a pattern closely consistent with Takamatsu and Wier, 1990 description where Ca\(^{2+}\) diffused circularly around Jurkat cells. Other experiments revealed that sustained elevated Ca\(^{2+}\) and lasting Ca\(^{2+}\) oscillations are required for T cell gene expression (Negulescu et al., 1994). We are the first to examine the effect of PIP3 on SOCE in single cell imaging.
**Phosphoinositol 3 kinase Inhibition**

Wortmannin, a fungal toxin is a potent PI3K inhibitor (Arcaro and Wymann, 1993). Another such inhibitor is LY294002. They bind to the catalytic subunit of the enzyme inhibiting it (reviewed in Deane and Fruman, 2004). We therefore hypothesized that, since PIP₃ mediates cytoplasmic Ca²⁺ influx, a decrease in PIP₃ production due to the absence of PI3K activity will elicit a decrease in cytoplasmic Ca²⁺. Free cytoplasmic [Ca²⁺] was previously measured using the fluorescent Ca²⁺ indicator quin 2 by Tsien, (1980) in mouse thymocytes.
II. HYPOTHESES

Our intent is to scrutinize the interplay of Ca\(^{2+}\) and pH, in the regulation of CRAC channels in Jurkats T cells. This project was prompted by our finding that SOCE in Jurkat T cells is short-lived (see introduction). Here we elucidate the regulatory relationship of changes in pH and intracellular [Ca\(^{2+}\)], with regards to Orai1 and Orai3. Previous studies (Grinstein and Cohen 1985; Gukovskaya and Zinchenko 1986) suggest that a rise in intracellular [Ca\(^{2+}\)] is correlated with intracellular alkalinization. We hypothesize that changes in intracellular pH will lead to changes in intracellular [Ca\(^{2+}\)]. Furthermore, we anticipate that Orai1 and Orai3 will differ in their pH sensitivity; providing unique Ca\(^{2+}\) signals in response to pH changes. *This is significant as this may point to the relative importance of individual Orai proteins in T-cell differentiation and proliferation.*

**Experimental objective 1**

To confirm the difference between Orai1 and Orai3 in response to 2-APB. This was done in both the Jurkat T cell line and a recombinant system using HEK293 cells. Additionally, to differentiate 2-APB mediated effect on CRAC channel activity with respect to pH.

**Experimental objective 2**

To experimentally change intracellular pH using propionate and NH\(_4\)\(^+\), as a weak acid and base respectively, and then measure the intracellular pH and [Ca\(^{2+}\)].

**Experimental objective 3**

To experimentally change cytoplasmic [Ca\(^{2+}\)] using PHA, PIP\(_3\), and wortmannin, then measure intracellular pH and [Ca\(^{2+}\)] in a store dependent and independent manner.
III. SIGNIFICANCE OF STUDY

CRAC channel studies have immediate profound significance to severe combined immune deficient (SCID) patients. The first indication that CRAC current is important in immunity came from the case in France (Partiseti et al. 1994; Le Deist et al., 1995) where a SCID patient lacked measurable CRAC current in their lymphocytes. Several years later, a family was discovered in Germany (Feske et al., 2005, Feske et al., 2006, reviewed in Feske et al., 2007) lacking CRAC current and suffering from SCID. Ten years later, the genetic mutation causing this defect was found to be in the Orai1 gene. Their Orai1 protein is homozygous for the C-T missense mutation due to a mutant E250Q and E160Q as determined by using RNA interference (RNAi) screening and single nucleotide polymorphism (SNP) genome wide analysis (Feske et al., 2006, Zhang et al., 2006, reviewed in Feske et al., 2007). The patients easily succumb to viral and fungal infections. Our intention is to elicit Orai protein function during SOCE. In this study we look at Orai1 and Orai3 only, and we also describe the effect of pH on them.
IV. MATERIALS AND METHODS

MATERIALS

Chemicals and Abbreviations Used
Fura-2 AM, fura-2 acetoxymethyl ester (Invitrogen Molecular Probes), 2’,7’-bis-(2-carboxyethyl)-5-(and 6-)carboxyfluorescein acetoxymethyl ester (BCECF-AM) (Invitrogen Molecular Probes), PHA; Phytohaemagglutinin (PHA; Calbiochem), 2-APB; 2-aminoethoxy diphenyl borate (Sigma-Aldrich), CPA; Cyclopiazonic acid (CPA; Sigma- Aldrich), TG; thapsigargin (TG; Sant Cruz Biotechnology), di-C8-PI(3,4,5)P3, 1-O-(1,2-di-O-octanoyl-sn-glycero-3-O-phosphoryl)-D-myoinositol 3,4,5-trisphosphate (Echelon), Ionomycin (IONO; Invitrogen Molecular Probes), Prop; Propionate; EIPA; 5(N-etyl-N-isopropyl) amiloride, HEPES; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, [ ], extracellular concentration; [ ], intracellular concentration; PI(3,4,5)P3, phosphatidylinositol 3,4,5 trisphosphate (PIP3); PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate (PIP2); PI3K, phosphoinositide 3-kinase; Phospholipase C, PLC; DPBS (Dulbecco’s phosphate buffered saline); Nig, nigericin (Fluka); Wortmannin (Echelon), Poly-L-lysine (Poly-K; Sigma-Aldrich).

Tissue Culture
Human embryonic kidney cells (HEK293; UC Irvine) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; HyClone, Thermo Scientific Inc) to which 10% fetal bovine serum (FBS); (PAA) and 50 µg/mL streptomycin/ 50 U/mL penicillin (MP
Biomedicals, LLC) were added. Jurkat cells (kindly provided by Dr. Thomas Brown Lab, Wright State University) were cultured in RPMI-1640 (HyClone, Thermo Scientific Inc) into which 10% fetal bovine serum (FBS; PAA) and 50 µg/mL streptomycin/ 50 U/mL penicillin (MP Biomedicals, LLC) were added. The day prior to transfection, cells were cultured without antibiotics. All cells were grown in an incubator conditioned at 5% CO₂, and 37°C. Jurkats cells were activated with either 2 µg/mL or 5 µg/mL PHA. Before superfusion, cells were attached to poly-L-lysine (poly-K; Sigma-Aldrich ) coated chambers. Plates were coated by adding a drop of 1 mg/mL poly-K to each chamber and allowed to sit at room temperature for 15-30 min after which it was rinsed with PBS. Cells were allowed to adhere to the glass for 5-10 min. Jurkat cells look unhealthy in poly-K after approximately 30 min.
**Transfection**

Orai and STIM1 proteins were transiently transfected into HEK293 cells. hOrai (UC Irvine) and STIM1 (UC Irvine) were transfected at a ratio of 1: 0.5 Orai/STIM1 or a ratio of 1:1Orai/STIM1. Polyfect Transfection Reagent (Qiagen) was used.

**Solutions**

Unless otherwise stated, all solutions were maintained at pH 7.30-7.33 and osmolality at 300 -310 mOsmol. All solutions are a modification of Tyrode’s solution.

**2 mM Ca (2Ca) solution:** 150 mM NaCl, 2 mM CaCl₂, 4.5 mM KCl, 1 mM MgCl₂, 10 mM Glucose, 5 mM HEPES.

**0Ca solution:** 150 mM NaCl, 2 mM EGTA, 4.5 mM KCl, 1 mM MgCl₂, 10 mM Glucose, 5 mM HEPES.

**1 mM NH₄Cl solution:** 149 mM NaCl, 1 mM NH₄Cl, 2 mM CaCl₂, 4.5 mM KCl, 1 mM MgCl₂, 10 mM Glucose, 5 mM HEPES.

**20 mM NH₄Cl solution:** 130 mM NaCl, 20 mM NH₄Cl, 2 mM CaCl₂, 4.5 mM KCl, 1 mM MgCl₂, 10 mM Glucose, 5 mM HEPES.

**37.5 mM NH₄Cl solution:** 112.5mM NaCl, 37.5 mM NH₄Cl, 2 mM CaCl₂, 4.5 mM KCl, 1 mM MgCl₂, 10 mM Glucose, 5 mM HEPES.

**75 mM NH₄Cl solution:** 75 mM NaCl, 75 mM NH₄Cl, 2 mM CaCl₂, 4.5 mM KCl, 1 mM MgCl₂, 10 mM Glucose, 5 mM HEPES.
Addition of drugs

**CPA solution:** 0Ca solution + 10 µM CPA. Unless otherwise stated, 10 µM CPA/0Ca was used for every experiment. When used 2Ca/CPA solution was made with 2Ca solution. The TG solution was made in the same way with sonication.

**50 µM 2-APB solution:** 2Ca solution + 50 µM 2-APB.

**100 µM 2-APB solution:** 2Ca solution + 100 µM 2-APB.

**37.5 mM NH₄Cl / 50 µM 2-APB solution:** 37.5 mM NH₄Cl + 50 µM 2-APB.

**37.5 mM NH₄Cl / 100 µM 2-APB solution:** 37.5 mM NH₄Cl + 100 µM 2-APB.

**75 mM NH₄Cl / 50 µM 2-APB solution:** 75 mM NH₄Cl + 50 µM 2-APB.

**75 mM NH₄Cl / 100 µM 2-APB solution:** 75 mM NH₄Cl + 100 µM 2-APB.

**Ionomycin solution:** 2Ca solution + 2 µM ionomycin.

**High K⁺ / nigericin solution:** 20 mM NaCl, 2 mM CaCl₂, 130 mM KCl, 1 mM MgCl₂, 10 mM Glucose, 5 mM HEPES and 10 µM nigericin with sonication.

**Low K⁺ / nigericin solution:** 150 mM NaCl, 2 mM CaCl₂, 4.5 mM KCl, 1 mM MgCl₂, 10 mM Glucose, 5 mM HEPES and 10 µM nigericin with sonication.

**5 µM di-C8-PIP₃ solution:** 2Ca solution + 5 µM di-C8-PIP₃ with sonication

**Wortmannin solution:** 2Ca solution + 1 µM wortmannin.

**Dye Loading**

For [Ca]ᵢ measurements, HEK293 and Jurkat cells were loaded with 1 µM/mL Fura-2 (Invitrogen Molecular Probes) for 45 min at room temperature. For pHᵢ measurements, HEK293 and Jurkat cells were loaded with 4 µM/mL 2’,7’-bis-(2-carboxyethyl)-5-(and 6-)-carboxyfluorescein acetoxyethyl ester (BCECF-AM); Invitrogen Molecular Probes)
for 45 min respectively at room temperature. Dye was diluted in Tyrode’s solution (150 mM NaCl, 2 mM CaCl₂, 4.5 mM KCl, 1 mM MgCl₂, 10 mM Glucose, 5 mM HEPES at pH 7.3). Cells were washed twice with the same solution after dye loading and initial recording was performed in this solution. In some experiments, we assessed possible problems in dye loading by superfusing cells with a 2 µM ionomycin-containing solution at the end to the experiment.

**Instrumentation**

The fluorescent imaging instrument is comprised of Olympus CKX41 inverted microscope, to which a Pixelfly 270XS 12737 charge-coupled device (CCD) camera (PCO, Germany) is attached. It has wider dynamic range and with no geometric distortion (reviewed in Hayashi and Miyata, 1994). The light source was a 175 Watt Xenon Lamp (Intracellular Imaging INC) and Lambda 10B/Smart Shutter (Sutter Instrument). We used the XF2058 filter for Fura-2 and the XF3011 filter for BCECF (Omega Opticals) dichroic filter which reflects unwanted portion of light and transmit the remainder.

**METHODS**

**Transfection**

Transient transfection of HEK293 with Orai proteins was performed. STIM1 (UC Irvine), hOrai3 (UC Irvine) and hOrai (UC Irvine) were all used at a concentration of 1 µg/mL. STIM1 was cotransfected with either hOrai3 or hOrai1 in either a ratio of 1:0.5 or 1:1.
Each pair of proteins was mixed in 64 µL serum free DMEM. The DNA mixture was equilibrated with 13 µL Polyfect Reagent and incubated at room temperature for 10 min before being added dropwise to wells containing 60 - 75% confluent cells grown in 1 mL serum- containing DMEM with no antibiotics. Cells were transfected for at least 20 hours after which they were rinsed with PBS, trypsinized and plated on lysine-coated chambers for same day experiments.

**Fluorescent Probes**

Fura-2 and BCECF are ratiometric fluorescent dyes used for Ca$^{2+}$ and pH measurements, respectively. Ratiometric dyes correct for unequal dye loading, bleaching and focal-plane shift. Fura-2 is a Ca$^{2+}$ chelator that changes according to free Ca$^{2+}$ concentration (Tsien, 1981; Tsien et al., 1982, Gryniewicz, et al., 1985). We chose an excitation wavelength at 340 nm and 380 nm with peak emission wavelength at 510 nm for fura-2. Ca$^{2+}$ concentration is measured as two fluorescent intensities taken at the two excitation wavelengths. Therefore, the ratio does not depend on the absolute intensity of the two signals. We chose fura-2 over quin-2 (another Ca$^{2+}$ indicator) because it has higher fluorescent intensity with little or no photobleaching and better selectivity to Ca$^{2+}$ over Mg$^{2+}$ (Gryniewicz et al., 1985). The [Ca$^{2+}$] is calculated as shown below from the two excitation wavelengths and so does not depend on the cell thickness, the dye concentration and the intensity of the excitation light (Gryniewicz et al., 1985):

$$[\text{Ca}^{2+}] = K_d \times \beta \times (R - R_{\text{min}})/(R_{\text{max}} - R)$$

Where $R$ = the ratio of fluorescence intensity from the cell at 340 nm and 380 nm excitation wavelengths; $R_{\text{min}}$ = the fluorescence ratio of fura-2 free acid recorded in the
absence of Ca\(^{2+}\); R\(_{\text{max}}\) = the fluorescence ratio of fura-2 free acid recorded at saturating [Ca\(^{2+}\)]; K\(_d\) = the Ca\(^{2+}\) dissociation constant of the dye; \(\beta\) = the ratio of the fluorescence of fura-2 free acid in the Ca\(^{2+}\)-free form to the Ca\(^{2+}\)-saturated form recorded at the wavelength used in the denominator of the ratio.

Both dyes were loaded in their acetoxyethyl ester form as BCECF-AM and fura-2 AM which are relatively lipophilic. BCECF and fura-2 were rendered hydrophobic by esterification with acetoxyethyl (AM) groups using acetomethyl bromide and diisopropylethylamine (Tsien, 1981). BCECF intensity changes according to the concentration of H\(^+\) ions. The excitation wavelengths for BCECF were chosen at 440 nm and 490 nm with an emission wavelength of 535 nm (Tsien et al., 1982). Upon entry into the cytoplasm, the cell’s endogenous esterases cleave the ester bonds (five in the case of fura-2) linking the AM groups to the COO\(^-\) groups rendering the dye negatively charged. The cleaved and charged forms of BCECF and fura-2 can no longer exit the cytoplasm where they are trapped for the entire duration of the experiment. As mention earlier, dye compartmentalization could be minimized by reducing loading temperature and dye concentration. This explains why we used 1 \(\mu\)M fura-2 AM and then performed loading and observations performed at room temperature for homogeneity (Malgaroli et al., 1987; Poenie et al., 1986).
Reaction 1: Lipophilic membrane-permeant fura-2AM is converted to ionic membrane impermeant fura-2 by endogenous intracellular esterases.

Reaction 2: Lipophilic membrane permeant BCECF-AM is converted to ionic membrane impermeant BCECF by endogenous intracellular esterases.

Intracellular pH (pHi) and in vivo calibration was performed using the high K⁺/nigericin method (Thomas et al., 1979). The principle behind the use of the H⁺/K⁺ antiporter (exchanger), nigericin is that it equilibrates the pH across the membrane by setting \([H^+]_i/\,[H^+]_o = [K^+]_i/\,[K^+]_o\). [K⁺]₀ is set approximately equal to [K⁺]ᵢ by superfusing...
the cells with a high $K^+$ and nigericin-containing solution. The nigericin exchanger then exchanges the internal $K^+$ for the external $H^+$. A high $[K^+]$ of 130 mM was used in our nigericin-containing solutions unless otherwise stated (Putnam and Grubbs, 1990; Thomas et al., 1979). This prevented the $K^+$ gradient from driving the pH gradient formed (Lassen et al., 1971). This technique was used for pH-sensitive BCECF dye calibration as well as for control purposes to show the effectiveness of the indicator to change with pH. For calibration purposes, the $pH_i$ of a BCECF loaded cell could be determined by imposing a nigericin-containing solution with a known pH value inside the cell and then obtaining the corresponding pH ratio at the 490 nm and 440 nm wavelength.

![Figure 4: pH measurement of 2 ug/mL PHA –activated Jurkats with low (4.5 mM ) $K^+$ in 10 µM nigericin (n = 3).]
In Fig. 4, low K\(^+\)/nigericin-containing solution was used. This experiment was performed to confirm Lassen et al., 1971 finding that low K\(^+\) drives the pH gradient.

**BCECF Calibration**

In order to reduce the effect of environmental factors on the indicator dyes, calibration was performed on the living cells to obtain curves of the dye that reflect its environment (Maxfield, 1989). Once loaded, cells were washed twice with 2Ca solution containing 4.5mM K\(^+\) (Low) with no nigericin. Jurkat cells were superfused with high (130 mM) K\(^+\)/10 \(\mu\)M nigericin-containing solutions with pH sequentially changed from 6.02, 7.00, 7.51 and 8.00 (Fig. 5a). WT HEK293 cells were superfused with high (130 mM) K\(^+\)/10 \(\mu\)M nigericin-containing solution with pH sequentially changed from 6.14, 6.99, 7.50, 8.00 and 8.48 (Fig. 6a). Standard curves were obtained by averaging the ratio (490/440) at saturation intervals and plotting them against the known pH values (Fig. 5b and 6b). Table 3a and 3b show pH values with their corresponding ratios (490/440) in WT HEK293 and Jurkat cells respectively.

Fura-2 was calibrated by Intracellular Imaging INC. and all graphs obtained were based only on the ratio of \(\lambda_1 = 340\) nm and \(\lambda_2 = 380\) nm (340/380) and emission of 510 nm. That is, results for Ca\(^{2+}\) are not expressed in [Ca\(^{2+}\)] but in the fluorescence ratio (340/380). In vivo calibration of fura-2-loaded cells is performed with the use of Ionomycin, a Ca\(^{2+}\)-ionophore (Liu and Hermann, 1978). This antibiotic equilibrates the intracellular and the known extracellular Ca\(^{2+}\) concentrations. Corresponding 340/380 fluorescence ratios obtained are then plotted against its known pH value.
Figure 5: (a) In situ calibration of BCECF in Jurkat T cells (n = 22). Cells were superfused with High (130 mM) K⁺ / 10 µM nigericin - containing solution with pH sequentially changed from 6.02, 7.00, 7.51 and 8.00. (b) Standard curve showing pH with corresponding ratio (490/440) obtained by averaging the ratio (490/440) at saturation intervals of pH 6.02, 7.00, 7.51 and 8.00.
Figure 6: (a) Insitu calibration of BCECF in HEK293 cells (n = 43). Cells were superfused with High (130 mM) K⁺ / 10 µM nigericin - containing solution with pH sequentially changed from 6.14, 6.99, 7.50, 8.00 and 8.48. (b) Standard curve showing pH with corresponding ratio (490/440) obtained by averaging the ratio (490/440) at saturation intervals of pH 6.14, 6.99, 7.50, 8.00 and 8.48.
NB: The corresponding ratio for pH 8.48 is not shown in table 3a because the BCECF dye is not effective at this pH as seen from the slight change in Fig.6a.

**Statistical Analyses**

Since we averaged data points for a group of cells at any given time in an experiment, we incorporated the standard error of the mean (SEM) in the graph as a measure of variability at any particular data point. For experiments where we compared the Ca$^{2+}$ levels, we used an analysis of variance. ANOVA was used to compare the average outcome; the outcomes considered were the SOCE peak and the store depletion transient peak. In the case of statistical significant ANOVA results, the Tukey (Honestly Significant difference; HSD) multiple comparism procedure was applied to determine how the means differed among the conditions. For all the conclusions, the 5% level of significance was used.
V. RESULTS

10 µM CPA is sufficient to deplete the ER stores.

In order to be certain that we completely deplete the stores in all our experiments that required SOCE, we performed a CPA dose response experiment (Fig. 7a and 7b) for both HEK293 cells and Jurkat T cells respectively. Jurkat cells were loaded with fura-2 and washed with Tyrode’s HEPES-buffered saline solution containing 2 mM Ca$^{2+}$. Superfusion started with the 2Ca solution, followed by 0Ca solution and then various concentrations of CPA solution were added to deplete the ER store. We show that 10 µM CPA is sufficient to deplete the ER store of calcium. Notice that almost all of the Ca$^{2+}$ is depleted by 0.1 µM CPA and by the time 50 µM CPA is added, there is no Ca$^{2+}$ left. Thus, we are certain that 10 µM CPA depletes the store completely.

Time course for SOCE in Jurkat T cells is transient

In one of our experiments with naïve Jurkat T cells we noticed that the time course for SOCE was short-lived (Fig. 8a and 8b). Previous findings have shown that Ca$^{2+}$ influx is short-lived (see Introduction) but this is not SOCE which occurs through CRAC channels. Here we looked at SOCE and show that its time course for decay is close (τ = 3.4 min for 47 Jurkat T cells; SD = 2.2 min) to that obtained from CPA mediated store depletion (τ = 3.3 min for 44 Jurkat T cells; SD = 1.9 min). This novel finding is quite important because Jurkats have been used for over 30 years to model T lymphocytes. We conclude here that, store-operated Ca$^{2+}$ influx-dependent NFAT nuclear translocation may only partially be accomplished by CRAC channels.
Figure 7: 10 µM CPA is sufficient to deplete the ER Ca\(^{2+}\) store. (a) CPA dose response for WT HEK 293 cells (n = 22). (b) CPA dose response for Jurkat T cells (n = 7). 0Ca / (0.1 µM, 1 µM, 10 µM and 50 µM CPA) solutions were sequentially superfused after 2Ca solution.
Previous findings report that 2-APB blocks Orai1 and potentiates Orai3 (Zhang, Kozak et al., 2008). We wanted to confirm these findings specifically with Orai1 and Orai3-expressing HEK293 cells while testing these proteins’ expression in a recombinant system and to find out which of these two Orai proteins participate. In order to test our recombinant system, WT HEK cells were transfected with Orai1 /STIM1 in a 1:1 ratio for 20 hours (Fig. 9a) as described in the Methods. Ca^{2+} measurements were also

**Orai response to 2-APB in our recombinant corroborates previous findings.**

Figure 8: SOCE is sustained for 3-4 min in naïve Jurkat T cells (n = 51). (a) SOCE was attained after 10 μM CPA store depletion.
performed as previously described. In Fig. 9a and 9b, store depletion was achieved with 2 
µM TG in a recombinant system and 10 µM CPA in WT HEK respectively. After SOC 
entry, 5 µM 2-APB activates Orai1 while 50 µM 2-APB blocks Orai1 as previously

![Graph showing time course for store depletion and SOCE with exponential fit parameters.]

**Table: Exponential Fit Parameters**

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<td>SEM</td>
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<td>Tau 1</td>
<td>Store Depletion</td>
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<td></td>
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</tr>
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<td></td>
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</tr>
<tr>
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<td>2.92</td>
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Figure 8 continued: The time course for store depletion and SOCE are not significantly 
different. (b) A single cell representation of the time course for the decay of store 
deployment transient (τ1) (n = 44) and SOCE (τ2) (n = 47) using an exponential fit with the 
equation $y = y_0 + Ae^{-(t/\tau)}$. 

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Figure 9: 5 µM 2-APB potentiates while 50 µM 2-APB blocks Orai1 as previously known (a) 20 hr Orai1/STIM1 (1:1) transfected HEK (n = 4). After 2 µM TG depletion of the ER store, 5 µM 2APB activates and 50 µM 2APB inhibits Orai1 as previously known. (b) The same results were obtained with 10 µM CPA mediated depletion of stores on WT HEK293 cells (n = 5). 2 µM ionomycin solution was added at the end of both experiments to validate proper dye functionality.
shown (Lioudyno et al, 2008). Ionomycin was added at the end of both experiments to confirm dye functionality. Both Orai1-expressing (Fig. 9a) and WT HEK293 (Fig. 9b) show the same 2-APB response. This means that WT HEK cells have predominantly endogenous Orai1 as previously known. The above experiments confirm previous findings and the proper functionality of our recombinant system.

Since store depletion activates CRAC, we expect that in like manner, store repletion should inactivate CRAC. We wanted to know if the mechanism of 2-APB mediated Orai1 block is by means of store replenishment. Since 2-APB distinguishes between Orai homologs (Zhang, Kozak et al., 2008), we decided to test 2-APB effect on Orai1 and Orai3-expressing HEK cells and the effect compared between these proteins. We also wanted to know which protein participates and to what extent in Ca influx and a description of their resultant pH dependence.

We began by comparing the effect of 2-APB with and without ER store depletion on these Orai. The same conditions were used to compare Orai1-transfected HEK (Fig. 10) to Orai3-transfected HEK (Fig. 12 and 13) as well as the control WT HEK cells (Fig. 11). In all these experiments we used CPA solution (unless otherwise stated, 10 µM was used in all experiments) to fully deplete the store (Fig. 10a, 11a, 12a and 13a) and in the absence of CPA we used 0Ca solution (Fig. 10b, 11b, 12b and 13b). After store depletion or no store depletion in all experiments we superfused the cell with 2Ca solution proceeded by 50 µM 2-APB solution. 50 µM 2-APB inhibited Orai1 in both cases (Fig. 10a and 10b). This means that 2-APB mediated Orai1 block is independent of store depletion and that the mechanism of block is not as a result of store replenishment.
Figure 10: 50 µM 2-APB inhibits Orai1/STIM1 (1:0.5) with and without store depletion as seen in previous findings (see Introduction).  (a) Store depletion-dependent Ca\(^{2+}\) influx was achieved with 10 µM CPA solution on Orai1/STIM1 (1:0.5) - transfected HEK (n = 8).  (b) In store depletion-independent Ca\(^{2+}\) influx, 0Ca solution was used on Orai3 /STIM1 (1:0.5) transfected HEK (n = 8).
Figure 11: 50 µM 2-APB inhibits endogenous Orai1 in WT HEK293 with and without store depletion. (a) Store depletion was achieved with 10 µM CPA solution on WT HEK (n = 21). (b) 0Ca solution was used on WT HEK without SOCE (n = 4).
Figure 12: 50 µM 2-APB activates Orai3/STIM1 (1 : 0.5) with and without store depletion. (a) Store depletion was achieved with 10 µM CPA solution on Orai3/STIM1 (1:0.5) transfected HEK (n = 7). (b) 0Ca solution was used on Orai3/STIM1 (1:0.5) transfected HEK without SOCE (n = 4).
Figure 13: 50 µM 2-APB also activates Orai3/STIM1 (1:1) with and without store depletion. (a) Store depletion was achieved with 10 µM CPA solution on Orai3/STIM1 (1:1) transfected HEK (n = 5). (b) 0Ca solution was used on Orai3/STIM1 (1:1) transfected HEK without SOCE (n = 5)
since Orai1 was still blocked when the store was full. Therefore, we confidently performed our experiments knowing that store remained depleted and that 2-APB mediated Orai1 block is manifested by another mechanism. As shown in previous findings, in Fig. 10b Orai1 block is preceded by an initial potentiation (Prakriya and Lewis, 2001). WT HEKs’ Orai proteins’ response to 2-APB was inhibition with and without store depletion (Fig. 11a and 11b) just like in Fig. 9b. This confirms previous findings that WT HEK cells have predominantly endogenous Orai1 protein or at least in response to 2-APB (Lioudyno, et al., 2008). Again, we see here that Orai1 block in WT HEK is preceded by an initial potentiation like in the Orai1 recombinant system.

50 µM 2-APB activated Orai3/STIM1 in both the 1:0.5 (Fig. 12a and 12b) and the 1:1 (Fig. 13a and 13b) ratios with and without store depletion. This means that 2-APB mediated Orai3 activation is independent of store depletion and that the mechanism of activation is not as a result of store depletion or replenishment. Therefore, 2-APB mediated Orai3 activation is achieved by some other mechanism.

NH₄⁺-induced alkalinization alone leads to increased cytoplasmic Ca²⁺ influx.

The next question we asked was, can changes in internal pH affect Ca signaling? Previous studies reported that alkalinization leads to increased cytoplasmic Ca²⁺ influx. A study of its effect on CRAC had not yet been expounded. In order to find out whether there is a connection between increased Ca²⁺ and CRAC, we superfused 24 hr transfected Orai/STIM1 (1:05) HEK293 cells with 75 mM NH₄Cl before store depletion (Fig. 14a) and after store depletion (Fig. 14b). Our results show a significant rise in store-independent cytoplasmic Ca²⁺ influx and a subsequent transient Ca²⁺ release during
Figure 14: NH₄Cl activates Orai1 in a store-independent manner but its effect is however, dependent on the level of Ca²⁺ in the cell. (a) NH₄Cl added on 24 hr Orai1/STIM1 (1:0.5) transfected HEK prior to CPA store depletion (n = 5). (b) NH₄Cl added on 24 hr Orai1/STIM1 (1:0.5) transfected HEK after to CPA store depletion (n = 6).
Figure 15: 75 mM NH₄Cl elicits increased intracellular Ca²⁺ by a mechanism independent of store depletion. Superfused Orai1/STIM1 transfected HEK cells (n = 7) with CPA-containing solutions to kept the ER stores depleted.

Figure 16: NH₄Cl acidifies the cell after 2-APB addition. After CPA mediated store depletion and subsequent SOCE, Orai3/STIM1 (1:0.5) transfected HEK cells (n = 3) were superfused with 50 µM 2-APB followed by 75 mM NH₄Cl.
CPA mediated store depletion (Fig. 14a).

On the other hand, Ca\(^{2+}\) increase after SOCE was slight which could lead one to suppose that NH\(_4^+\) instigates cytoplasmic Ca\(^{2+}\) entry by means of store depletion which is an alternative reason why we depleted the store after adding NH\(_4^+\). This way, if the NH\(_4^+\) had already depleted the store, then CPA addition would have revealed empty stores: no transient Ca\(^{2+}\) released. To further validate the evidence that NH\(_4^+\) does not partake in ER store depletion, we kept the store depleted by adding 10 µM CPA to all solutions except in the initial 2Ca solution (Fig. 15). NH\(_4^+\) still elicited small Ca\(^{2+}\) influx irrespective of emptying the store. We noticed a comparatively lower Ca\(^{2+}\) influx in experiment 14b and 15 following SOCE as opposed to the high store-independent Ca\(^{2+}\) influx (Fig. 14a) prompted by NH\(_4^+\).

Figure 17: 2-APB-activated Orai3 is inhibited in the presence of NH\(_4^+\)Cl. Upon store depletion with CPA and subsequent SOCE, HEK293 cells (n = 5) transfected with Orai3/STIM1 were activated with 50 µM 2-APB. While keeping 2-APB present, 75 mM NH\(_4^+\)Cl (Stale) was added. We observed a sharper response when the solution was 3 or more days old).
Figure 18: NH₄Cl also blocks CRAC following 2-APB activation of Orai3. (a) After CPA mediated store depletion and subsequent SOCE, Orai3/STIM1 (1:0.5) transfected HEK cells (n = 3) were superfused with 100 µM 2-APB followed by 37.5 mM NH₄Cl (fresh). (b) Repeated the same experiment in 17a to confirm findings.
Figure 19: \( \text{NH}_4\text{Cl} \) educed acidification of Jurkat cells after 2-APB inhibition of endogenous Orai1 is not CPA dose-dependent. (a) 1 \( \mu \text{M} \) CPA \((n = 29)\), (b) 3 \( \mu \text{M} \) CPA \((n = 38)\), (c) 10 \( \mu \text{M} \) CPA \((n = 32)\) CPA.
$\text{NH}_4^+$ alkalinization inhibits CRAC in the presence of 2-APB.

We proceeded to study the effect of alkalinization further with several different scenarios in the presence of 2-APB. First, to our great surprise, following 2-APB mediated activation of Orai3, 75 mM $\text{NH}_4^+$-containing 50 μM 2-APB solution inhibited Orai3 which was slightly regulated by the cell leading acidification after 2Ca wash (Fig. 16). A very interesting phenomenon about this novel finding is that separately, $\text{NH}_4^+$ and 2-APB will elicit increase $\text{Ca}^{2+}$ influx through Orai3 but together, they inhibit Orai3 instead of enhancing their effects.

Figure 20: The inhibitory effect of $\text{NH}_4\text{Cl}$ requires the presence of 2-APB but prior 2-APB response is not necessarily required. Upon store depletion with CPA and subsequent SOCE, WT HEK293 cells ($n = 8$) were directly superfused with a 50 μM 2-APB and 75 mM $\text{NH}_4\text{Cl}$-containing solution, followed by 2Ca wash.
The observed inhibition of both Orai1 and Orai3 channels by \( \text{NH}_4^+ \) however, depends on the preceding 2-APB (Fig.16 and 19) or on the continuous presence of 2-APB (Fig. 17 and 18). It is worth mentioning here that, we observed a sharper response when the \( \text{NH}_4\text{Cl} \) solution was 3 or more days old. This enhanced inhibition could be attributed to the easier diffusion of \( \text{NH}_3 \) through the plasma membrane into the cell cytoplasm to be converted \( \text{NH}_4^+ \) in the presence of \( \text{H}^+ \) ions. \( \text{NH}_3 \) is probably favored over \( \text{NH}_4^+ \) in the \( \text{NH}_3/\text{NH}_4^+ \) equilibrium depending on the environmental factors like cooling (stored solutions at +4\(^0\)C) according to Le Châtelier’s principles.

We proceeded to study the CPA dose response of 1\( \mu \text{M} \) CPA, 3 \( \mu \text{M} \) CPA, 10 \( \mu \text{M} \) CPA (Fig 19a, 19b and 19c respectively) on Jurkat T cells to see if there is a correlation between the \( \text{NH}_4^+ \) -induced block of CRAC and partial or complete store depletion in these cells. Following SOCE and 50 \( \mu \text{M} \) 2-APB inhibition of endogenous dominant Orai1 in Jurkats, we studied the effect of 75 mM \( \text{NH}_4\text{Cl} \). Results show that \( \text{NH}_4^+ \) inhibits endogenous Orai1 following 2-APB addition (Fig.19) as seen in Fig. 16 with Orai3. This means that the 2-APB/\( \text{NH}_4^+ \) effect does not depend on store depletion (in agreement with results shown in recombinant Orai1 experiments in Fig. 14 and 15) as well as the Orai protein.

In Fig. 20 we tested if a preceding 2-APB is necessary for the subsequent \( \text{NH}_4^+ \) inhibitory response. Upon store depletion with CPA and subsequent SOCE, WT HEK293 cells (n = 8) were directly superfused with a 50 \( \mu \text{M} \) 2-APB and 75 mM \( \text{NH}_4\text{Cl} \)-containing solution, followed by a 2Ca wash. As shown, the result in Fig. 20 is analogous to those in which there was prior 2-APB addition. **Conclusively, the inhibitory effect of \( \text{NH}_4\text{Cl} \)**
requires the presence of 2-APB but prior 2-APB response is not necessarily required.

After the extensive examination of the possible scenarios surrounding the observed NH$_4^+$ inhibition of Orai proteins in the presence of 2-APB we moved ahead to describe the pH dependence of these phenomena. We began by showing 2-APB effect on Orai3 Ca$^{2+}$ influx and the proceeded to measuring is corresponding pH as described in the Methods section. Following CPA store depletion, SOCE and 100 μM 2-APB activation, we superfused the Orai3-transfected HEK cells with 75 mM NH$_4^+$-containing 100 μM 2-APB solution (Fig. 21a and 21b). The pH measurements were carried out on cells from the same transfection well and these cells were also superfused with the same solutions used in Ca$^{2+}$ measurements. Fig. 21b (pH not measured) shows a replica confirmation experiment from another transfection well for further validation of these substantial novel findings. The pH measurement experiment in Fig. 22a with corresponding pH ratios in Fig. 22b represents cells taken from the same well as in the experiment in Fig. 21a.

As shown above, following 2-APB mediated activation of Orai3, 75 mM NH$_4^+$-containing 100 μM 2-APB solution inhibited Orai3 which was slightly regulated by the cell leading to acidification after 2Ca wash (Fig 21a and 21b). We see here another interesting and significant novel finding in regards to intracellular pH measurements. We show here that 2-APB mediated Ca$^{2+}$ influx is accompanied by a gentle and steady acidification of the cytoplasm (Fig. 22a and 22b). This 2-APB mediated Ca$^{2+}$ influx in Orai3 changes to inhibition (Fig. 21) accompanied by a sharp alkalinization of the cytoplasm (Fig. 22a and 22b) when 75 mM NH$_4^+$ is added in its presence.
Figure 21: 2-APB in NH₄⁺ inhibits Orai3. (a) After CPA mediated store depletion and then SOCE, 100 µM 2-APB was added, followed by the sequential addition of 100 µM /75 mM NH₄Cl and 2Ca solutions on 24 h ORAI3/STIM1 (1:0.5) transfected HEK (n = 5). (b) The same above experiment was performed to confirm findings using the same solutions on the 24 h ORAI3/STIM1 (1:0.5) transfected HEK (n = 4).
Figure 22: pH measurement reveals that 2-APB activates Orai3 by acidification and in the presence of NH$_4^+$ Orai3 is inhibited by alkalinization. (a) Shows pH when Orai3/STIM1 (1:0.5) transfected HEK (n = 28) superfused with 2Ca, CPA, 100 µM 2APB and 100 µM 2APB/75 mM NH$_4$Cl solutions. (b) Shows pH ratio of the same experiment.
Figure 23: 2-APB in NH4+ very slightly activates WT endogenous Orai1 (a) and transiently activates transfected Orai1(b). (a) After CPA mediated store depletion and then SOCE, 100 µM 2-APB was added, followed by the sequential addition of 100 µM/75 mM NH4Cl and 2Ca solutions on WT HEK (n = 36). (b) The same above experiment was performed on 24 h Orai1/STIM1 (1:1) transfected HEK (n=6).
Figure 24: pH measurement reveals that 2-APB activates WT endogenous Orai1 by acidification and in the presence of NH₄⁺ Orai1 is slightly activated by alkalinization. (a) Shows pH when WT HEK293 (n =33) were superfused with 2Ca, CPA, 100 µM 2APB and 100 µM 2APB/ 75 mM NH₄Cl solutions. (b) Shows pH ratio of the same experiment.
Although the mechanism of alkanized 2-APB mediated inhibition of Orai3 is not clear, we however conclude that whatever the mechanism, pH definitely plays a very significant role in concert with 2-APB. This is the first demonstration of 2-APB’s effect on cytosolic pH. It was worth mentioning here that 2Ca and CPA solutions show no change in cytoplasmic pH prior to 2-APB and 2-APB/NH₄Cl addition which indeed stand as an internal control and also confirms that CPA mediated block of the SERCA pump is not due to pH change (Figs. 22 and 24).

Ca²⁺ measurements reveal that 2-APB in NH₄⁺ inhibits WT endogenous Orai1 with an initial very slightly activation (Fig. 23a) and inhibits transfected Orai1 with a transient potentiation (Fig. 23b) unlike Orai3. Again, WT HEK untransfected cells show a similar response as Orai1 transfected cells. However, the pH response (Fig. 24a and 24b) of WT HEK untransfected cells is the same as that of Orai3 since 100 µM 2-APB promotes acidification of the cytoplasm during endogenous Orai1 inhibition and during recombinant Orai3 activation. Also, 100 µM 2-APB/75 mM NH₄⁺ promotes alkalinization during the inhibition of endogenous orai1 in WT HEK following the very slight potentiation (Fig. 24a and 24b) similar to the alkalinization during recombinant Orai3 inhibition. This suggests that acidification can activate one protein (Orai3) while it also inhibits the other (Orai1) when 2-APB is present. In other words, different responses on different channels are mediated by the resultant pH responses. We show next (Fig. 25a, 25b and 26) that pure acidification (i. e. without 2-APB) can only inhibit both proteins. This means that 2-APB mediated effect is regulated by pH.
Propionate acidification inhibits CRAC

Next, we examined the effect of propionate on HEK cells transfected with Orai. After CPA-mediated store depletion and subsequent SOCE, we superfused the cells with 75 mM sodium propionate solution and then subsequently washed it off with a 2Ca solution (Fig 25a, 25b and 26). We observed a sharp inhibition of Orai1 (Fig. 25a and 25b) and Orai3-mediated Ca\(^{2+}\) influx (Fig. 26). Unlike in Fig. 22 and 24 where we showed contrasting results with 2-APB-mediated acidification with the different Orai proteins, propionate mediated acidification leads to only inhibition in both proteins. This suggests that, the 2-APB’s acidification signal is interpreted differently by these different Orai proteins. Meaning that 2-APB might alter a pH-sensitive sight on these Orai proteins.

In order to confirm that propionate was actually causing acidification, we performed pH measurements on WT HEK cells by sequentially adding increasing concentrations (1 mM, 20 mM, 37.5 mM, 75 mM) of sodium propionate (Fig. 27). After that, we alkalinized the cells with 75 mM NH\(_4\)Cl to reverse the acidic effect which was washed with 2Ca at the end of the experiment. Results showed a stepwise decrease in pH as pH was increasingly lowered. This was completely reversed with NH\(_4^+\) alkalinization. We noticed that as we increasingly acidified the cells with increasing [propionate], there was a decreasing change in pH; cells showed the most regulation (slight alkalinization) after the most acidic solution was added. This means that intracellular pH changes are limited by the cell.
Figure 25: Weak acid, propionate inhibits Orai1. (a) After CPA mediated store depletion and then SOCE, 75 mM sodium propionate solution was used to superfuse 22 hr Orai1/STIM1 (1:0.5) transfected HEK cells (n = 4). (b) The same was done for 24 hr Orai1/STIM1 (1:0.5) transfected HEK cells (n = 3).
Figure 26: Weak acid, propionate also inhibits Orai3. (a) After CPA mediated store depletion and then SOCE, 75 mM sodium propionate solution was used to superfuse 22 hr Orai3/STIM1 (1:1) transfected HEK cells (n = 8).

Figure 27: pH measurement showing the effect of different mM concentrations of propionate and NH4Cl on WT HEK293 cells (n = 37).
*Orai3-transfected HEK293 cells show different population subtypes with pH changes.*

Several reports showed alkalinization of T cell cytoplasm during activation. We therefore, examined the effect on NH₄Cl alkalinization on CRAC proteins. **We hypothesized that alkalinization may provide the prolonged Ca signal needed for NFAT translocation into the nucleus.** First we tested the pH dependence of Orai1 and Orai3 in the HEK293 recombinant system. In this experiment, we sequentially added

Figure 28: HEK293 transfected Orai3 show different populations with change pH. See color-coded separated subpopulations in Fig. 28a/b and 29a. We sequentially superfused transfected cells with 1 mM, 20 mM, 37.5 mM, 75 mM NH₄Cl, then 75 mM sodium propionate, 2Ca, CPA and 2Ca (see population subtypes in Fig. 28a, 28b and 29b for labels). The lower red and blue graphs represent actual Orai3 response without endogenous WT response (activated Orai1) obtained by subtracting the low expressing cells (black) from the high (top blue) and medium (top red) expressing cells.
Figure 29: (a) HEK cells with high Orai3 expression are inhibited by propionate \((n = 2)\) while (b) HEK cells with medium expression of Orai3 show no visible effect \((n = 3)\).
Figure 30: Orai1 is activated by propionate in WT HEK293 cells after alkalinization. (a) HEK cells with low Orai3 expression are activated by propionate (n = 17). (b) A different experiment on WT HEK (n = 30) shows response similar to HEK cells that express low Orai3. This control confirms that Fig. 29a is the population with low Orai3 expression (high endogenous Orai1).
increasing concentrations (1 mM, 20 mM, 37.5 mM, 75 mM) of NH$_4$Cl to Orai3 (Fig. 28, 29 and 30) and Orai1-transfected cells (Fig. 31). Note that Fig. 29 and 30 shows groups of cells with the same population characteristics from one experiment shown together in Fig. 28 (color-coded).

After alkalinization, we acidified the cells with 75 mM sodium propionate to reverse the alkaline effect. Propionate was washed with 2Ca solution and then the ER stores were depleted with CPA solution followed by SOCE. We show here that high expressing-Orai3 cells (Fig. 29a) similar to Orai3 (Fig. 26) and Orai1 (Fig. 31) are inhibited by propionate however, WT HEK cells show increased Ca$^{2+}$ in propionate. This could imply that propionate mediated acidification is dependent of number of functional channels present; i.e. less Orai1 in WT HEK cells are activated while a high Orai1
Figure 32: Jurkat cells were activated with 2 µg/mL PHA for 2 days. (a) Ca\(^{2+}\) measurements showing store depletion by CPA, followed by SOCE with 2Ca solution superfusion (n = 14). (b) pH measurement showing pHi a little higher than 7.35 but lower than 7.70. Measured pH by superfusing cells (n = 16) with Tyrode’s 2Ca solution at pH 7.30 and then with two successive 10 µM nigericin/high (130 mM) [K\(^+\)]-containing solutions at pH 7.35 and 7.70 were superfused.
recombinant system is inhibited. In other words CRAC channel function is cumulative and depending on the cell type, pH elicits regulation based on the number of channels present where fewer channels are activated and vice versa. We think HEKs have an endogenous low pH-activated Ca$^{2+}$ influx i.e. HEK cells have a channel activated by acidification following alkalinization. It is worth mentioning again that cells internally regulate their pH after experimental pH manipulation where after Ca$^{2+}$ influx due to alkalinization, there is a gentle decrease in Ca$^{2+}$. Likewise there is a small increase in Ca$^{2+}$ after acidification (Fig. 31). Fig. 29b show medium Orai3-expressing cells with their endogenous low pH-activated Ca$^{2+}$ influx masking their Orai3 inhibition causing no significant change in Ca$^{2+}$ influx. A subtraction of the endogenous low pH-activated Ca$^{2+}$ influx reveals Orai3 inhibition (Fig. 28; lowers red curve) in the medium Orai3-expressing cells and an even higher inhibition in the high Orai3-expressing cells (Fig. 28; lowers blue curve).

**PHA-activated cells show variations in Ca$^{2+}$ influx and intracellular pH.**

Once we had characterized the intricate effects of internal pH changes on the Orai1 and Orai3 channels in a recombinant system and in Jurkat T cells, we proceeded to look at its effect on Jurkat T cells in their *activated* state since several findings strongly suggest that CRAC channels are the only channel responsible for Ca$^{2+}$ influx in T cell activation (reviewed in Rao et al 1997; Lewis, 2001). Jurkat cells were activated as described in the Methods section. Experimental results shown in Figs. 32-40 were performed as follows: For Ca$^{2+}$ measurement experiments, activated (with either 2 μg/mL or 5 μg/mL PHA) and naïve Jurkat cells loaded with fura-2 and washed twice with Tyrode’s 2Ca solution at pH 7.30 were superfused with 2Ca, then 10 μM CPA for store
Figure 33: Naïve 2 days control. (a) Ca^{2+} measurements showing store depletion by CPA, followed by SOCE with 2Ca solution superfusion (n = 26). (b) pH measurement showing pHi below 7.35 and 7.70. Measured pH by superfusing cells (n = 15) with Tyrode's 2Ca solution at pH 7.30 and then with two successive 10 µM nigericin/high (130 mM) [K+] -containing solutions at pH 7.35 and 7.70 were superfused.
depletion and subsequent activation of CRAC, followed 2Ca solutions for SOCE through CRAC. At the end of experiments shown in Figs. 34-40 Jurkat cells were superfused with \(\text{NH}_4\text{Cl}\) to study pH response in their activated state which all showed a similar initial increase in \(\text{Ca}^{2+}\) influx followed by a decrease.

For each consecutive pH measurement experiment, Jurkat cells from the same flask as its preceding \(\text{Ca}^{2+}\) experiment were used. Activated and naïve Jurkat T cells loaded with BCECF and washed twice with Tyrode’s 2Ca solution at pH 7.30 (close to the cells physiological pH) were first superfused at the start of the experiment with the same 2Ca solution (pH 7.30) previously used for washing in order to measure intracellular pH (pHi). Recall that the 2Ca solution has a low 4.5 mM \([\text{K}^+]\) similar to the cell’s physiological concentrations. Following this solutions, were two successive 10 \(\mu\text{M}\) nigericin/high (130 mM) \([\text{K}^+]\)-containing solutions at different known pH values (but same for each group of experiments) were superfused.
Table 4 Mean levels for store depletion transient and SOCE in Jurkats.

<table>
<thead>
<tr>
<th>Cell State</th>
<th>CPA store depletion mean value</th>
<th>SOCE mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group1 Day 2 P = 0.6472</td>
<td>Group1 Day 2 P = 0.0017</td>
</tr>
<tr>
<td></td>
<td>Group 2 Day 1-2 P = 0.0002</td>
<td>Group 2 Day 2-3 P = 0.001</td>
</tr>
<tr>
<td>Naïve</td>
<td>1.2944</td>
<td>2.4169</td>
</tr>
<tr>
<td>2 µg/mL</td>
<td>1.2893</td>
<td>2.0666</td>
</tr>
<tr>
<td>PHA</td>
<td>1.2015</td>
<td>2.1389</td>
</tr>
<tr>
<td>5 µg/mL</td>
<td>1.3587</td>
<td>1.9289</td>
</tr>
<tr>
<td>PHA</td>
<td>0.9289</td>
<td>1.0127*</td>
</tr>
<tr>
<td></td>
<td>1.2505*</td>
<td>0.9350#</td>
</tr>
<tr>
<td></td>
<td>2.4123*</td>
<td>2.4508#</td>
</tr>
</tbody>
</table>

The time of measurement was variable due to difficulties in adhering activated Jurkat to plate using poly-K.

*Measured earlier. # Measured later. Mean values in faint print represent Ca2+ influx levels that were not significantly different from each other down the column. However, they are significantly different from those in bold print and bold print are significantly different from each other. The level of significance is based on the P values shown in the table.

Table 5: Approximate pHi for PHA-activated Jurkats.

<table>
<thead>
<tr>
<th>Cell State</th>
<th>Day 2 pH1</th>
<th>Day 1-2 pH2</th>
<th>Day 2-3 pH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>Between 7.35 and 7.70</td>
<td>Between 7.30 and 7.69</td>
<td>Between 7.30 and 7.66</td>
</tr>
<tr>
<td>2 µg/mL PHA</td>
<td>Lower than 7.35 and 7.70</td>
<td>Between 7.30 and 7.69</td>
<td>Between 7.30 and 7.66</td>
</tr>
<tr>
<td>5 µg/mL PHA</td>
<td>No data</td>
<td>Lower than 7.30 and 7.69</td>
<td>Lower than 7.30 and 7.66</td>
</tr>
</tbody>
</table>

1 Group 1 set of experiments. 2 Group 2 set of experiments.
Figure 35: Naive 1-2 days control. (a) \(\text{Ca}^{2+}\) measurements showing store depletion by CPA, followed by SOCE with 2Ca solution superfusion \((n = 25)\). (b) pH measurement showing pHi between 7.30 and 7.69. Measured pH by superfusing cells \((n = 51)\) with Tyrode’s 2Ca solution at pH 7.30 and then with two successive 10 \(\mu\text{M}\) nigericin/high (130 mM) [K+] -containing solutions at pH 7.30 and 7.69 were superfused.
Figure 36: Jurkat cells were activated with 2 µg/mL PHA for 1-2 days. (a) Ca\(^{2+}\) measurements showing store depletion by CPA, followed by SOCE with 2Ca solution superfusion (n = 16). (b) pH measurement showing pHi a little higher than 7.30 and lower than 7.69. Measured pH by superfusing cells (n = 13) with Tyrode’s 2Ca solution at pH 7.30 and then with two successive 10 µM nigericin/high (130 mM) [K\(^+\)] -containing solutions at pH 7.30 and 7.69 were superfused.
These latter two solutions were used to show the effectiveness of the pH changes where
the pH readings should reflect the pH of the external solution added. A measure of pH
change with the high K+/nigericin-containing solution served as an indication of proper
dye functionality and for calibration.

Results in Figs. 32-34 represent a group of experiments performed under the same
experimental conditions except that the Jurkat cells in Fig. 32 were activated with 2
µg/mL PHA, whereas those in Fig. 34 were activated with 5 µg/mL PHA. Cells in Fig. 33
served as a naïve Jurkat control. Despite several attempts in this group of experiments we
were unable to perform pH measurements on the 5 µg/mL PHA-activated Jurkat cells due
to their highly fragile state. Comparing the mean Ca^{2+} influx for CPA- induced ER store
depletion the of naive Jurkats to that of 2 µg/mL PHA activated Jurkats and to that of 5
µg/mL PHA activated, the results (Table 4) show that there was no significant difference
in the group 1 experiments (1st column are all grey). The lowest SOCE was seen in 2
µg/mL PHA-activated Jurkats for the day 2 Jurkat T cells in group 1 in comparism to the
naïve and 5 µg/mL PHA-activated Jurkats which, showed no significant difference from
each other. A look at the approximate corresponding pH values (Table 5) reveals an
increase in pH for the 2 µg/mL PHA-activated Jurkats with respect to the naïve. Even
though there is no available data for our 5µg/mL PHA-activated Jurkats, it could still be
speculated that there is no direct correlation between the pH and Ca^{2+} influx in this group
since the SOCE for the 2 µg/mL PHA-activated Jurkats is significantly lower than that of
the naïve Jurkats but its pH is significantly higher as unexpected.
Figure 37: Jurkat cells were activated with 5µg/mL PHA for 1-2 days. (a) Ca\textsuperscript{2+} measurements showing store depletion by CPA, followed by SOCE with 2Ca solution superfusion (n = 4). (b) pH measurement showing pHi much lower than 7.30 and 7.69. Measured pH by superfusing cells (n = 4) with Tyrode’s 2Ca solution at pH 7.30 and then with two successive 10 µM nigericin/high (130 mM) [K\textsuperscript{+}] -containing solutions at pH 7.30 and 7.69 were superfused.
Figure 38: Naïve 2-3 days control. (a) Ca\(^{2+}\) measurements showing store depletion by CPA, followed by SOCE with 2Ca solution superfusion (n = 17). (b) pH measurement showing pHi higher than 7.30 but lower than 7.66. Measured pH by superfusing cells (n = 7) with Tyrode’s 2Ca solution at pH 7.30 and then with two successive 10 μM nigericin/high (130 mM) [K+] -containing solutions at pH 7.30 and 7.66 were superfused.
Figure 39: Jurkat cells were activated with 2 µg/mL PHA for 2-3 days. (a) Earlier Ca$^{2+}$ measurements showing store depletion by CPA, followed by SOCE with 2Ca solution superfusion (n = 7). (b) Another Ca$^{2+}$ measurement performed later to compare to earlier changes (n = 5).
For the group 2 experiments (Fig. 35-40), a comparison of CPA-induced store depletion shows no significant difference between the 1-2 day naïve Jurkats and the 1-2 day 2 µg/mL PHA-activated Jurkats between the naïve and the 2 µg/mL PHA activated Jurkats. This implies that there was no significant change in total transient Ca$^{2+}$ that leaked from the ER store, namely the store content. The 5 µg/mL PHA activated Jurkats however show a significantly lower CPA transient compared to the other two. The CPA mediated transient for the 2-3 day activation again showed no significant difference between the naïve Jurkats and the 2 µg/mL PHA-activated Jurkats meaning that there was no significant change in total transient Ca$^{2+}$ that leaked from the ER store.
For the 2-3 days 5 µg/mL PHA-activated Jurkats, its CPA transient is significantly lower compared to the naïve and 2 µg/mL PHA-activated Jurkats as seen with the 5 µg/mL PHA-activated Jurkats in group 1. A comparison between the day 2-3 early and late Jurkats cell activated with 5 µg/mL PHA show no significant difference. This of interest because the transient measured on day 1-2 also show a low CPA transient mean which is very close to the later measurement for the 5 µg/mL PHA-activated Jurkats on day 2-3. This could mean that the more activated the T cell, the smaller the Ca\(^{2+}\) content in the ER store which probably coincided with the release of Ca\(^{2+}\) for signaling purposes. The comparatively high CPA transient at the earlier stage of the 2-3 day 5 µg/mL PHA-activated Jurkats could coincide with the initial filling of the store in preparation for later release which, could be provided through CRAC channel influx. Interestingly, till today there are no reports linking store content and Ca influx through SOCE.

Now, a comparative look at the SOCE of the 2\(^{nd}\) group’s 1-2 day-2 µg/mL PHA-activated Jurkats reveals that the significantly lower mean Ca\(^{2+}\) influx occurs with the 5 µg/mL PHA-activated Jurkats in comparison to the naïve and 2 µg/mL PHA-activated Jurkats which show no significant difference between each other. The Jurkat cells activated with 5 µg/mL PHA show a significant difference by day 2-3 where the SOCE influx measured earlier and later are significantly higher than that of the naïve and the earlier 2 µg/mL PHA-activated Jurkats but not higher than the later 2 µg/mL PHA-activated Jurkats. The earlier and later measurements of the 5 µg/mL PHA-activated Jurkats show no significant difference between each other. The 2 µg/mL PHA-activated Jurkats and the naïve this time were not significantly different in their SOCE influx.
Figure 40: Jurkat cells were activated with 5µg/mL PHA for 2 -3 days. (a) Earlier Ca\(^{2+}\) measurements showing store depletion by CPA, followed by SOCE with 2Ca solution superfusion (n = 12). (b) Another Ca\(^{2+}\) measurement performed later to compare to earlier changes (n = 7)
Figure 40 continued: (c) pH measurement showing pHi much lower than 7.30 and 7.66. Measured pH by superfusing cells (n = 7) with Tyrode’s 2Ca solution at pH 7.30 and then with two successive 10 µM nigericin/high (130 mM) [K⁺] -containing solutions at pH 7.30 and 7.66 were superfused.

One could draw from this that for the 2-3 days group of experiments, the naïve Jurkats are significantly lower in Ca²⁺ than the earlier and later 5 µg/mL PHA-activated Jurkats and the later 2 µg/mL PHA-activated Jurkats as expected. However, the 1-2 days group 2 experiments show no direct correlation.

A combined look at the corresponding pH (Table 5) for the 1-2 days and 2-3 days activated cells show a general trend where there is a decrease in pH from the naïve to the 2 and 5 µg/mL PHA-activated Jurkats, respectively. On the other hand, there is no direct correlation between the pH and the SOCE as expected in which increasing alkalinity leads to higher Ca²⁺ influx. For example, on the day 2-3 of activation, the 2µg/mL PHA-
activated Jurkats showed a SOCE significantly lower than that of the 5 µg/mL PHA-activated Jurkats but the pH of the 2 µg/mL PHA-activated Jurkats is rather higher than that of the 5 µg/mL PHA-activated Jurkats. From the seemingly haphazard trends seen in PHA activation of Jurkat T cells, we conclude that it is neither the content of the store that determines the activation process nor did we see a direct correlation of increasing cytoplasmic Ca\(^{2+}\) and its corresponding alkalinity. **The activation process probably relies on specific Ca\(^{2+}\) signaling pattern mediated by variable pH changes and not just on the amount of Ca\(^{2+}\) influx.**

**PIP3 is effects on Ca\(^{2+}\) entry in Jurkat T cells.**

PI(3,4,5)P3 has been reported to cause elevated cytoplasmic Ca\(^{2+}\). **CRAC involvement in this process however, has not been previously been clarified.** We started by confirming past findings by showing the elevation of cytoplasmic Ca\(^{2+}\) with the addition of 5 µM di-C8-PI(3,4,5)P3 – containing solution on Jurkat cells initially superfused with 2Ca without store depletion (Fig. 41).
Ca\textsuperscript{2+} waves were observed with a seemingly visible change in Jurkat shape from round to crescent when PIP3 was added which, was reversed after waves subsided. This revolving change in shape was probably the visible pattern of Ca\textsuperscript{2+} location where the other half-moon areas without Ca\textsuperscript{2+} appeared dark (image not shown). So, when the localized oscillations stopped, Ca\textsuperscript{2+} dispersed in the entire cytoplasm revealing the round shape of the Jurkat T cells.

Figure 41: Elevation of cytoplasmic Ca\textsuperscript{2+} by di-C8-PI(3,4,5)P\textsubscript{3} with observed moon-shaped rotating oscillations on Jurkat T cells (n = 4).
Next we examined the role of CRAC channels in PIP3 mediated Ca\textsuperscript{2+} rise. Since Orai1 appears to play the principal role in Jurkats, we tested 5 µM di-C8-PI(3,4,5)P\textsubscript{3} effect on this channel over-expressed in HEK293 cells without store depletion (Fig. 42). The results show that store-independent Ca\textsuperscript{2+} influx are inhibited by di-C8-PI(3,4,5)P\textsubscript{3} in an Orai1/STIM1 (1:0.5) recombinant HEK cells with waveform oscillations. This means that PIP3 mediates its effect in a store-independent manner by inhibiting Orai1 when over-expressed. We therefore conclude that, PIP3 plays a regulatory role on Ca\textsuperscript{2+} which is dependent on the Orai1 activity where it causes decreased Ca\textsuperscript{2+} when Orai1 is over-expressed. This means that PIP3 –mediated increase in Ca\textsuperscript{2+} in Jurkat T cells occurs through another pathway.
Figure 43: wortmannin inhibits SOCE in Jurkat T cells in an Orai1-dependent manner. (a) 50 µM 2-APB distinguished Orai1 (inhibition after an initial potentiation) as the dominant player after the treatment Jurkats (n = 6) with 1 µM wortmannin for 30-40 min. SOCE mean value = 1.4263. (b) Control; in the absence of wortmannin, control Jurkats (n = 4) show higher SOCE (mean value = 3.8685) after CPA mediated store depletion. P = 0.0003
To further distinguish and elucidate the functional role of the Orai proteins with regards to PIP3, an inhibitor of PI3K, wortmannin was used. After depleting the ER stores with 10 µM CPA to activate CRAC, 1 µM wortmannin-treated Jurkat cells (Fig. 43a) and untreated Jurkat cells (Fig. 43b) were sequentially superfused with 2Ca and 50 µM 2-APB. This result show that SOCE significantly (P = 0.003) decreased from a mean value of 3.8685 in the control to 1.4263 with PI3K inhibition in comparism to the control Jurkat cell. Both experiments however, showed a 50 µM 2-APB inhibition preceded by an initial potentiation. This confirms the fact that Ca^{2+} influx through CRAC with Orai1 being dominant is dependent on PIP3.
VI. DISCUSSION

When an antigenic peptide presented by antigen presenting cells (APC) is recognized by the specific T-cell receptor (TCR), a mobilization of T cell surface molecules occurs and signaling molecules are recruited to the immunological synapse (IS) (Grakoui et al, 1999; Bromley et al., 2001). Among various transmembrane proteins, recruited to this synapse are Orai1 and STIM1 which colocalize with TCR and costimulatory molecules (Lioudyno et al., 2008). Other channels like the voltage-gated potassium channel (Kv 1.3) have been shown to be accumulated at the IS of activated human cytotoxic T lymphocytes (CTLs) (Panyi et al, 2004). The sustenance of signals required for the complete T cell activation is provided by the prolonged interaction of T cell/APC at the immunologic synapse (Huppa et al., 2003) and presence of SOCE. The present study proposes several possibilities for the regulation of CRAC channels in native and recombinant systems. It also opens the door to new areas of research in the field of pH regulation of calcium signaling.

Calcium influx provided by SOCE is probably not enough to sustain a long-duration, high intracellular [Ca$^{2+}$] necessary for the immune response. This suggests that SOCE through CRAC (Orai) channels cannot be solely responsible for NFAT translocation, and consequently, proliferation and differentiation of T-cells. There are other sources of calcium such as the ER Ca$^{2+}$ release channel, the ryanodine receptor (RyR). There are 3 types expressed in Jurkats, which may also contribute to the Ca$^{2+}$ homeostasis (Lioudyno et al, 2008). Additionally, there are several reports of cyclic ADP-ribose (cADPR) activated Ca$^{2+}$ influx pathway in lymphocytes, (Guse et al., 1995;
Guse et al., 1997; Guse et al., 1999). It is also likely that Orai channels are gated by unknown second messenger or regulated by them. So far the only physiological stimulus known to activate Orai channels is the depletion of ER calcium stores.

NFAT requires ~2 hrs (Shaw et al., 1988, Crabtree et al., 1989) for the completion of its entire translocation into the nucleus. This means that the 3-4 min long transient SOCE we observed may not be long enough to fully account for NFAT translocation. This Ca\(^{2+}\) signal, however, may be necessary for the initiation phase of NFAT translocation such as early steps of calmodulin-Ca\(^{2+}\) sensing (Weinstein and Mehler, 1994) but not the entire NFAT translocation process. The numerous disparate findings on calcium signal duration could more be closely explained by Dolmetsch et al. (1997). According to this study, the variation in amplitude and duration of Ca\(^{2+}\) signal promote activation of genes differentially. We observed that the duration of SOCE varied significantly from experiment to experiment following activation of Jurkat T-lymphocytes. This can possibly be explained by cyclical time-dependent changes in calcium signaling that are not synchronized. Even though we could not see a clear pattern of cyclical calcium changes due to a relatively small number of cells studied, a future and more detailed examination of a large number of peripheral human T cells may allow us to describe them.

Jurkat CRAC and recombinant Orai1 current inhibition by 2-APB is preceded by an initial potentiation (Prakriya and Lewis, 2001; DeHaven et al., 2008). Prakriya and Lewis, (2001) reported that low micromolar concentrations of 2-APB potentiate CRAC current whereas high (50-100 µM) concentrations inhibit it. When superfusing high 2-APB solutions we observed an initial potentiation followed by an inhibition. We suggest
that this initial potentiation could be explained by short-lived low 2-APB concentration resulting from an initial dilution during superfusion where the incoming 2-APB-containing solution mixes with the outflowing normal 2 mM calcium solution. A close look at Fig. 9b (5µM 2-APB solution added prior to 50µM 2-APB solution) in comparison to Figs. 10b and 11 (50µM 2-APB solution only) reveals a compelling similarity. Essentially, the speed of superfusion is a contributing factor.

One recent study on 2-APB’s mechanism of action on Orai3 revealed that it may occur through the reversal or prevention of STIM1 localization near the plasma membrane “puncta” (DeHaven et al., 2008). It was previously shown that upon store depletion the STIM1 molecules aggregate near the plasma membrane forming areas of high density which are referred to as “puncta” (Zhang et al., 2005). Dehaven and colleagues show that 2-APB at 50 µM concentrations disperses STIM1 aggregates (seen after TG store depletion) into diffused distribution, as seen with filled Ca\(^{2+}\) stores. However, this study also presents evidence that potentiation can occur in a manner that is independent of STIM1 re-localization. One can speculate that Orai3 transmembrane segments are separated just enough to fit the aggregated configuration of STIM1, observed in the physiological store-dependent activation without 2-APB, while Orai1 transmembrane segments require a close STIM1 arrangement at all times. If this is the mechanism, then it ties in with the suggestion that 2-APB mediates it effect by gating CRAC channels (Navarro-Borelly., 2008).

An earlier paper demonstrated that store depletion is in fact not indispensable for human CRAC channel activation (Zhang, Kozak et al., 2008). They show that mutations in the STIM1 EF-hand domain make the protein Ca\(^{2+}\)-insensitive (Zhang et al., 2005).
The authors demonstrate that the C-terminus of STIM1 (C-STIM1) is sufficient to pre-activate Orai channels without prior store depletion. C-STIM1 was shown to be the effector domain that interacts with Orai proteins.

Three recent studies suggest that 2-APB may distort the architecture of CRAC channel thus altering its biophysical properties. (Zhang, Kozak et al., 2008; DeHaven et al., 2008, Peinelt, Lis et al., 2008). They demonstrate that 2-APB-mediated Orai3 activation elicits a change in its ion selectivity by drastically increasing the monovalent Cs⁺/Na⁺ permeability ratio so that extracellular divalent cations at millimolar concentrations can no longer effectively block monovalent cation flow (Zhang, Kozak et al, 2008). By contrast, the native CRAC channels do not conduct measurable currents carried by Cs⁺ (Kozak et al, 2002). Here, we use NH₄⁺ in external solutions to extensively study its known alkalinity-inducing and possibly the monovalent cation effects. To our surprise we found that NH₄⁺ inhibits 2-APB-mediated activation of Orai3 but not 2-APB-mediated inhibition of Orai1. In other words, NH₄⁺ reverses the stimulatory effects of 2-APB but not its inhibitory effects. We also found that application of 2-APB at **100 µM concentrations resulted in significant acidification of the cytosol**. The acidification was slow and reached its plateau in minutes. This acidification is not thought to be mediated by calcium since in the same experiment neither CPA, to deplete stores, nor reintroduction of external calcium affected the pH. Overexpression of Orai3 and STIM1 also did not noticeably change the acidification induced by 2-APB. Even though NH₄⁺ in combination with 2-APB inhibited Orai3-mediated Ca²⁺ influx, its ability to rapidly alkalinize the cytosol was not diminished in WT or Orai3/STIM1-transfected HEK cells. The inhibitory effect of 2-APB in Jurkat T cells was magnified by the
addition of NH$_4^+$, similar to the situation with Orai1/STIM1 in HEK cells. We have not tested the effect of 2-APB on pH in Orai1/STIM1-overexpressing HEK cells or Jurkat T cells. We think it unlikely, however, that the presence of Orai1 would interfere with the acidification observed in 2-APB or with alkalinization by NH$_4^+$, since Orai3 overexpression did not cause these changes. Orai1 and Orai3 (in presence of STIM1) are both inhibited by propionate mediated acidification and potentiated by NH$_4^+$ mediated alkalinization. Based on these combined observations we suggest that the inhibition of recombinant and endogenous Orai1 CRAC current and SOCE by high concentrations of 2-APB may in part be due to its acidifying effect on the cytosol.

The potentiating effect of 2-APB on Orai3 would be difficult to explain since acidification by propionate strongly inhibits this channel when it is activated by store depletion. In fact, one would expect that, under circumstances preventing cytosol acidification (increased buffering), 2-APB would be even more potent in its ability to activate Orai3. In other words, the observed activation is limited due to simultaneously occurring acidification. A proposed mechanism of action of 2-APB mediated intracellular response (acidification) that leads to variable changes in Ca$^{2+}$ influx in CRAC Orai1 and Orai3 channels is the alteration of a specific and unique pH-sensitive site on the proteins. So that, depending on the pH-sensitive site composition and subsequent 2-APB alteration, cytosolic acidification mediated by 2-APB promotes potentiation of Orai3 and inhibition of Orai1. These proteins may therefore have a binding site for 2-APB.

At present, we do not have a good explanation for the combined NH$_4^+$ and 2-APB effects on Orai1 and Orai3. If the combination of NH$_4^+$ and 2-APB resulted in changes in an unknown component of SOCE signaling and reversed the change in pH
“perceived” by the Orai3 channel thus resulting in its inhibition, then it would be expected to also change the pH “perceived” by Orai1 and a resulting activation by 2-APB. Yet, this is not observed: for both channels, a combination of 2-APB and NH$_4^+$ proves to be absolutely inhibitory. One possibility for NH$_4^+$+2-APB reversal of action would be that NH$_4^+$ ions flow through Orai3 channels previously modified by 2-APB (see above) and thus prevent alkanilization by external NH$_4^+$. This possibility is discounted, however, by our observation that in Orai3-overexpressing HEK cells NH$_4^+$ still alkanilizes the cytosol. Orai1 does not conduct monovalents (NH$_4^+$ among them) in the presence of 2-APB, therefore, this scenario could not occur in Orai1-expressing cells.

It is also possible that 2-APB exerts its effect by gating the CRAC channel directly (Navarro-Borelly et al., 2008; Zhang, Kozak et al, 2008). Thus using a chimeric approach, Zhang, Kozak and colleagues (2008) argue that 2-APB binds directly to the transmembrane domains 2-3 (TM2-3) of Orai3 and not STIM1. In this scenario pH would also play a role in gating regulation. The implication of this is that CRAC channel must have a pH-sensitive site or domain. 2-APB on the other hand could alter this pH-sensitive site by binding to its own site on the cell or protein (TM2-3) causing the Orai homologs to respond differently to acidification. If this is the underlying mechanism then, such modifications might be vital for Ca$^{2+}$ plateau sustenance or long repetitive spikes, depending on the pH value and its subsequent change in CRAC conformation. pH mediated conformational changes could also lead to changes in ion selectivity and permeability. We have not directly tested this possibility yet and these can only be assessed in patch-clamp studies.
pH is endogenously regulated after an acute experimental pH change. After acidification or alkalinization and then subsequent decrease or increase in Ca\(^{2+}\), there is an immediate reversal of the effect and return to steady state. pH changes are very quickly regulated by the cell’s Na\(^+\)/H\(^+\) antiporter especially when there is a concurrent change in [Na\(^+\)] (Paris and Pouysségur 1983 and 1984 Rosoff et al., 1984, Boron et al., 1984). In the same respect, the cell only allows for pH mediated Ca\(^{2+}\) change to a certain extent. This explains why increasing amounts of propionate and NH\(_4\)Cl show less [Ca\(^{2+}\)]\(_i\) and pHi transients (Rogers et al., 1983). It could be inferred that the NH\(_4^+\) effect depends on intracellular Ca\(^{2+}\) level.

We found that untransfected HEK293 cells that are commonly used for over-expression studies possess an unusual Ca\(^{2+}\) influx mechanism activated by cytosolic acidification. Thus, HEK cells transfected with Orai3 show different population subtypes. In one population where Orai3 expression levels are high, propionate reduces Ca\(^{2+}\) levels in the cell. In the second population, presumably expressing medium levels of Orai3, propionate has no significant effect on Ca\(^{2+}\). The third population exhibits a significant Ca\(^{2+}\) elevation in response to propionate application; we think that this population reflects the low Orai3 expressing cells. In agreement with this, untransfected HEK cells also show propionate-activated Ca\(^{2+}\) influx.

Our findings corroborate previous suggestions in the literature that alkalinization leads to Ca\(^{2+}\) influx while acidification leads to decrease Ca\(^{2+}\). However, we show here that under certain conditions, this is not always the case. For example, our novel findings show that 2-APB mediates its activation effect on Orai3 leading to increased Ca\(^{2+}\) influx by acidification meanwhile in the presence of NH\(_4^+\), 2-APB does the opposite (inhibition)
by alkalization. The mechanism of action of 2-APB-mediated acidification is not well understood, however, we suggest that if 2-APB acts by dispersing STIM1 then, it probably achieves this by activation of Na\(^+\)/H\(^+\) exchanger leading to acidification. This implies that STIM1, in addition to being a Ca\(^{2+}\) sensor may also be a pH sensor. A study of STIM2, an inhibitor of STIM1 could shed more light on 2-APB-mediated intracellular acidification. In this experiment, STIM1/STIM2/Orai3 could be cotransfected and one should see an attenuation of acidification as STIM2 inhibits STIM1.

Previous findings have shown that there is a substantial increase in CRAC-mediated cytoplasmic Ca\(^{2+}\) influx following T lymphocyte activation (Lioudyno et al., 2008). Various other studies have demonstrated that PHA-activated T cells exhibit higher resting calcium levels as well. Several publications have shown that during activation of T lymphocytes there is an increased Ca\(^{2+}\) influx accompanied by alkalization. Our present studies showed that PHA treatment could indeed alkalize Jurkat T cell cytosol. However, we show that, depending on the time of Ca\(^{2+}\) measurement and the concentration of the PHA, CRAC-mediated Ca\(^{2+}\) influx was variably lower or higher relative to naïve Jurkats. Two concentrations of PHA were tested for their ability to activate Jurkat T-cells but the higher concentration showed weaker alkalization effect compared to the low one. In like manner, for several experiments, depending on the duration of PHA treatment, we observed differences in the extent of pH changes. Due to this we were unable to ascertain a direct correlation between resting pH levels and resting calcium levels in Jurkats. It is clear to us that the PHA activation protocol in vitro needs further optimization to achieve consistent activation responses. Even though PHA and other plant lectins have been used for several decades in T cell research, they do not
**represent a physiological antigen presentation paradigm.** PHA is still used by several laboratories for activating T cells in vitro but many modern papers have abandoned this tool in favor of CD3/CD28-coated dishes and direct antigen presentation by an APC (see Lioudyno, et al, 2008). It may therefore be wiser to simply steer in this direction rather than extensively study what PHA does to Jurkats. Even though PHA has been useful in studies of calcium mobilization in Jurkat T cells, its effects on pH have been more puzzling. The changes in pH seen post mitogenic lectin activation of Jurkats, could possibly be explained by cell division (Gerson et al., 1982). The mouse lymphocytes were activated with concanavalin A (ConA) and lipopolysaccharide (LPS) and pH measured by the $[^{14}\text{C}]$DMO-microfuge method. They assert that the first stage of lymphocyte activation by ConA coincides with the 0-12 hr transient alkalinization. Others have reported that this first stage is accompanied by metabolite, RNA and protein synthesis (Larsson and Coutinho, 1979; Larsson et al., 1980). The second alkalinization occurs as the cell enters mitotic cycle accompanied by DNA synthesis which, decreases steadily after day 1 (Larsson and Coutinho, 1979). Decrease in pH inhibits DNA synthesis (Margolis et al., 1987). All these findings substantiate our finding that pH changes variably in PHA-activated Jurkat cells.

We observed a circular calcium wave which was confined to the cytosol area close to the plasma membrane. PIP2 hydrolysis leads to IP$_3$ generation which causes Ca$^{2+}$ oscillations. It is possible that IP$_3$ mediated alkalinization and subsequent Ca$^{2+}$ rise promote mitotic division in Jurkat T cells (Ciapa et al., 1994). This circular oscillation which lasted several minutes after PIP3 addition eventually reverts to diffused Ca$^{2+}$ elevation spanning the whole cytosol of the cell. As reviewed in (Oh-hora and Rao,
NFAT and NFκB respond to different frequencies of intracellular Ca$^{2+}$ oscillations where both are activated by high oscillation frequencies with NFκB preferring low frequencies. Ca$^{2+}$ oscillations may also be required for motility as a short-term function in Jurkat T cells as well as lymphocytes (reviewed in Oh-hora and Rao, 2008).

Wortmannin-treated cells (low PIP3 due to PI3K inhibition) showed significantly low SOCE compared to their naïve counterparts. This is however, a preliminary study and needs to be confirmed with further experimentation since our current result is a representation of a few cells. Also, wortmannin has been shown to be a non-specific inhibitor of PI3K and PI4K (Downing et al., 1996). Therefore, a decrease in SOCE may not definitively reflect an effect of low PIP3 since other molecules may be involved in calcium homeostasis in Jurkats which we are unaware of. Therefore, PIP3 alone and wortmannin treatment may not be equivalent. A comparison of Fig. 43a and 43b reveals a full scale decrease in Ca$^{2+}$ influx in the wortmannin treated cells. This led us to think that wortmannin could have an effect on fura-2. However, previous literature shows that wortmannin had no effect on increased [Ca$^{2+}$], levels resulting from either 1 µM thapsigargin or from 5 µM Ionomycin meanwhile wortmannin inhibited [Ca$^{2+}$]i plateau phase in Ca$^{2+}$-mobilizing agonists like bradykinin and angiotensin II (Nakanishi et al., 1994). This implies that wortmannin has little or no effect on fura-2 suggesting that the decrease in Ca$^{2+}$ influx is not a result of reduced fura-2 signal.

Finally, we also show that Ca$^{2+}$ influx through CRAC with Orai1 being the dominant player is dependent on PIP3. However, we show here that store-independent and dependent (data not shown) Ca$^{2+}$ influx is inhibited in Orai1-transfected HEK293. This result contradicts that seen with endogenous Orai1 in Jurkats. Could it be due to the
differences in Jurkat and HEK cell’s effect on the short chain PIP3 (from Echelon) as opposed to the endogenous long chain PIP3? Again, this preliminary finding needs to be confirmed. However, judging from the 50 µM 2-APB inhibition seen in both the naïve and the wortmannin-treated Jurkats one could say that Orai1 is consistently the dominant player in Jurkats whether or not PIP3 levels are high or low.

In summary, we have shown a novel method for the inhibition of CRAC in the presence of 2-APB and NH$_4^+$+. This could be another vital tool for the inhibition of CRAC. We have also shown that PHA mediated activation of Jurkats is pHi-dependent in an up and down manner leading to gene expression. HEK293 cells transfected with Orai3 show different population subtypes where acidification by propionate inhibits the Orai3 subtype while the endogenous Orai1 subtype is activated. Finally, PIP3 appears to modulate CRAC (Orai1)-mediated SOCE.
VII. CONCLUSIONS

1. NH$_4^+$ inhibits CRAC in the presence of 2-APB.

2. Acidic pH blocks CRAC

3. Orai1 and Orai3 are inhibited by acidic pH transfected HEK cells

4. Untransfected HEK cells show endogenous acidification-activated Ca$^{2+}$ influx

5. PHA- mediated activation elicits variable Ca$^{2+}$ influx and pH changes in Jurkat T cells.

6. PIP3 causes an increase in SOC influx but a decrease in HEK transfected Orai1

7. The decreases SOCE following wortmannin inhibition of PI3K and subsequent decrease in PIP3 confirms that it has a significant role in CRAC mediated Ca$^{2+}$ influx.
VIII. FUTURE DIRECTIONS

1. Elucidate the mechanism of action of NH₄⁺ mediated inhibition of CRAC in the presence of 2-APB.

2. In the future long-term effects of pH changes could be analyzed by measuring and quantifying changes in NFAT, NFκB and AP-1 levels.


4. Further description of Ca²⁺ and then pH changes in response to PIP3 mediated intracellular Ca increase in all Orai proteins with and without store depletion.

5. A description of Orai2 and STIM2 response to pH change with and without 2-APB.
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