POTENT AND SPECIFIC ACTIONS OF 2-AMINOETHOXYDIPHENYL BORATE (2-APB) DERIVATIVE ON ORAI CHANNEL FUNCTION

A Dissertation
Submitted to
the Temple University Graduate Board

In Partial Fulfillment
of The Requirements for the Degree of
Doctor of Philosophy

By
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August 2013

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ABSTRACT

In an effort to dissect the mechanism of SOCe activation, I used two novel 2-APB analogs (DPB162-AE and DPB163-AE) which are ~50-100 times more potent at modifying SOCe than 2-APB. In the presence of STIM1, both compounds (2 μM) differentially affected Orai subtypes, fully blocking endogenous Orai1, but not Orai2 or Orai3 mediated SOCe in DT40 Orai-specific knockout cells. Neither analog directly activated Orai3 over-expressed alone in HEK293 cells. Analysis of constitutively active Orai1 mutant, Orai1V102C, showed an increase in Ca^{2+} entry after application of DPB162-AE independent of STIM1. When STIM1 was co-expressed with Orai1V102C, there was no inhibitory effect of the analog on the mutant channel complex. DPB162-AE appeared to have a long term effect on the channel complex revealed a lack of SOCe 10 minutes after washout of the analog. STIM1ct-Orai1 Ca^{2+} entry was moderately increased by DPB162-AE yet constitutively active Stim1ct4EA-Orai1 Ca^{2+} entry was robustly inhibited. The activation of mutant Orai1V102C indicated the analogs are capable of interacting with Orai1, perhaps to widen the pore, and pointing to a putative mechanism of action for inhibition. FRET analysis indicated no effect on *STIM1-Orai1, STIM1ct-Orai1 or SOAR-Orai1* coupling. Thus, the inhibitory effect on STIM1-Orai may be through physical alteration of *Orai1 gating.* Previously reported as having biphasic effect on SOCe proteins, DPB163-AE appeared to effect its potentiation exclusively via STIM2 with no evident inhibition of STIM2 SOCe. Inhibition by both analogs was mediated by STIM1. DPB162-AE and DPB163-AE had remarkable specificity on Orai1 as opposed to

other Ca^{2+} permeant channels. Neither compound affected Ca^{2+} entry through TRPC3, TRPC6, or strontium entry through $Cav_{1.2}$ channels at concentrations (2 μM) that completely inhibited Orai1-mediated SOCe. In summary, DPB162-AE and DPB163-AE are highly specific inhibitors of Orai1 SOCe, with little effect on Orai2 and Orai3, and no effect on other Ca^{2+} channels. They do not disrupt STIM-Orai coupling but may modify functional Orai1 channel structure to effect their inhibitory action on SOCe.

ACKNOWLEDGMENTS

My journey to this finale commenced almost 20 years ago in the confines of St. Patrick's Grammar School, Armagh, Northern Ireland, and the science classes of Miss Cathy McVeigh. A fresh graduate from teacher training college she brought immense enthusiasm and passion for the classes she taught and made learning easy. In the intervening years, there have been many who've guided and influenced my journey as a scientist; a journey I hope has many years of fruitful insight to come.

My current mentor, Dr. Donald L. Gill, invited me to join his lab when I transferred from another program, and I hope I've been able to show him his investment in me was worthwhile. For driving me to progress, for challenging me and for providing an opportunity to complete my PhD when other matters seemed to be driving me away from science, I sincerely thank you.

Thank you to thesis advisory committee members, Drs. Rothberg, Soboloff and Chong, who guided and supported my research and helped shape my scientific thinking throughout my time at Temple University.

Fellow lab members, Salvatore Mancarella, Youjun Wang, Xiaoxiang Deng, Xi-Zhuo Wang who were always available to bounce ideas off, interrogate with questions and provide critique of my work though out my PhD.

Dr. James D. Stockand, professor of Physiology at University of Texas Health Science Center, San Antonio, Texas, who, in 2001, took me into his lab as an undergraduate intern. He gave me an opportunity few would have afforded an international undergraduate student they'd never met. He showed me first-hand the

intricacies of important and interesting research. Those nights on Bourbon Street, New Orleans, 2002, may or may not have played a part in my return to his lab to start my PhD in 2006. For the opportunity alone, I'm eternally grateful.

Thanks also to my family in Ireland for supporting my decision to move 'across the pond' as well as constantly driving me to be successful.

And last, but certainly not least, my beautiful wife, Karin, and daughter Aoibheann. Karin is the rock I've leaned on for the last six and a half years. She's been with me right from the start, teaching me, supporting me, guiding me, and most of all, putting up with my often difficult personality. I really wouldn't be here today if it was not for you. No words I write here will ever express my gratitude to you for being the brightest light on the darkest of days. For sticking by me during hard times and for constantly polishing the rough off me, to help me be the person I know I am.

Baby Aoibheann, my two and half years with you have been some of the most joyous I've experienced. Thank you for bringing me that happiness each day on our walks to and from the city, for your constant 'why' and 'what you doing papa', and for showing me with a smile, that no matter what, life goes on!

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate enzyme
BAPTA	(1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid)
B cell	White blood cell of the lymphocyte group
bleo	Bleomycin
bsr	Blasticidin S
Ca^{2+}	Calcium
$Ca^{2+}{}_{i}$	Intracellular calcium concentration
$Cav_{1.2}$	Voltage gate calcium channel
CPA	Cyclopiazonic acid
CT	C Terminal region
DAG	Diacylglycerol
DBHQ	2,5-Di-tert-butylhydroquinone
DKO	Double Knock Out
DPB's	Dimeric 2-APB analogs DPB162-AE and DPB163-AE
EGTA	ethylene glycol tetraacetic acid
FBS	Fetal Bovine Serum
G418	*O*-2-Amino-2,7-didesoxy-D-glycero-α-D-gluco-heptopyranosyl-(1→4)-*O*-(3-desoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl- (1→6))-D-streptamin
H+	Hydrogen ion
IBC	IP_3 Binding Core
IP_3	Inositol trisphosphate

IP_3R	Inositol trisphosphate receptor
KO	Knock Out
MAPK	Mitogen activated protein kinase
mM	Millimolar
μM	Micromolar
ms	Millisecond
mV	Millivolt
NT	N Terminal region
OAG	1-oleoyl-2-acetyl-sn-glycerol
PDB	Protein data bank
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein Kinase C
PKD	Protein Kinase D
PLCβ	Phospholipase C beta
PLCγ	Phospholipase C gamma
PM	Plasma membrane
PMCA	Plasma membrane Ca2+ ATPase
RAS	Small GTPase in the MAPK pathway
RyR	Ryanodine Receptor
SAM	Sterile alpha motif
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
SOAR	STIM-Orai Activating Region
SOCe	Store operated Ca^{2+} entry
SOCs	Store Operated Channels
SPCA	Secretory pathway Ca^{2+} Mn^{2+} ATPase's

SR	Sarcoplasmic reticulum
STIM	Stromal Interaction Molecule
T cell	White blood cell of the T lymphocyte group
TG	Thapsigargin
TRPC	Transient Receptor Potential Canonical ion channel
TRPV	Transient Receptor Potential Vallinoid ion channel

CHAPTER 1
CALCIUM SIGNALING

STORES, CHANNELS AND PHARMACOLOGICAL MODIFIERS

Calcium (Ca^{2+}), one of the most abundant elements by mass on earth, is an essential nutrient for all living organisms (Peacock 2010), including plants (White and Broadley 2003). For vertebrates, its functions are varied and wide ranging: it is important for maintaining the potential difference across excitable cell membranes, and is the primary constituent of bone, the main calcium store of the body. It acts as a signaling molecule for cellular processes including muscle contraction, fertilization of eggs, neurotransmitter release from neurons, transcription and growth, among many processes (Clapham 2007).

Intracellular Ca^{2+} fluxes are mediated by ion channels and transporters of various types. Calcium concentration within the cytoplasm is maintained at ~100 nM, in contrast to an extracellular concentration of ~1-3 mM, which allows for a strong gradient across membranes. Environmental stimuli can initiate ion channel activation, by voltage, ligands, stretch or intracellular second messengers, resulting in cytoplasmic Ca^{2+} concentration increases, either via influx of extracellular Ca^{2+}, or release of Ca^{2+} from intracellular stores. Once in the cytoplasm, Ca^{2+} is then sequestered by one if it's many binding partners (phosphate, sulfate, or calcium binding proteins), or, it acts to regulate ion channels or cellular pathways as a second messenger by participating in critical cellular responses such as gene transcription, growth and proliferation. Most Ca^{2+} binding

proteins are activated when the cytoplasmic Ca^{2+} concentration rises. Upon the cessation of environmental stimuli, cytoplasmic Ca^{2+} levels are decreased via active transportation of Ca^{2+} across the plasma membrane (PM) and endoplasmic reticulum (ER) membrane, or into mitochondria. The latter two act as intracellular Ca^{2+} stores, sequestering Ca^{2+} from the cytoplasm, while simultaneously maintaining a concentration gradient across their membranes to facilitate Ca^{2+} re-entry into the cytoplasm in response to cellular signals. Expectedly, while the Ca^{2+} transporters can be activated by a number of molecules, the inhibition of their function is also important. Pharmacological inhibition or genetic manipulation (accidental or otherwise) of components of the transport machinery can lead to catastrophic physiological consequences for the cell. Thus, understanding mechanisms of pharmacological modification of Ca^{2+} transport machinery, provides insight into the action of these proteins, as well as the potential to develop for use, compounds capable of regulating aberrant ion channels or other transport proteins and their downstream pathways.

1.1. Activation of the Inositol-1,4,5-trisphosphate (IP_3) pathway

Ca^{2+} is the most tightly regulated ion in physiology, with the propensity to bind thousands of proteins resulting in changes in association, function and cellular localization, thus, providing a signal to different compartments and processes occurring within a cell (Clapham 2007). The idea that Ca^{2+} could act as a signaling molecule was first proposed by Sydney Ringer in elegant experiments on the beating hearts of frogs (Ringer 1883). Since then, intracellular Ca^{2+} signaling has been shown to be a highly regulated, spatio-temporal phenomenon employing numerous components; proteins, enzymes, and, of course, Ca^{2+} itself. Stringent regulation is required to maintain low cytoplasmic Ca^{2+} concentrations, and most importantly, to avoid unwanted activation of Ca^{2+}-dependent processes within the cell. Without such a concerted effort to maintain very low intracellular calcium concentration, [$Ca^{2+}{}_i$], the cellular processes would run amok, resulting in unnecessary and undesirable side effects and disease states.

The signaling process can generally be described as a 4 step process (Berridge, Lipp et al. 2000)

a) Triggering of Ca^{2+} mobilizing signals

b) Entry of Ca^{2+} into the cytoplasm raising $Ca^{2+}{}_i$ above resting levels

c) Stimulation of Ca^{2+} sensitive processes

d) Extrusion of Ca^{2+} from the cytoplasm

Extracellular stimuli acting on various types of receptors/ion channels in the plasma membrane can either allow Ca^{2+} to directly enter the cell, as is the case with Ca^{2+} permeant ion channels or transporters, or activate a signaling cascade to mobilize Ca^{2+}. One of the most common receptor mediated Ca^{2+} mobilization pathways is hydrolysis of Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) by phospholipases (PLCγ activated by

Tyrosine Kinase Receptors; PLCβ activated by G-Protein Coupled Receptors) to generate Diacylglycerol (DAG) and Inositol trisphosphate (IP_3). IP_3 activates the IP_3 Receptor (IP_3R), releasing Ca^{2+} from the Endoplasmic Reticulum. DAG regulates Serine/Threonine kinases including Protein Kinases, C and D (PKD, PKC) and Ras (a participant in the Mitogen Activated Protein Kinase (MAPK) cascade), thus affecting many different downstream pathways (Finlay and Cantrell 2011). The release of Ca^{2+} from intracellular stores can trigger a multitude of events, not least of which is Store Operated Calcium Entry (SOCe), which is most relevant to this work, and essential for providing Ca^{2+} for longer term responses such as gene transcription, cellular movement and proliferation.

1.2. Endoplasmic Reticulum (ER)

While the cytoplasm has a very low concentration of free Ca^{2+}, intracellular organelles serve as Ca^{2+} stores, retaining most of the unbound Ca^{2+} within the cells. Thus, the stores provide a pool of Ca^{2+} with a strong gradient across the organelle membranes, important for temporal signaling processes. The main storage compartment is the ER, or in muscle cells, the sarcoplasmic reticulum (SR). The primary function of the ER is in the synthesis and folding of proteins (supported by high luminal Ca^{2+}) (Zhang and Kaufman 2008), lipids and steroids, metabolism of carbohydrates, storage of Ca^{2+} and drug detoxification. While all are important, the storage of Ca^{2+} is most relevant to this study.

The ER is regulated by a series of channels and pumps, which permit the flow of Ca^{2+} across its membrane. Primary among these are the Sarco/Endoplasmic Retiulcum Calcium ATPase family (SERCA), IP_3 receptors (IP_3R) and Ryanodine Receptors (RyR). The coordinated influx and efflux of Ca^{2+} via these channels allows spatio-temporal changes in $Ca^{2+}{}_i$ and modification of downstream signaling pathways. Almost simultaneous to ER store emptying, plasma membrane Store Operated Channels (SOC's) are activated to allow calcium to enter the cell to facilitate store refilling as well as provide Ca^{2+} for signaling process. This concerted cycle of emptying and refilling is essential to the process of Store Operated Calcium entry (SOCe), discussed in Section 1.7 and Fig 4.

1.2.1. Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA)

First discovered in muscle (Ebashi and Ebashi 1962), the Sacroplasmic/Endoplasmic Reticulum Calcium ATPase family is one of the most extensively studied membrane proteins. Multiple isoforms, in three groups, (SERCA1, 2 & 3) are generated by alternative splicing of three paralogous gene transcripts, with the ubiquitous 'housekeeping' SERCA2b and more specialized SERCA2a isoforms being most prevalent (Vandecaetsbeek, Vangheluwe et al. 2011). SERCA2b has a dual function in the ER membrane; to maintain a low cytoplasmic Ca^{2+} concentration 100 nM by translocating Ca^{2+} into the ER lumen, while simultaneously keeping the ER luminal Ca^{2+} concentration high 500 μM. During each catalytic event, SERCA transports two Ca^{2+} ions via two transport sites on the protein while counter transporting a single H+ for each ATP hydrolyzed, a unique feature of this subgroup, as other related calcium pumps (PMCA; SPCA) each only pump a single Ca^{2+} ion.

Many years have been devoted to understanding the functional mechanisms underlying SERCA activity, and early work used inhibitors to garner such information. Inhibition of SERCA induces passive ER store depletion by blocking Ca^{2+} reuptake from the cytoplasm. The use of irreversible inhibitors facilitates a state where SOCe is continually active, as Ca^{2+} entering the cell cannot be pumped into the ER. In fact, many of the early inhibitors are still in use today; so much so, they are important for enabling the understanding of numerous cellular signaling processes (including SOCe) by virtue of the fact they alter Ca^{2+} homeostasis by blocking SERCA activity. Common inhibitors include Thapsigargin (TG) (from roots of *Thapsia garginica*), Cyclopiazonic Acid (CPA) (produced by Penecillinum and Aspergillus) and 2,5-di-(tbutyl)-1,4-hydroquinone (DBHQ). TG is known as a tumor promoter and appears to have a specific, non-

competitive effect on SERCA over other closely related ATPase's (Thastrup, Cullen et al. 1990; Bokkala, el-Daher et al. 1995; Wootton and Michelangeli 2006). A synthetic pro-drug, G-202, related to TG, is currently being tested in clinical trials for cancer treatment by virtue of its ability to cause Ca^{2+} imbalance in prostate cancer cells, thus inducing apoptosis (National Cancer Institute).

For this study, TG was the primary SERCA inhibitor used. Its actions are irreversible, the result of TG locking the pump in an irreversible inactive state (Sagara, Fernandez-Belda et al. 1992). Thus, this useful attribute of TG is sufficient to maintain an ER constantly depleted of Ca^{2+}. While there is no doubt SERCA plays an integral role in SOCe, more recently it has become evident that it may be more closely involved with Stromal Interaction Molecule (STIM) and Orai plasma membrane Ca^{2+} channels than previously imagined, perhaps through close interactions with STIM (Sampieri, Zepeda et al. 2009; Alonso, Manjarrés et al. 2012)

1.2.2. IP_3 Receptors

IP_3 receptors, expressed in the majority of cells, are responsible for Ca^{2+} signals resulting from activation of Plasma Membrane (PM) receptors. There are three IP_3R subtypes in vertebrates often with a particular subtype having multiple splice variants (Regan, Lin et al. 2005). The receptors bind IP_3 at the IP_3 binding core (IBC) at the N-terminus (Bosanac, Alattia et al. 2002), with the pore of the channel towards the C-Terminus (Schug, da Fonseca et al. 2008).The binding of IP_3 results in conformational changes to the channel, thus gating the pore (Chan, Whitten et al. 2007; Rossi, Riley et al. 2009). IP_3R's are also regulated by cytosolic Ca^{2+}, evidenced by rapid stimulation and slow inhibition (Taylor and Laude 2002; Taylor, da Fonseca et al. 2004; Foskett, White et al. 2007). PLC hydrolysis of $PI(4,5)P_2$ to IP_3 and DAG provides the substrate to activate the ER membrane IP_3R, releasing Ca^{2+} from ER stores, triggering multiple events including rearrangement of Stromal Interaction Molecules (STIMs), and a key process for SOCe activation.

1.3. Protein Components of Store Operated Calcium Entry (SOCe)

First conceived by Jim Putney as capacitive calcium entry (Putney 1986), SOCe has only fairly recently been brought to the forefront with the discovery and more recent crystallization of its molecular components, *Stromal Interaction Molecule* (STIM) (Liou, Kim et al. 2005; Roos, DiGregorio et al. 2005; Yang, Jin et al. 2012) and Orai channel proteins (Feske, Gwack et al. 2006; Vig, Peinelt et al. 2006; Zhang, Yeromin et al. 2006; Hou, Pedi et al. 2012). SOCe involves a highly concerted sequence of events initiated by ligand interaction with G-Protein Coupled Receptors (GPCR) or Receptor Tyrosine Kinases and activation of the IP_3/DAG pathway, resulting in Ca^{2+} release from intracellular stores and subsequent activation of plasma membrane Orai channels. As perhaps the nomenclature suggests, the discovery STIM in stromal cells (Parker, Begley et al. 1996), and later their characterization as tumor suppressor molecules (Sabbioni, Veronese et al. 1999), predated any notion they were involved in SOCe. STIM proteins, type 1A single pass transmembrane proteins, are the ER Ca^{2+} store sensors, oligomerizing in response to depleted ER calcium levels. There are two vertebrate homologues, STIM1 and STIM2, as well as highly conserved homologues in multiple classes including mammals, lizards, fish and flies. The role of STIM proteins, and their physiological importance has been the subject of many reviews, most recently from the Gill Lab (Soboloff, Rothberg et al. 2012). It’s important to note that STIM has been implicated in sensing other physiological stresses, including temperature (Xiao, Coste et al. 2011), pH and hypoxia (reviewed in (Mancarella, Wang et al. 2011), and oxidative stress (Hawkins, Irrinki et al. 2010), aside from its Ca^{2+} sensing ability. Thus, STIM proteins appear to be involved in many diverse cellular functional pathways, some of which are only just coming to light.

STIM1 Luminal Domain

STIM1 Cytoplasmic Domain

318-322 EEELE

382-387 KIKKKR

SP cEF hEF SAM TM IH SOAR ID PS K

Coiled Coil

ER Lumen

Cytoplasm

SP – Signal Peptide
cEF – canonical EF Hand
hEF – hidden EF Hand
SAM – Sterile Alpha Motif
IH – Inhibitory Helix
SOAR – Amino Acids 344-442
ID – Inhibitory Domain
PS – Proline/Serine rich region
K – Lysine Rich region

Figure 1: Schematic representation of STIM1 protein domains

Simple schematic representation of STIM1 protein structure with many of the important domains utilized in the this work highlighted and described. Glutmates (E) in the 318-322 region were mutated to alanine (A) in a STIM1CT construct, rendering it constitutively active. Lysines (K) 384-386 were mutated to glutamine (Q) in STIM1-3KQ mutant, resulting in abolition of putative electrostatic interactions with C-Terminal acidic residues on Orai1.

1.4. STIM1

Predominantly found in the ER, but also located in the PM (Williams, Manji et al. 2001; Spassova, Soboloff et al. 2006; Hauser and Tsien 2007), STIM1, an ER calcium store sensor, is responsible for transducing the depleted Ca^{2+} store signal to PM Orai channels. STIM1 has been implicated as a sensor of pH, temperature and redox state of the cell (reviewed in (Soboloff, Rothberg et al. 2012). One of the two intraluminal EF-Hand domains, the canonical, cEF-Hand, binds Ca^{2+} when stores are full, the other EF-hand, known as the hidden, hEF-Hand, does not bind Ca^{2+}, but is still necessary for oligomerization of STIM1, (Liou, Kim et al. 2005; Spassova, Soboloff et al. 2006; Wu, Buchanan et al. 2006). Dissociation of Ca^{2+} from cEF-hand upon store depletion, results in destabilization and subsequent aggregation of STIM1, mediated by luminal Sterile Alpha Motif (SAM) and cytoplasmic coiled-coil domains (Stathopulos, Li et al. 2006; Stathopulos, Zheng et al. 2008; Stathopulos, Zheng et al. 2009). The cEF-hand sensitivity of STIM1 is in the range of K_d ~200 μM, with STIM2 much more sensitive to smaller Ca^{2+} fluctuations in ER levels (K_d ~500 μM) (Stathopulos, Li et al. 2006; Zheng, Stathopulos et al. 2008) . Three amino acids in STIM1 (A79, N80 & D82) are different in STIM2 at the cEF-Hand region, which account for the differences in Ca^{2+} affinity between the proteins (Brandman, Liou et al. 2007; Stathopulos, Zheng et al. 2008; Stathopulos, Zheng et al. 2009). These different Ca^{2+} affinities between STIM1 and STIM2 are important for spatiotemporal Ca^{2+} signaling within the cytoplasm. Differential activation of Receptor Tyrosine Kinases (RTK) and PLCγ can be slower than G-Protein Coupled Receptor (GPCR) activation of PLCβ (Kar, Bakowski et al. 2012), thus, with superficial RTK mediated Ca^{2+} release from the ER, STIM1 and STIM2 may be activated. This is in contrast to the speedy GPCR mediated Ca^{2+} release which

predominantly activates STIM1 to assist with recovering the rapidly depleting intracellular stores. Therefore, STIM2 is more likely to participate in maintaining ER Ca^{2+} homeostasis on a smaller scale, while STIM1 is responsible for large scale store refilling and other more pertinent cytoplasmic Ca^{2+} signals.

Intra-ER EF-hand mutation at aspartate^{76} (D76A), or complete removal of the EF-hand, reduces STIM1 Ca^{2+} sensitivity even further by destroying the Ca^{2+} binding ability of the EF-hand (Liou, Kim et al. 2005; Li, Lu et al. 2007). This renders STIM1 constitutively active and capable of binding with Orai independent of ER store content. Upon store depletion and STIM aggregation, STIM puncta become captured in ER-PM junctions at approximately 20-40nm from the PM (Liou, Kim et al. 2005; Luik, Wu et al. 2006; Wu, Buchanan et al. 2006; Liou, Fivaz et al. 2007; Luik, Wang et al. 2008). These aggregated STIM 'puncta' directly interact with plasma membrane Orai channel proteins to form functional, activated channels (Lewis 2007; Muik, Frischauf et al. 2008), discussed in more detail in section 1.7.

1.5. STIM2

Found in all vertebrates, including rats, mice, horses, chickens and cattle (Cai 2007), STIM2 (Liou, Kim et al. 2005; Roos, DiGregorio et al. 2005) is ubiquitously expressed across all human tissues, but at higher levels than STIM1 in dendritic (Bandyopadhyay, Pingle et al. 2011) and brain cells (Berna-Erro, Braun et al. 2009). Yet, STIM1 and STIM2 have many subtle, and important, differences in terms of their role within the SOCe pathway. The STIM1 and STIM2 amino acid sequences display ~61% homology, particularly in essential functional regions such as EF-hand, SAM, transmembrane domain and C-Terminal coiled coil regions. STIM2 has a longer C-terminus characterized by a highly variable region different from that seen in STIM1. Furthermore, evidence suggests the N-Terminal region of STIM confers different kinetics in the activation of Orai1 (Zhou, Mancarella et al. 2009). Despite similarities between STIM1 and STIM2, there is relatively little known about the latter. It has an inhibitory role over endogenous SOCe when over expressed in HEK293 cells (Soboloff, Spassova et al. 2006) and, upon store depletion mediates activation of Orai1 with kinetics different to that of STIM1 (Parvez, Beck et al. 2008; Zhou, Mancarella et al. 2009), suggesting that while STIM proteins can activate SOCe, both homologues act to tightly control each other and the level of calcium entry via Orai. Furthermore, STIM2, with its lower sensitivity for Ca^{2+} in the EF-hand, is active at higher ER Ca^{2+} levels and thus earlier than STIM1 in the SOCe initiation process. It has been postulated that perhaps STIM2 controls basal ER Ca^{2+} by being partially active at resting Ca^{2+} levels (Brandman, Liou et al. 2007), and can mediate store independent activation of Orai1. STIM2 knock down reduces basal ER and cytosolic Ca^{2+} levels (Brandman, Liou et al. 2007), further

indication of its role, with its apparent lower activation threshold, as a tight modulator of resting cellular Ca^{2+} content.

1.6. Orai– 'Keepers of heaven's Gate'

Orai proteins, the pore forming subunits for the Calcium Release Activated Calcium Current (I_{CRAC}), were discovered by a similar means to STIM proteins – whole-genome screen of Drosophila S2 cells (Feske, Gwack et al. 2006; Vig, Peinelt et al. 2006; Zhang, Yeromin et al. 2006), combined with gene mapping of a small cohort of individuals who suffered immunodeficiency due to a loss of functional I_{CRAC} (Feske, Gwack et al. 2006). This loss of function was encoded genetically by a single amino acid substitution Arginine^{91} to Tryptophan (R91W) in the first transmembrane domain of the Orai1 channel (Feske, Gwack et al. 2006). Other groups were able to confirm the screening reports by over-expressing STIM and Orai in heterologous systems which upon store depletion recapitulated currents with pharmacological and biophysical characteristics of I_{CRAC}, albeit on a larger scale (50x bigger currents) (Mercer, Dehaven et al. 2006; Peinelt, Vig et al. 2006; Soboloff, Spassova et al. 2006). Thus, the molecular identity of capacitative calcium entry was complete, with multiple isoforms of STIM (STIM1 & STIM2) and three Orai proteins (Orai1, Orai2 & Orai) identified (Feske, Gwack et al. 2006). Also known as CRACM1 (Vig, Peinelt et al. 2006), Orai1 appears to be the primary channel subtype involved in the SOCe pathway, either alone as homomultimers, or via heteromeric interactions with the other Orai subtypes. All three Orai subtypes are activated via interaction with STIM in response to store depletion, yet Orai2 and Orai3 have smaller current amplitudes, possibly reflecting differences in expression profiles and channel characteristics (Mercer, Dehaven et al. 2006; Lis, Peinelt et al. 2007). Orai proteins appear to be expressed at similar mRNA and protein levels (Gross, Wissenbach et al. 2007; Gwack, Srikanth et al. 2007).

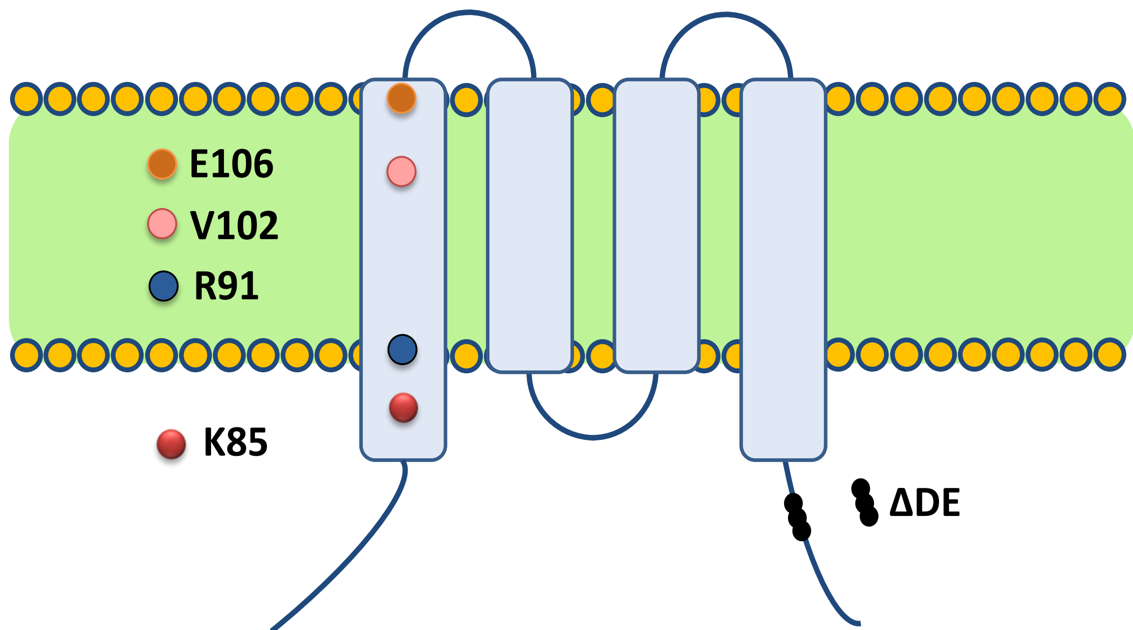


Figure 2: Schematic model of the membrane topology of a single Orai1 subunit

Simple model of Orai protein in plasma membrane. Depicted are 4 transmembrane domains as well as highlighted residues, mutations at which either confer loss of function of the channel (K85, R91, E106, ΔDE) or gain of function (V102). K85 is important for gating of Orai1, while 3 aspartate and 3 glutamate residues (indicated by ΔDE) in Orai1, are involved in STIM1 interactions with Orai1. Mutations at all residues except R91 and E106 were used in this work.

Furthermore, only Orai3 shows robust, fast Ca^{2+}-dependent inactivation (Orai1 and Orai2 – moderate), yet slow Ca^{2+} dependent inactivation is only evident for Orai1 (Zweifach and Lewis 1995; Lis, Peinelt et al. 2007). Inactivation is evident in current traces as a sharp reduction (fast inactivation) in current, followed by a leveling off of the reduction in current (slow inactivation). There are a number of key residues in the Orai1 channel, important for the high selectivity of the channel for Ca^{2+}, for fast and slow inactivation (Fig 3.), and also for STIM interactions, both at the N- and C-termini (Fig. 2&3).

A series of acidic residues at the distal end (extracellular) of TM1, in the TM1-TM2 extracellular loop, and one in TM3, form the calcium selectivity filter (Fig. 3), with Glutamate^{106} (E106) (Fig. 2) absolutely essential for ion conduction. A conserved mutation of E106 to Asparate (D) causes a loss of high selectivity for calcium, resulting instead in the flux of monovalent ions across the pore (Prakriya, Feske et al. 2006; Vig, Beck et al. 2006; Yeromin, Zhang et al. 2006). Mutation of E106 to the non charged, hydrophobic residue Alanine (E160A), or polar Glutamine (E106Q), results in a pore dead mutant completely incapable of conducting ions of any nature across the pore of the Orai1 channel (Prakriya, Feske et al. 2006; Vig, Beck et al. 2006; Gwack, Srikanth et al. 2007). Furthermore, the dominant negative effect of Orai1 E106Q is also prevalent over Orai2 and Orai3, supporting the idea of heteromultimeric channels (Vig, Beck et al. 2006; Lis, Peinelt et al. 2007), which likely form a hexameric assembly in the plasma membrane, given the Drosophila Orai1 crystal structure is hexameric (Hou, Pedi et al. 2012).

In addition to E106, three aspartate residues in the TM1-TM2 loop (D110/112/114) further contribute to the ion selectivity. D110/112A mutations result in increased permeation of monovalent ions (Vig, Beck et al. 2006; Yamashita, Navarro-Borelly et al. 2007), particularly Cs^{+}, a likely indication of a widening of the entrance to

the pore to accommodate this large ion. Furthermore, these mutations reduce the moderate, fast calcium-dependent inactivation of Orai1, an essential feedback mechanism to control channel function (Hoth and Penner 1993; Zweifach and Lewis 1995; Fierro and Parekh 1999). In addition to fast inactivation, calcium entering through Orai1 also plays a role in slow calcium-dependent inactivation, a process likely also mediated by store refilling, even in the presence of TG (Zweifach and Lewis 1995).

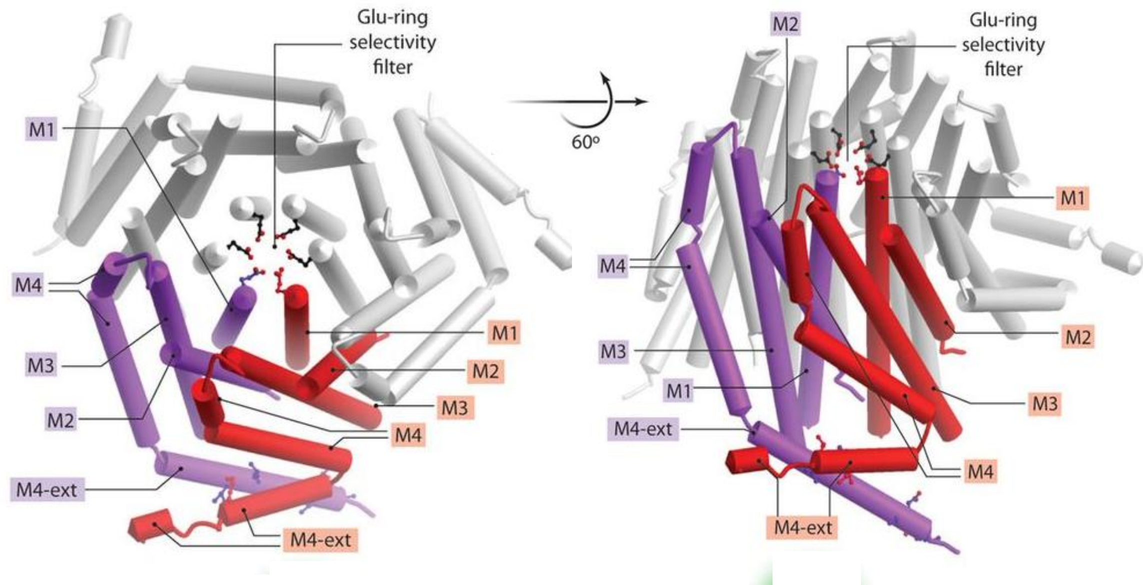


Figure 3: Model of Orai1 Channel complex

Model of Drosphila Orai1 crystal structure adapted from Rothberg, Wang et al. 2012, and based on Protein Data Bank entry: #4HKS). Depicted are 6 subunits with M1 forming the pore lining interface of the channel, the sidechains forming the glutamate ring of the selectivity filter at the top of the pore and an essential Leucine in M4, required to maintain Orai1 C-terminal integrity.

1.7. Key Features of STIM activation of Orai.

SOCe through CRAC channels, the primary source of Ca^{2+} signals in T cells, is the result of $PI(4,5)P_2$ hydrolysis by PLC, IP_3 activation of IP_3R and depletion of ER stores. Upon activation of store depletion, either naturally (cell surface receptors) or pharmacologically (by TG, Ionomycin), Ca^{2+} fluxes from the ER into the cytoplasm reducing the intra-ER Ca^{2+} concentration. Ca^{2+} is cleared from the cell by the Plasma Membrane Calcium ATPase (PMCA), as well as some pumped back into ER in the absence of TG, thus maintaining the low cytoplasmic Ca^{2+} concentration, while the remaining Ca^{2+} participates in cellular signaling processes. The ER luminal decrease in Ca^{2+} is sensed by the EF-Hand domains of the STIM proteins (STIM2 then STIM1), resulting in dissociation of Ca^{2+} from the EF-Hand, allowing STIMs to oligomerize to multimers. The STIM1 SAM domains within the ER (Zheng, Stathopulos et al. 2011), the cytoplasmic coiled-coil domains in the cytoplasm play a role in STIM1 oligomerization. Activated STIM move to areas of the ER closely associated with the PM – the ER-PM junctions, where they interact with Orai oligomers, forming puncta, tethering Orai channels and activating SOCe. The Ca^{2+} entering the cells is used for a variety of purposes, including signaling cascades, as well as to refill the ER stores.

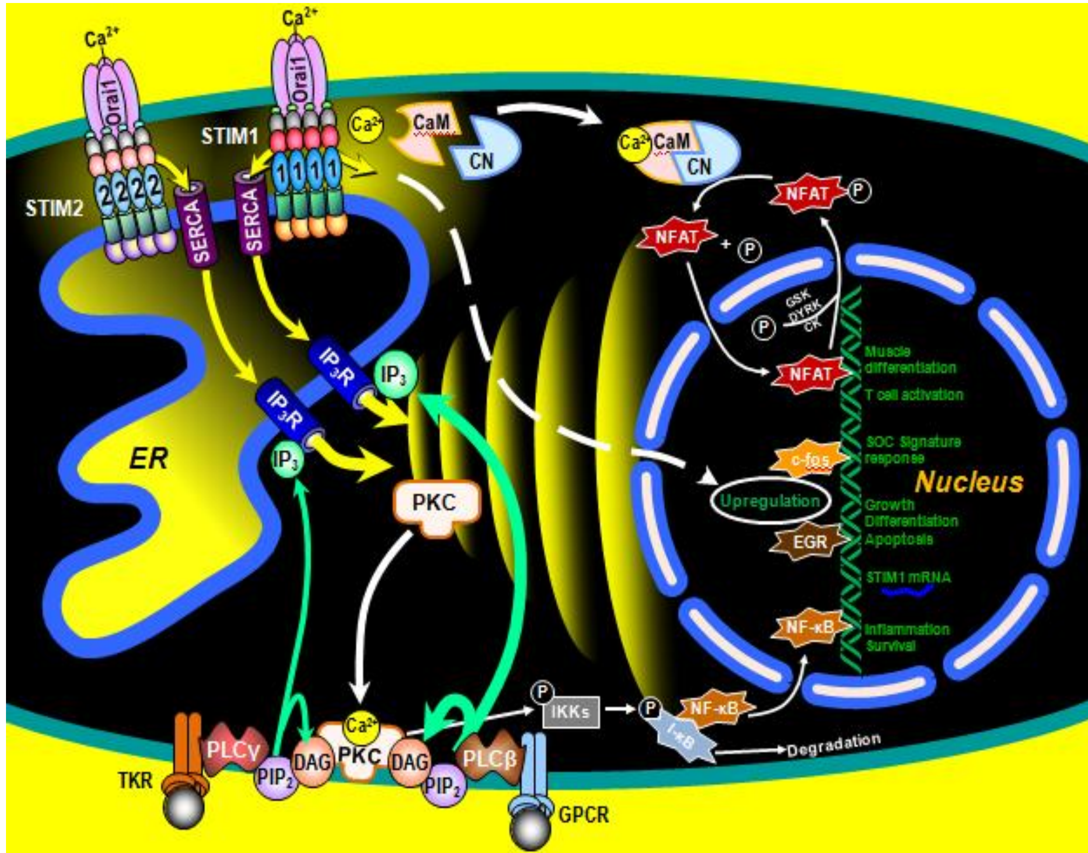


Figure 4: Schematic diagram of events associated with Store Operated Ca2+ Entry in a typical cell

Schematic depicting events which initiate SOCe and the downstream pathways affected by ER Ca^{2+} release and extracellular Ca^{2+} entry events associated with SOCe. The process is described in detail in chapter 1.7. Figure reproduced with permission from Dr Donald L. Gill.

1.8. SOCe and Disease – The significance of this research

One of the most prolific disease states resulting from loss of functional SOCe helped investigators solve the conundrum of the proteins involved in the process. A rare mutation in the Orai1 gene, Arginine^{91} to Tryptophan (R91W) which manifest itself in the form of Severe Combined Immunodeficiency Disease (SCID) in a small cohort of individuals, enabling the discovery of Orai1 as the channel responsible for SOCe (Feske, Gwack et al. 2006). These patients were deficient in several pathways important for T-Cell function, including decreased SOCe and immune response, and reduced Nuclear Factor of Activated T-Cell (NFAT) mediated gene transcription (Feske, Giltnane et al. 2001; Feske, Prakriya et al. 2005; Feske, Gwack et al. 2006).

T cells are not alone in being affected by the SOCe pathway. Immune cell types, including B cells (Limnander, Depeille et al. 2011; Matsumoto, Fujii et al. 2011), Natural Killer (NK) cells, macrophages/monocytes and neutrophils, SOCe, and consequently loss of SOCe, also affects their immune function. Orai1 knockout mice show decreased B cell proliferation and cytokine production (Gwack, Srikanth et al. 2008). Furthermore, Orai1 deficient mice display defective mast cells (Vig, Dehaven et al. 2007), with STIM1 also important in mast cell activation (Baba, Nishida et al. 2008), an indication of the role of SOCe in allergic responses, and presenting putative targets to control such conditions.

Immunodeficiency's due to amino acid mutations in STIM1 and Orai1 are quite rare, with the R91W being the most famous. There are some frame shift missense mutations which result in abolition of STIM protein expression, rather than synthesis of non-functional proteins (Feske, Picard et al. 2010). Logically, with no STIM proteins, there is no SOCe in those cells. Interestingly, lack of expression of STIM can also have

protective effects, in the case of mice without STIM1 or STIM2, which appear to be protected against development of multiple sclerosis (Ma, McCarl et al. 2010).

In addition to immune cell function, it has become apparent that SOCe is an important mediator of the function of many other cell types and pathophysiological conditions. Thus, SOCe plays a role in the vasculature (Trebak 2012) and the cardiovascular system (Hulot, Fauconnier et al. 2011), platelets (Braun, Varga-Szabo et al. 2008; Varga-Szabo, Braun et al. 2011; Braun, Vogtle et al. 2012), and some brain tissue (Klejman, Gruszczynska-Biegala et al. 2009), as well as inflammatory diseases such as psoriasis, rheumatoid arthritis and inflammatory bowel disease (Huang, Hoebe et al. 2008; Dellis, Mercier et al. 2011) and breast cancer (Motiani, Abdullaev et al. 2010; Faouzi, Hague et al. 2011) (reviewed in detail, (Soboloff, Rothberg et al. 2012)).

It is apparent that defects in calcium entry/homeostasis in innate and adaptive immune cell types play a multifaceted role in pathophysiological phenotypes in individuals where SOCe is deficient. Interestingly, most of the phenotypes presented were caused by aberrant function of STIM or Orai, an indication that specific targeting of these proteins with pharmacological agents may be a useful tool in the fight against disease.

Therefore, mechanistic understanding of SOCe modifiers, either excitatory or inhibitory, will provide important data about potential targets for treatment of autoimmune diseases, cancer vascularization, progression and metastasis, thrombosis and many other diseases dependent on calcium homeostasis.

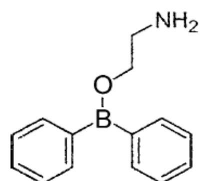
1.9. Pharmacology of SOCe

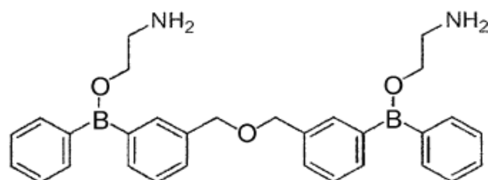
Pharmacological modification of SOCe has the potential to provide relief to patients with aberrant intracellular calcium signaling pathways, particularly in immune cells. SOCe is critically important for immune cell functions including mast cell degranulation as well as cytolytic T cell clearance of infected targets. Furthermore, SOCe is required for longer term responses initiated by B cells (including their differentiation) and T cells (cytokine production). Many of these functions are regulated by NFAT upon its dephosphorylation by calmodulin-calcineurin complex in response to intracellular calcium concentration increases via SOCe (Fig. 4) (reviewed in (Hogan, Lewis et al. 2010).

Thus, since the discovery of SOCe and its constituent proteins, there has been a great deal of research and development into discovering highly potent modifiers of I_{CRAC} and SOCe (Zhou, Iwasaki et al. 2007; Derler, Fritsch et al. 2008; Sweeney, Minatti et al. 2009; Goto, Suzuki et al. 2010; Suzuki, Ozaki et al. 2010). Many pharmaceutical companies, including Abbot Labs, Astellas Pharma, Boheringer Ingelheim, Synta Pharmaceuticals & GlaxoSmithKlein as well as some independent labs, have proposed structures for molecules which inhibit SOCs, yet specificity for STIM/Orai has remained elusive (Sweeney, Minatti et al. 2009). While SOCe inhibitor discovery has been quite extensive, I will focus my attention to compounds most relevant to this work, i.e. those synthesized from 2-Aminoethoxydiphenyl borate (2-APB).

1.10. 2-Aminoethoxydiphenyl borate - 2-APB

2-APB (Fig. 5, top) is a boron containing compound with non-specific effects on multiple ion channels. 2-APB inhibits gap junctions (Tao and Harris 2007), though it is probably most famous for its inhibitory action on IP_3 receptors (Maruyama, Kanaji et al. 1997; Diver, Sage et al. 2001), some Transient Receptor Potential Canonical (TRPC) channels (TRPC3 and TRPC6) (Bootman, Collins et al. 2002; Lievremont, Bird et al. 2005; Xu, Zeng et al. 2005), its activation of TRP Vallinoid (TRPV) channels (Chung, Lee et al. 2004; Hu, Gu et al. 2004), inhibition of SERCA pumps (k_d ~200 μM) (Missiaen, Callewaert et al. 2001; Bilmen, Wootton et al. 2002; Peppiatt, Collins et al. 2003), and its transient potentiation and subsequent rapid inhibition of Orai subtypes (Prakriya and Lewis 2001; Ma, Venkatachalam et al. 2002; Dehaven, Smyth et al. 2008; Peinelt, Lis et al. 2008; Schindl, Bergsmann et al. 2008). Evidence suggests the Boron-Oxygen Core (BOC) of the molecule is absolutely responsible for SOCe potentiation (Dobrydneva and Blackmore 2001; Dellis, Mercier et al. 2011). Furthermore, the number of phenyl rings is directly related to the potentiation/inhibition capacity of 2-APB (Dellis, Mercier et al. 2011). Bisboron containing 2-APB molecules have ~20-45 fold increase in inhibitory capacity (Suzuki, Ozaki et al. 2010), thus, further development of drugs using this structural idea resulted in the Dimeric 2-APB analogs, DPB162-AE and DPB163-AE (Fig. 5) at the center of this work (Goto, Suzuki et al. 2010). 2-APB has been particularly useful in delineating some of the more subtle aspects of SOCe and as such has been used as a tool to help define the mechanism of action of STIM-Orai interactions (Lis, Peinelt et al. 2007; Peinelt, Lis et al. 2008; Schindl, Bergsmann et al. 2008; Wang, Deng et al. 2009), as well as define interacting protein partners of STIM1 (Wang, Deng et al. 2010).





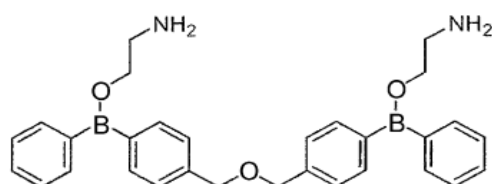


Figure 5: 2-APB, DPB162-AE and DPB163-AE

Adapted from Goto *et al*. 2010*,* representative images of 2-APB, DPB162-AE and DPB163-AE. Note, the only difference between the DPB's is the position of the linker.

1.11. DPB162-AE and DPB163-AE

Early screens of 2-APB analog libraries revealed the importance of the dimeric configuration of the compound (Dobrydneva and Blackmore 2001; Zhou, Iwasaki et al. 2007; Goto, Suzuki et al. 2010). Thus, Mikoshiba's group set about synthesizing dimeric 2-APB analogs which were stable (Suzuki, Ozaki et al. 2010), and highly specific for SOCe, resulting in the formulation and analysis of DPB162-AE and DPB163-AE (Goto, Suzuki et al. 2010) (Fig. 5). Initial work with these compounds indicated different inhibitory profiles in different cell types, possibly due to a degree of specificity among STIM and Orai subtypes as well as protein expression across different cells. It appeared that different Orai subtypes responded differently to DPB162-AE and DPB163-AE. In terms of efficacy, both analogs were inhibitory at concentrations approximately 50-100 times less than 2-APB (Chinese Hamster Ovary (CHO) cells - 2-APB IC_{50}= 2.9±0.1 μM; DPB162-AE IC_{50}=190±6 nM; DPB163-AE IC_{50}= 210±20 nM), depending on cell type assayed. Interestingly, DPB163-AE was also found to have a potentiation effect at concentrations lower than the inhibitory concentration (HeLa Cells (immortalized cervical cancer cell line) - EC_{50}=30±6.8 nM; no potentiation evident in CHO cells), making it a similar compound to 2-APB in terms of its biphasic activity profile. This potentiation was suggested to be dependent on relatively higher expression levels of STIM2 vs. STIM1. Furthermore, DPB162-AE disrupted over-expressed STIM1 clustering. However, this effect may have been an artifact due to lack of over expressed Orai1 in that assay (Goto, Suzuki et al. 2010). Armed with a bevy of cellular and protein 'tools' as well as the two DPB compounds, I set about defining the specificity DPB162-AE and DPB163-AE for STIM and Orai proteins, with the hypothesis:

Inhibition by DPB162-AE and DPB163-AE is highly specific for STIM1-Orai1-mediated SOCE, while SOCe potentiation by DPB163-AE is mediated exclusively via STIM2

CHAPTER 2

MATERIALS AND METHODS

2.1 Imaging Buffers

A standard, nominally calcium free, 4x imaging buffer was prepared periodically, from which 1x buffer was used for all imaging experiments.

4x Stock imaging buffer:

12.5g NaCl; 1.07g KCl; 0.49g $MgCl_2.6H_2O$; 4.15g Glucose; 9.53g Hepes, 500ml H_2O; final pH 7.2. (Final concentrations of buffer salts as follows: 107 mM NaCl, 7.2 mM KCl, 1.2 mM $MgCl_2$, 11.5 mM glucose, 20 mM Hepes-NaOH, pH 7.4)

For 1x buffer, 12.5ml 4x stock was diluted with ddH_2O to 50ml on the day of the experiment. Addition of calcium (50µl 1M $CaCl_2$ to 50ml 1x buffer) resulted in 1 mM calcium-containing imaging buffer. The highest concentration of calcium-containing imaging buffer used was 3 mM final concentration calcium (150µl 1M $CaCl_2$ in 50ml calcium free buffer) for DT40 cells.

2.2 Tissue culture and plating of cells for imaging experiments

2.2.1 Human Embryonic Kidney (HEK293) Cells

HEK293 cells stably over-expressing STIM and Orai were grown to ~90% confluency at 37°C in 5% CO_2, on 100 mm tissue culture dishes in DMEM supplemented with Fetal Bovine Serum (FBS) (10% v/v), Pen/Strep antibiotics (1% v/v), as well as antibiotics for selection of the proteins of interest; G418 for Orai, and Puromycin for STIM in cells over-expressing those proteins. The STIM and Orai plasmids contained resistance cassettes for the respective antibiotics, therefore cells expressing the plasmid also had resistance to the antibiotic, and were positively selected for their expression. 24 hours before experiments, cells were trypsinized and plated on circular 35mm glass coverslips in 6 well dishes, containing 2ml of supplemented media (previously described) to maintain positive selection.

2.2.2 RBL-2H3 cells

RBL-2H3 cells were cultured as HEK cells except DMEM did not contain the positive selection markers G418 and Puromycin.

2.2.3 DT40 and Jurkat cells

DT40 and Jurkat suspension cells were grown in RPMI media supplemented with FBS 10% (v/v), and Penicillin/Streptomycin 1% (v/v). When at ~90% confluency, 500µl of cells was pipetted on to glass coverslips coated with 0.01% poly-*l*-lysine to ensure

attachment of the cells to coverslips for downstream Ca^{2+} imaging experiments where regular solution changes occurred.

2.3 Cell Transfection

2.3.1 Transfection of HEK cells

For some experiments it was necessary to transfect plasmids encoding mutant or truncated STIM or Orai. Thus, for each plasmid to be transfected (as indicated in Chapter 3), the following protocol was used:

Cells were grown to ~90% confluency and trypsinized as described previously. Trypsin activity was neutralized by addition of 10ml FBS supplemented DMEM, and cells were pipetted to a 15ml conical tube for centrifugation at 3000rpm for 5 minutes. The resulting cell pellet was resuspended in 4ml OPTI-MEM media containing Penicillin/Streptomycin (1% v/v) but *without FBS*. A 650µl volume of resuspended cells was transferred to a 4mm gap electroporation cuvette, to which ~10µg of the plasmid DNA of interest was added. The cuvette was capped, gently vortexed to mix the plasmid DNA and cells effectively, then electroporated under the following conditions on a Biorad Gene Pulser xCell electroportation machine:

Voltage: 180V
Pulse Length: 25ms
# of pulses : 1

Following the electroportation protocol, cells were plated on 35mm round glass coverslips in 6 well dishes; 100µl of cells from the electroporation cuvette per coverslip. Thus, one cuvette was sufficient to provide cells at sufficient density for 6 coverslips. After plating, a further 100µl of OPTI-MEM with 1% (v/v) Pen/Strep media was added

to each coverslip. Transfected cells were left at 37°C for approximately 2 hours to allow the cells to settle on the coverslips and to recover from the electroportation event. After this time, 2ml of OPTI-MEM with 1% (v/v) Penicillin and Streptomycin and 10% (v/v) FBS, were added to each well of the 6 well dish, and the cells incubated overnight at 37°C in 5% CO_2**.** Experiments were performed within 24 hours of transfection.

2.3.2 Orai3-CFP transfection of DT40 cells

DT40 cells were cultured as previously described and collected by centrifugation at 3000rpm in 15ml tubes. The pellet was resuspended and aliquoted to electroporation tubes as described for HEK cells. As DT40 cells are difficult to transfect, it was necessary to use a high concentration of DNA to ensure enough cells that survived the transfection procedure resulted in expression of the plasmid. Thus, 50µg of Orai3-CFP plasmid DNA per transfection cuvette was used for each transfection. The following electroporation protocol was employed for DT40 cells:

Voltage: 210V

Capacitance: 200µF

Resistance: ∞Ω

Following electroportation, cells were plated in 100mm tissue culture dishes as for HEK cells. 24 hours after transfection, cells were centrifuged and resuspended in 1.2 ml of OPTI-MEM media with 1% (v/v) Penicillin and Streptomycin and 10% (v/v) FBS. 200µl of the resuspended cells were plated on poly-*l*-lysine coated coverslips in 6 well dishes and allowed to settle for 30

minutes at 37°C. After this time, transfected DT40 cells were loaded with Fura-2 as described next.

2.4 Fura-2 ratiometric imaging

50μg of membrane permeable Fura-2-AM (Life Technologies) was dissolved in 25μl DMSO, to give a final concentration of 2 μg/μl. 12μl of this Fura-2-AM stock was dissolved in 12 ml 1 mM calcium imaging buffer (or 300 μM buffer for cells expressing constitutively active proteins – STIM1D76A, Orai1V102C, SOAR1). 6 well dishes containing coverslip plated cells were removed from 37°C incubator and the culture media aspirated off. 2ml of imaging buffer containing Fura-2-AM was added to each well of the 6 well dish and left for 30 minutes at room temperature in the dark. After 30 minutes, the buffer was aspirated off and replaced with fresh buffer containing no Fura-2-AM, to allow for deesterification and uniform distribution of Fura-2 inside the cells. After 1 hour of loading and deesterification, experiments commenced. Calcium entry measurements were performed on a Leica DMI 6000B fluorescence microscope controlled by Slidebook Software (Intelligent Imaging Innovations). Fura-2 was excited at 340nm and 380nm and intracellular calcium concentrations are represented by the ratio of the fluorescence intensities measured at 340nm and 380nm from groups of single cells. Measurement traces show mean ± SEM from groups of 10-50 cells. Each graph is representative of at least 3 individual and independent repeats.

2.5 Electrophysiology protocol and solutions

Whole cell current recordings were collected and analyzed using HEKA Pulsetools, Patchmaster, Fitmaster, and Origin software. Automatic capacitive and series resistance compensation was achieved with HEKA EPC10 amplifier. After establishing a whole-cell current, a voltage ramp was applied as follows: from 0V holding potential, a 50ms step to -100mV, followed by a 50ms voltage ramp from -100 to +100mV, every 2 seconds.

Solutions used to measure various currents are described below:

2.5.1 Extracellular solution

The extracellular solutions contained (mM): 145 NaCl, 10 $CaCl_2$, 10 CsCl, 2 $MgCl_2$, 2.8 KCl, 10 HEPES, 10 glucose, pH 7.4. A 10mV junction potential compensation was applied.

2.5.2 CRAC Current recording solution

Intracellular solutions (mM):

145 CsGlu, 10 HEPES, 8 NaCl, 6 $MgCl_2$, 2 MgATP (total 8 mM Mg^{2+}), 0.03 IP_3, 10 EGTA (or BAPTA where specified), pH 7.2. TRPM7 activity was suppressed by the presence of 8 mM Mg^{2+} and ATP

2.6 Förster Resonance Energy Transfer (FRET)

All experiments were performed in HEK cells stably expressing Orai1-CFP and STIM1-YFP (Wildtype or mutated as indicated in figures), or stably expressing Orai1-CFP and transfected with YFP-tagged truncation construct as indicated in the figures.

Fluorescence was recorded on a Leica DMI6000B microscope running Slidebook software for image analysis, and equipped with filters for CFP (436Ex/480Em), YFP (500Ex/535Em)), $FRET_{raw}$ ($438_{Ex}/542_{Em}$).

Images were obtained every 20 seconds at room temperature with a 40x oil objective lens (N.A.1.35; Leica). CFP, YFP and FRET were recorded and three-channel corrected FRET was calculated as follows:

$FRET_c = F_{raw} - F_d/D_d * F_{CFP} - F_a/D_a * F_{YFP}$

$FRET_c$ is the total corrected energy transfer

F_d/D_d is the measured bleed-through of CFP through the YFP filter (0.447)

F_a/D_a the measured bleed-through of YFP through the CFP filter (0.049)

F_{raw} is fluorescence through the CFP/YFP filter cube

F_{CFP} is measured CFP fluorescence

F_{YFP} is measured YFP fluorescence

FRET data shown is representative of at least three independent experiments, carried out on separate days.

2.7 Hamamatsu µCell 384 well plate reader

DT40 cells were cultured as previously described. On the day of experiment, prior to plating, cells were loaded, in 15ml tubes, with Fluo-4 (as described previously for Fura-2) while in suspension. Following Fluo-4 loading, cells were counted on Biorad Cell Counter and resuspended in 3 mM calcium imaging buffer, to provide a final cell density 55,000 cells per well on the 384 well plate. 20µl resuspended cells were then plated on each well of 384 well poly-*l*-lysine coated plate. The plate was centrifuged briefly (5 minutes @3000RPM) to assist with settling the cells on the bottom of the dish. Plates were then transferred to the Hamamatsu µCell machine for commencement of the experiment.

2.7.1 Preparation of compound addition plates

For each experiment, two compound addition plates were prepared for automatic loading and solution change by the imaging system. The first plate contained all applicable drugs (TG; DPBs) at 6x desired final concentration (12 µM), 20µl per well. The automatic solution change added 4µl of each solution to the cell plate, thus establishing the final concentration of 2 µM for TG and 2 µM for DPBs (4µl of 12 µM into 20µl = 24µl of 2 µM TG and DPB).

For the second compound addition plate, 3 mM calcium imaging buffer was added at 5x. Thus, 20µl of 15 mM calcium imaging buffer was deposited in each well (384) of the second compound addition plate. Upon automatic addition to the recording chambers, 6µl of 15 mM calcium imaging buffer was added to 24µl already in the well, resulting in a 5x dilution. Thus, 6µl of 15 mM diluted into 24µl = 30µl of 3 mM calcium.

2.8 Generation of DT40 knockout cells

Highly replicative DT40 chicken (*Gallus gallus*) B cells present an opportunity to easily generate gene knockouts (Baba, Hayashi et al. 2006) in cells which are functionally relevant and can be used to study SOCe. The chicken B cells have a very high level of homologous recombination enabling easy knock-in or knockout of genes. Furthermore, the chicken genome contains only two Orai genes, Orai1 and Orai2, further simplifying the system. The Kurosaki lab kindly provided DT40 cell lines with specific STIM or Orai genes knocked out. Gene knock out (KO) was achieved using either a Bleomycin (*bleo)* or Blasticidin S *(bsr)* cassette inserted in to exon 1 of the gene of interest. Bleomycin induces DNA breaks and Blasticidin S blocks peptide bond formation. Thus, when cells are tranfected with plasmids for a STIM or Orai subtype containing either resistance cassette in exon one of the gene of interest, the new gene recombined into the chicken genome and was selected for with the resistance marker *bleo* or *bsr*, providing cells which are deficient in the gene of interest, in this case, Orai1 or Orai2. An identical process was involved in construction of the STIM specific knock out cells (STIM1KO or STIM2KO) utilized later in this report, (Baba, Hayashi et al. 2006). The end result is a clean background on which to study the gene not knocked out. Double knockout (DKO) cells for STIM's or Orai's were generated by transfecting two plasmids for genes containing *bleo* or *bsr* into DT40 cells and selecting cells with resistance to both cassettes, i.e. those containing neither functional gene of interest, resulting in STIM1-STIM2 DKO cells or Orai1-Orai2 DKO cells

2.9 Preparation of glass coverslips for imaging experiments

35mm glass coverslips were washed in 100% ethanol and allowed to air dry under UV light in tissue culture hood. After 1 hour, coverslips were removed and stored aseptically.

2.9.1 Poly-*l*-lysine coating of coverslips

It was necessary to coat coverslips for use with cells grown in suspension as the positive charge from the lysine provides a substrate to which the negative charge of the plasma membrane of the cells can 'attach' via electrostatic attraction.

200µl of 0.01% poly-*l*-lysine was added to the center of coverslips and spread around with a pipette tip to increase the coverage area. Coverslips were then placed in 37°C incubator for one hour. After this time, excess solution was aspirated off and the coated coverslips were stored aseptically at room temperature prior to attachment of cells.

CHAPTER 3

RESULTS

FUNCTIONAL ANALYSIS OF DPB'S ON CALCIUM PERMEANT CHANNELS, AND ENDOGENOUS SOCE IN HEMATOPOIETIC CELL LINES

3.1 Calcium Permeant Channels - TRPC3 TRPC6 and $Cav_{1.2}$

To test the hypothesis that DPBs are highly specific for Orai subtypes, I first analyzed their effects on other Ca^{2+} permeant channels (Fig. 6), utilizing HEK293 cells stably expressing $Cav_{1.2}$, TRPC3 or TRPC6. After loading of cells with Fura-2, Ca^{2+} entry through TRPC3 or TRPC6, was not inhibited by DPB162-AE or DPB163-AE 2 μM (Fig 6a, b) following channel activation by 1-oleoyl-2-acetyl-sn-glycerol (OAG), a membrane-permeable DAG analog. This was in contrast to a strong inhibitory effect of 2-APB 50 μM on Ca^{2+} entry though the same channels (Figs. 2&3). To analyze DPB action on $CaV_{1.2}$, I assessed activation of strontium entry through the voltage gated calcium channel, $Cav_{1.2}$, known to be reciprocally modulated by STIM1 (Park, Shcheglovitov et al. 2010; Wang, Deng et al. 2010). SOCs are highly selective for calcium over strontium, thus, the passage of strontium was used to distinguish $Cav_{1.2}$ over Orai channels in HEK293 cells. Strontium entry through $Cav_{1.2}$ was not affected by DPB162-AE, or DPB163-AE (fig 6c), a strong indication DPBs are specific for the widely established STIM and Orai SOCe machinery. As expected, the $Cav_{1.2}$ channel blocker

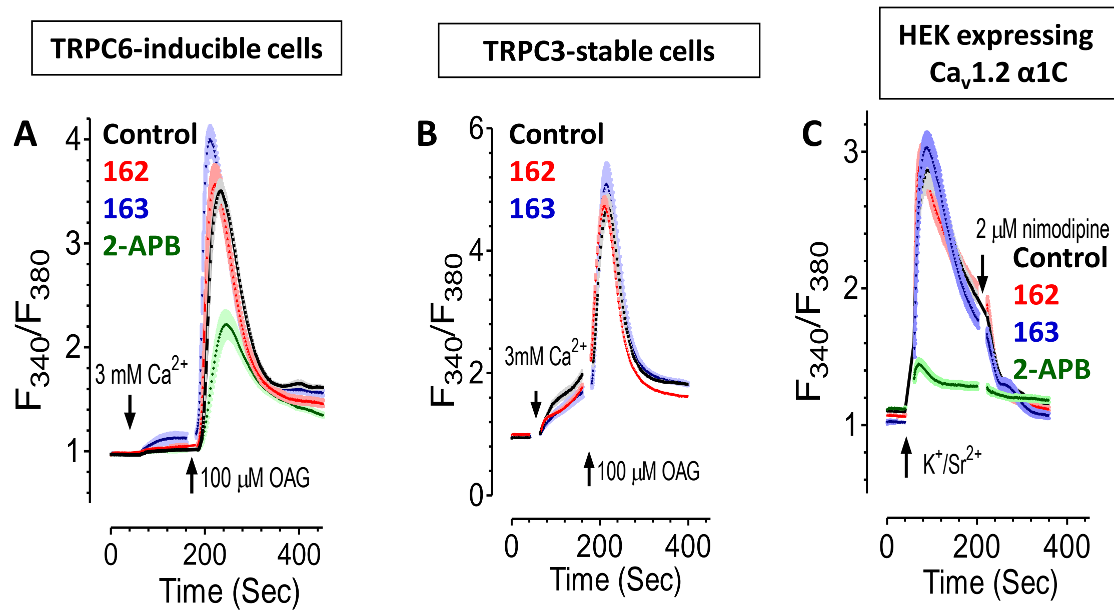


Figure 6: The action of DPB's on non-CRAC channels

A) TRPC3 and B) TRPC6 channel activity was induced with OAG, while DPB's were present. Upon addition of calcium to the recording chamber, neither analog inhibited calcium entry through these channels. C) Activation of $Cav_{1.2}$ by high potassium also resulted in a lack of inhibition of strontium entry through the $Cav_{1.2}$ channel. (Black traces – no drug; red traces – DPB162-AE; Blue Traces – DPB163-AE, all 2 μM; green traces – 2-APB 50 μM.

nimpodipine 2 μM actively inhibited $Cav_{1.2}$, indicating no competition of DPB's with established inhibitory sites on $Cav_{1.2}$ channels.

3.2 Hematopoietic cell lines – RBL-2H3, Jurkat and CEM3.71 cells.

Goto *et al.* reported differential expression of STIM and Orai proteins in different cell types could account for the differences they saw in the inhibitory profile of the DPB analogs (Goto, Suzuki et al. 2010). In line with this observation, I analyzed the effects of DPB's on endogenous SOCe in three hematopoietic cell lines, RBL-2H3 (Rat Basophilic Leukemia cells), Jurkat (T cells) and CEM3.71 (a CD3+ clone of human T lymphoblast-like cell line) (Fig. 7). In all cell types, TG induced endogenous SOCe was robustly inhibited when either DPB162-AE or DPB163-AE was present from the start of the experiment (T=0), indicating SOCe in all three cell lines is likely mediated by STIM1 and Orai1.

Given a clear lack of effect on ion channels known to permeate Ca^{2+} which are not involved in the SOCe pathway, I focused my attention on SOCe proteins, utilizing a unique combination of knock out cells and molecular mutations/deletions to STIM and/or Orai proteins in an effort to resolve the role of DPB's on inhibition/potentiation of SOCe.

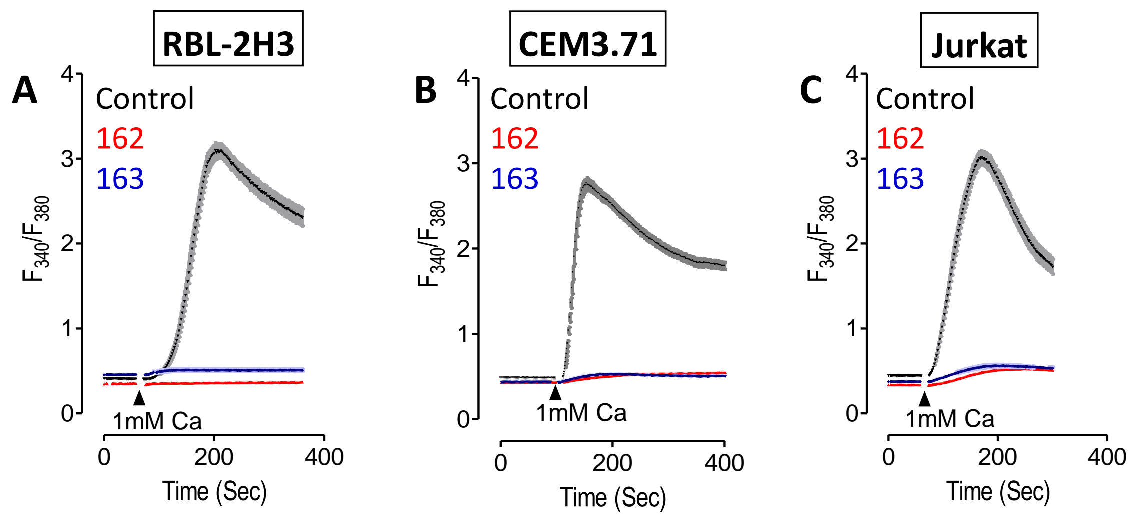


Figure 7: Inhibition of endogenous SOCe in hematopoietic cell lines

DPB162-AE and DPB163-AE inhibit native SOCe in hematopoietic cell lines. Intracellular calcium stores were depleted with Thapsigargin for 10 minutes prior to the start of the experiment. DPB's were added at T=0. (Black traces – no drug; red traces – DPB162-AE; Blue Traces – DPB163-AE; all 2 μM

3.3 Selective inhibition of Orai1, but not Orai2 by DPB162-AE and DPB163-AE

Orai1 Knockout (KO), Orai2KO or Orai1/2 double KO (DKO) cells, enabled analysis of DPB’s on SOCe mediated by different Orai proteins. Each Orai knockout cell line still expresses both endogenous STIM proteins. Fura-2 imaging revealed strong Orai1-mediated SOCe (in Orai2KO cells) following store depletion by TG and in the absence of DPB's. Addition of DPB162-AE or DPB163-AE 2 μM two minutes prior to addition of calcium 1 mM, revealed complete inhibition of SOCe in Orai2KO cells. (Fig. 8a). Similarly, in HEK cells stably over expressing STIM1 and Orai1, a comparable inhibitory effect was observed. However, STIM and Orai over-expressing cells required a 12 minute pretreatment with DPB’s was required to see inhibition of SOCe (Fig. 8b). This increase in time to inhibit is likely due to the increased expression of STIM1 and Orai1 proteins over endogenous levels (DT40, Jurkat, RBL-2H3). Furthermore, the inhibitory effect of DPB’s in HEK293 cells stably over-expressing STIM1-Orai1 was sustained when calcium was re-added to the recording chamber 10 minutes after removal of DPB's (Fig. 8c). Thus, DPB’s have a strong and prolonged action on STIM1-Orai1 mediated SOCe in both endogenous cells and in over-expression systems.

Analysis of standard logarithmic scale doses (0.01-3 μM) of both DPB162-AE and DPB163-AE on SOCe in HEK cells expressing STIM1 and Orai (Fig. 8d) resulted in IC_{50} for both compounds in the range previously reported (DPB162-AE IC_{50}=190±6 nM; DPB163-AE IC_{50}= 210±20 nM in CHO cells) (Goto, Suzuki et al. 2010).

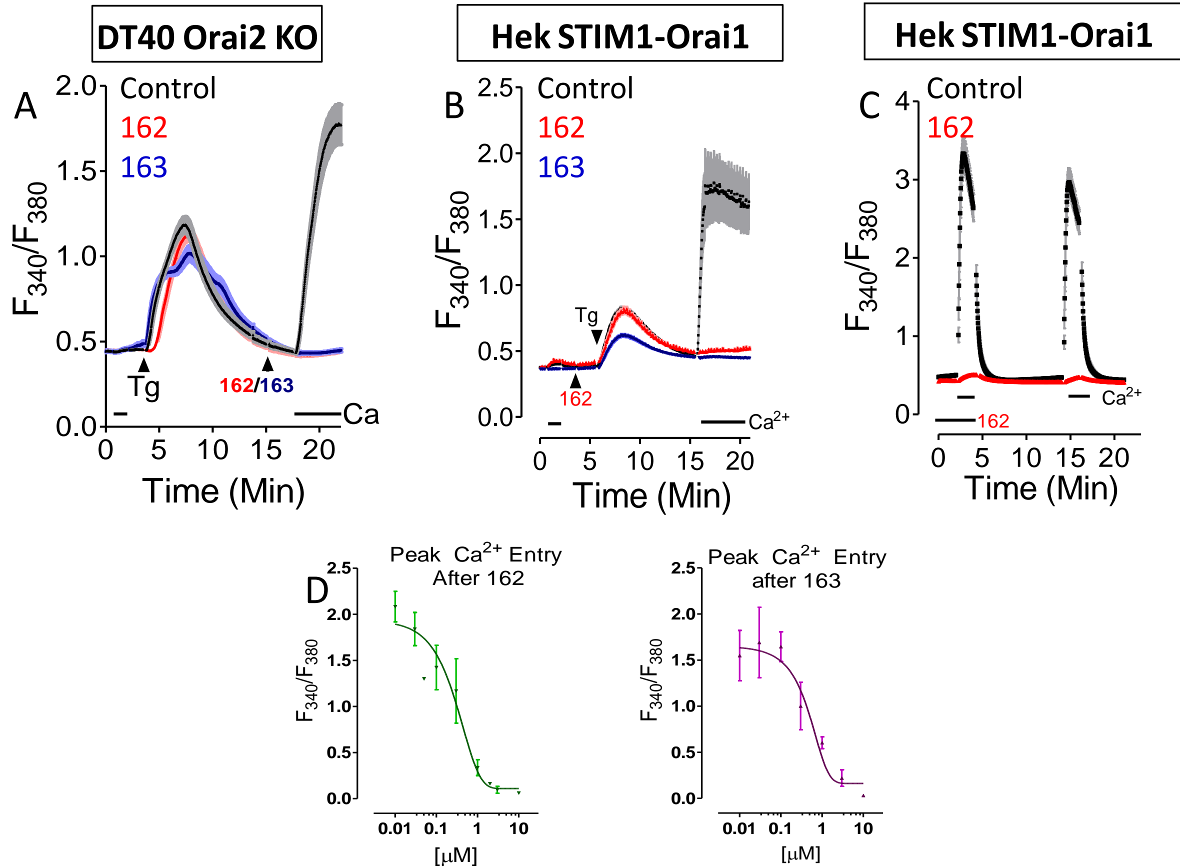


Figure 8: Selective inhibition of Orai1 mediated SOCE

A) DPB-162-AE and DPB-163-AE inhibit native SOCe in DT40 Orai2 knockout cells. B) DPB's inhibit SOCe from over expressed STIM1 and Orai1 in HEK cells. C) Sustained inhibition of STIM1-Orai1 SOCe in HEK cells after 10 minutes washout of the initial DPB162-AE treatment. D) Dose response curves for DPB162-AE and DPB163-AE inhibition of SOCE in HEK cells stably expressing STIM1 and Orai1 (Black traces – no drug; red traces – DPB162-AE; Blue Traces – DPB163-AE; all 2 μM.

Orai2-mediated SOCE (in Orai1KO cells) (Fig. 9a) was not inhibited to the same extent as Orai1 mediated SOCe in Orai2KO (Fig. 8a) cells when assessed with Fura-2 Ca^{2+} imaging. Analysis of the same cells on a Hamamatsu FDSS μCell high throughput screening system, using Fluo-4 as a reporter for Ca^{2+} concentration, revealed a somewhat stronger inhibitory effect of DPB's when cells were treated with either drug for 12 minutes prior to addition to 1 mM Ca^{2+} (Fig. 9b,c). Yet, even at 3 μM for either DPB162-AE or DPB163-AE, there was as little as ~50% inhibition of Orai2- mediated SOCe when compared to the inhibitory effect of DPB's on Orai1. Thus, DPB162-AE and DPB163-AE display strong selectivity for Orai1 over Orai2 in endogenous expression systems. What of their activity on Orai3?

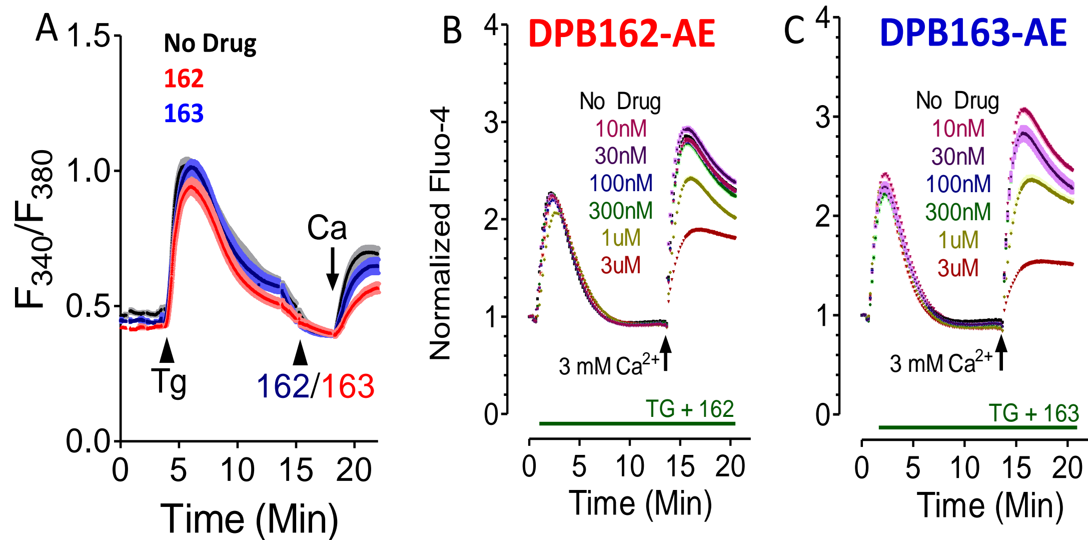


Figure 9: Inhibition of Orai2 in DT40 Orai1 knockout cells

A) Moderate inhibition of Orai2 mediated SOCe in DT40 cells but DPB's using FURA-2 as reporter for calcium entry (Black traces – no drug; red traces – DPB162-AE; Blue Traces – DPB163-AE; all 2μM. Dose response traces of the same cells in a high throughput screen for B) DPB162-AE and C) DPB163-AE, both displaying incomplete inhibition of Orai2 mediated SOCE (concentrations as indicated in B and C.

3.4 DPBs have no effect on Orai3 mediated SOCe nor STIM independent calcium entry through Orai3

3.4.1 Constitutive Ca^{2+} entry though Orai3

2-APB activates Orai3 'independently from STIM' (Schindl, Bergsmann et al. 2008), To assess whether STIM proteins were involved in this activation, Orai3-CFP was transfected into DT40 STIM1-STIM2 DKO cells. Addition of 50 μM 2-APB (the concentration at which Orai1-STIM1 SOCe is inhibited) revealed activation of calcium entry through Orai3 independent of any STIM involvement (Wang, Deng et al. 2009).

Therefore, I sought to determine whether DPB's could also potentiate Orai3 similar to the action of 2-APB. Orai3-CFP was exogenously expressed in HEK Wildtype (WT) cells and *store independent* calcium entry assessed with Fura-2. CFP positive cells, those expressing Orai3, displayed no activation of Ca^{2+} entry following application of DPB's 2 μM (Fig. 10a). Interestingly, DPB's did not prevent subsequent 2-APB activation of Ca^{2+} entry through Orai3, indicating DPB's and 2-APB have different sites or modes of action on Orai proteins (Fig. 10a).

3.4.2 Orai3-STIM1 mediated SOCe

Orai3 mediated SOCe in HEK Orai3-CFP stable cells transiently transfected with STIM1 revealed *incomplete* inhibition of Orai3 by DPBs, even after a 12

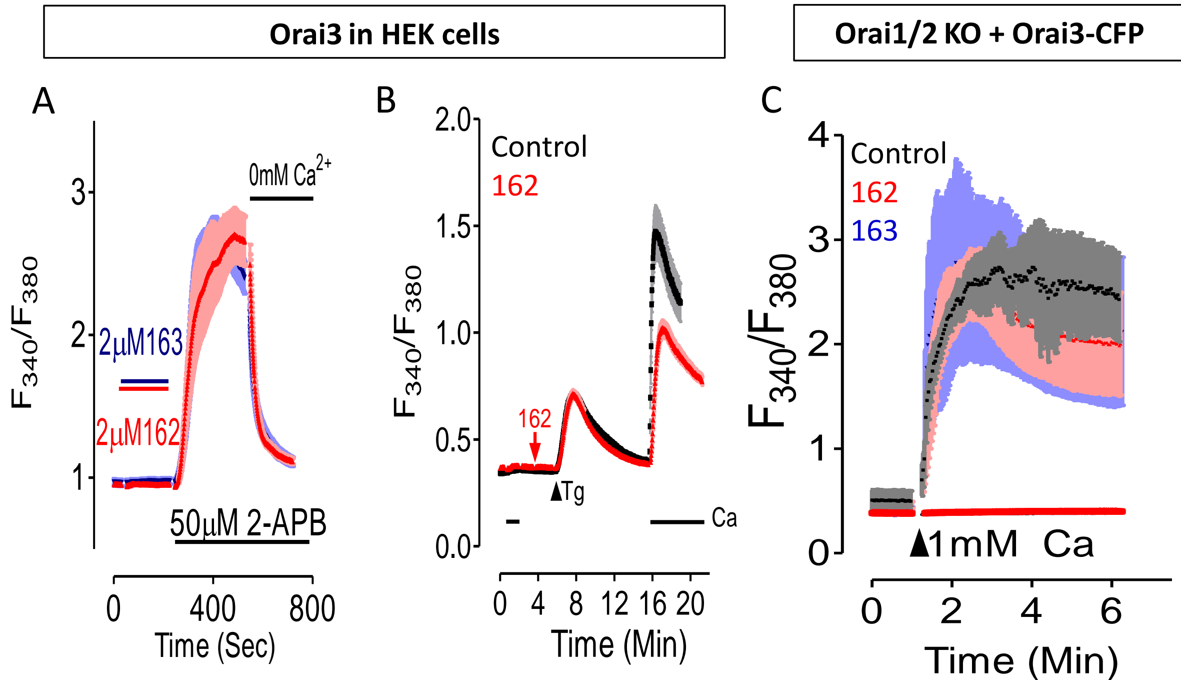


Figure 10: DPB effects on Orai3

A) In HEK cells transiently transfected with Orai3, DPBs were unable to effect the same robust calcium entry through Orai3 that has been widely reported for 2-APB, independent of STIM1. B) In HEK Orai3 stable cells, transiently transfected with STIM1, 12 minute pretreatment with DPB162-AE failed to completely inhibit Orai3 mediated SOCE. C) DT40 Orai1-Orai2 double knock out cells transiently transfected with Orai3. Stores were depleted for 12 minutes by TG in the presence of DPB162-AE or DPB163-AE. Neither analog inhibited SOCe. (Black traces – no drug; red traces – DPB162-AE; Blue Traces – DPB163-AE; all 2 µM

minute pretreatment with DPB162-AE or DPB163-AE (Fig. 10b). Given Orai1-STIM1 SOCe is robustly inhibited by both DPB's, it is entirely plausible the small inhibitory effect seen in this over expression system is due to the formation of a number of endogenous Orai1-STIM1 channel complexes. To test this idea I transfected Orai3-CFP into DT40 Orai1-Orai2 DKO cells. This model provides an excellent system where individual Orai proteins can be studied without additional Orai subunits present. Over expression of Orai3-CFP revealed no inhibition of SOCe after store depletion with TG for 10 minutes prior to the start of the experiment. DPB's were also present during the 10 minute store depletion phase, (prior to T=0; store depletion phase not recorded) (Fig. 10c). Cells displaying no CFP fluorescence (no Orai3 expression) were selected from the same coverslips as those displaying CFP. The non-expressing cells show no Ca^{2+} entry, confirmation that there are no endogenous Orai proteins expressed in the Orai1-Orai2 DKO cells (Fig 10c – three bottom traces are superimposed; one trace to represent untransfected cells from each of the experiments displayed above). Therefore, the Ca^{2+} entry in transfected cells is solely due to the presence of exogenous Orai3 (the chicken genome, from which DT40 cells are derived, contains only genes for Orai1 and Orai2). Furthermore, this assay also indicates the small degree of inhibition of Orai3 SOCe seen in the HEK293 cell assay (Fig 10b), is likely due to the presence of endogenous Orai1-STIM1 complexes in those cells.

These experiments present the first clear evidence of DPB discrimination between individual Orai subtypes in vitro, and that DPB's are highly selective for Orai1 with little effect on Orai2 and no effect on Orai3 channels. Furthermore, DPB's are not capable of

directly activating Orai3 independent of Ca^{2+} store content, and do not prevent 2-APB activation of Orai3 independent of STIM1 (Fig 10a). Given both analogs have quite similar inhibitory profiles, I focused my attention on DPB162-AE, which was more potent, and was reported to display only an inhibitory effect on SOCe as opposed to the reported biphasic activity of DPB163-AE (Goto, Suzuki et al. 2010).

3.5 Mutant Orai1V102C

3.5.1 Orai1V102C is potentiated by DPB162-AE independent of STIM1 interactions with the channel.

Orai1V102C, first described in cysteine accessibility scan of the Orai1 pore (McNally, Yamashita et al. 2009), and utilized to determine Orai1 pore architecture (Zhou, Ramachandran et al. 2010), is a non-selective, constitutively active Orai1 mutant which permits STIM1 independent ion flow through the Orai1 pore. Since wild type Orai1 is dominant negative and conducts no current when expressed alone (Soboloff, Spassova et al. 2006), it is possible, with this mutant channel, to discern drug effects directly on Orai1 independent of ER store content, i.e. when stores are full, and no STIM proteins are interacting with the channel.

In HEK Orai1V102C-CFP stable cells, DPB162-AE potentiated constitutive Ca^{2+} entry (Fig. 11a,b) and whole cell patch clamp current recordings through the mutant channel when intracellular Ca^{2+} stores were full (Fig 11d – cytoplasmic calcium buffered at 100 nM with BAPTA). This indicates DPBs may modify Orai1 pore architecture or the membrane configuration of the channel to allow an increased flow of ions through the pore. Similarly, in HEK Orai1V102C cells, when stores were depleted with TG, there was evidence of significant potentiation of Ca^{2+} entry through mutant Orai1V102C channel (Fig 11c). Yet, the potentiation was less than for cells with full stores. It is possible the lower degree of potentiation seen when stores are depleted is due to

endogenous STIM1-Orai1 complexes being inhibited, as these cells express endogenous Orai1, though at lower levels than the over-expressed mutant channel. Since intracellular stores are depleted, STIM1 is free to interact with all Orai1 channels available in the plasma membrane. Therefore, with DPB162-AE exhibiting robust inhibition of wildtype STIM1-Orai1 (Fig. 8a,b), the constitutive Ca^{2+} entry is not unreasonable when compared to the previous experiment when intracellular ER stores were full (Fig. 11b).

Similar to the experiment examining the duration of DPB162-AE effect on wildtype STIM1-Orai1 SOCe (Fig.8c), I analyzed the likelihood of sustained DPB162-AE alteration of constitutive Ca^{2+} entry (Fig 11.e) and current (Fig. 11f) through Orai1V102C. Calcium entry was activated upon addition of DPB162-AE in HEK Orai1V102C stable cells. Following washout of DPB162-AE, there was sustained calcium entry with little rundown to baseline (Fig 11e). Upon re-addition of DPB162-AE, 10 minutes after the first addition, there was no re-potentiation of calcium entry indicating DPB162-AE has a sustained interaction with Orai1V102C.

When whole cell currents through Orai1V102C were examined, there was a similar potentiation of the mutant channel (Fig. 11f), and complete blockade of the channel by Lanthanum (La^{3+}). Removal of La^{3+} reestablished the CRAC like current, but further application of DPB162-AE failed to elicit re-potentiation of the current, similar to the effect seen in the Ca^{2+} imaging experiment. Therefore, DPB162-AE, has the same sustained effect on mutant Orai1V102C as it does on the wildtype channel, whereby Orai1-STIM1 SOCe was robustly and permanently inhibited by DPB162-AE (Fig. 8c). Furthermore, La^{3+} inhibition is independent from DPB162-AE inhibition as neither drug

appeared to compete with the other in inhibiting the channel. This indicates DPB162-AE likely mediates it action from the intracellular side of the channel rather than the extracellular side synonymous with pore blockers like La^{3+}.

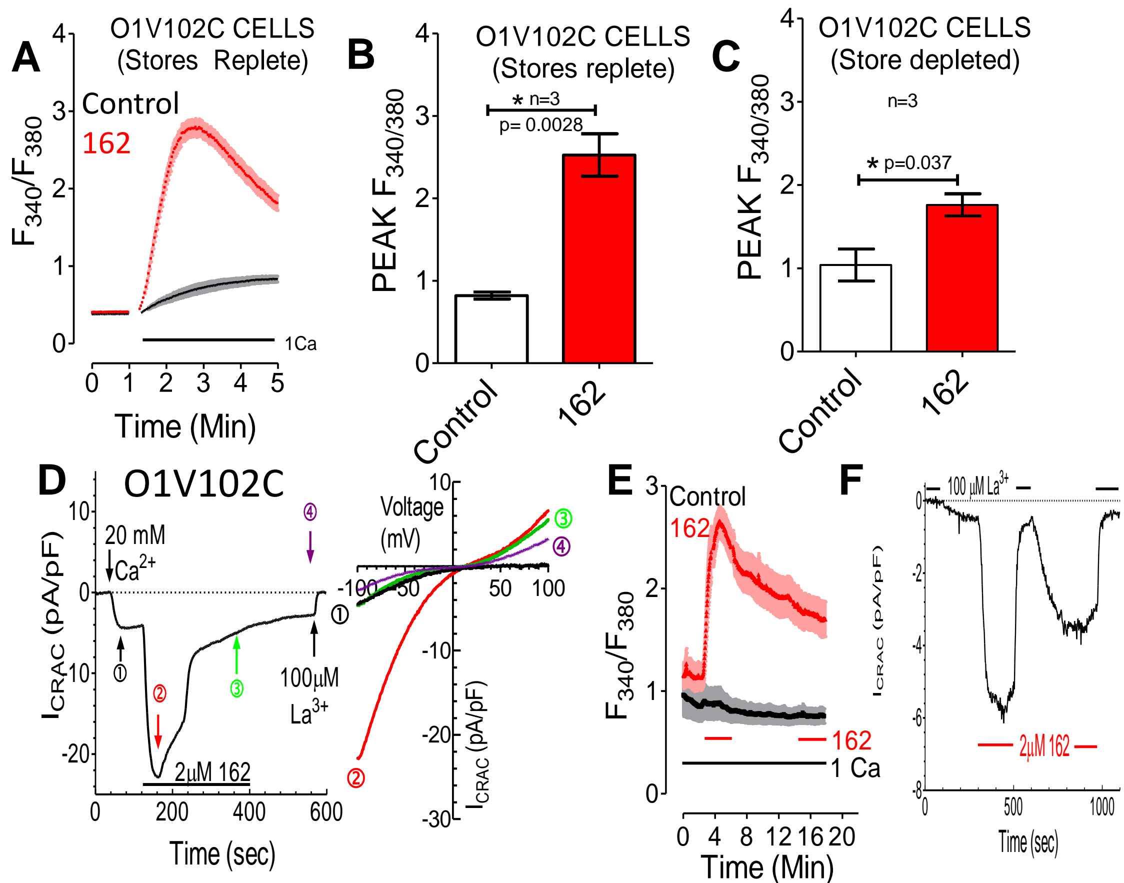


Figure 11: Constitutive activation of Orai1V102C by DPB162-AE in HEK Orai1V102C stable cells

(A, B) Activation of Ca^{2+} entry (stores replete) and C) Stores depleted. Cells were pretreated for 10 minutes with DPB162-AE prior to addition of calcium. E) Activation of I_{CRAC} through Orai1V102C. Cytoplasmic Ca2+ concentration was clamped at 100 nM, to maintain ER [Ca^{2+}], thus the current is the result of the action of DPB162-AE and not store depletion. E) Sustained effect of DPB162-AE on constitutive Ca^{2+} entry and current (F) following washout. There was no re-potentiation of Ca^{2+} entry or current following a second application of DPB162-AE in either experiment.

3.5.2 Lack of inhibition of STIM1-mediated SOCe through Orai1V102C

Subsequently, I analyzed DPB162-AE effects on co-expressed Orai1V102C and STIM1 when stores were depleted. In HEK293 Orai1V102C-STIM1 stable cells, *following passive store depletion* by EGTA 10 mM in the patch pipette, acute application of DPB162-AE 2 μM activated current through the mutant channel (Fig 12a, marked at 1). Upon removal of DPB162-AE, currents stabilized to the levels prior to DPB162-AE addition (Fig. 12a,b marked at 2 and 4), and were completely abolished upon application of the Orai1 pore blocker La^{3+} 10 μM (Fig.12a,b marked at 5). Previous work indicated STIM1 interaction with Orai1V102C reverted the nonselective channel back to a highly Ca^{2+} selective state (McNally, Somasundaram et al. 2012). There was a leftward shift in I_{CRAC} reversal potential following DPB162-AE application (Fig. 12b), suggesting DPB162-AE changes the selectivity of the mutant Orai1-STIM1 complex to make the channel non selective again. Furthermore, there was no evident inhibition of the constitutive ion flux through the mutant channel complex, suggesting Orai1V102C may not be inhibited by DPB162-AE (Fig.12a). When DPB162-AE was applied for 12 minutes prior to addition of Ca^{2+} (Fig. 12c), there was evidence of limited reduction in Ca^{2+} entry compared to control cells where no DPB162-AE was applied. In the over-expression system, there are still endogenous wildtype Orai1 channels expressed, which can interact with and be activated by STIM1. Thus, reduction in Ca^{2+} entry seen in these experiments is most likely due to the inhibition of Ca^{2+} entry through endogenous wildtype STIM1-Orai1 complexes. It is possible the lack of visible potentiation in the

Ca^{2+} entry experiments (Fig. 12c) is due the magnitude of the SOCe component of entry, which likely masks any potentiation mediated by DPB162-AE. This massive SOCe is not evident in HEK293 cells stably expressing only Orai1V102C due the lesser expression of only *endogenous* STIM1. In those cells, it is clear there is a reduction in the potentiation by DPB162-AE (Fig. 11c) when compared to the same cells when stores are full and no STIM1 interacting with Orai1V102C (Fig. 11b)

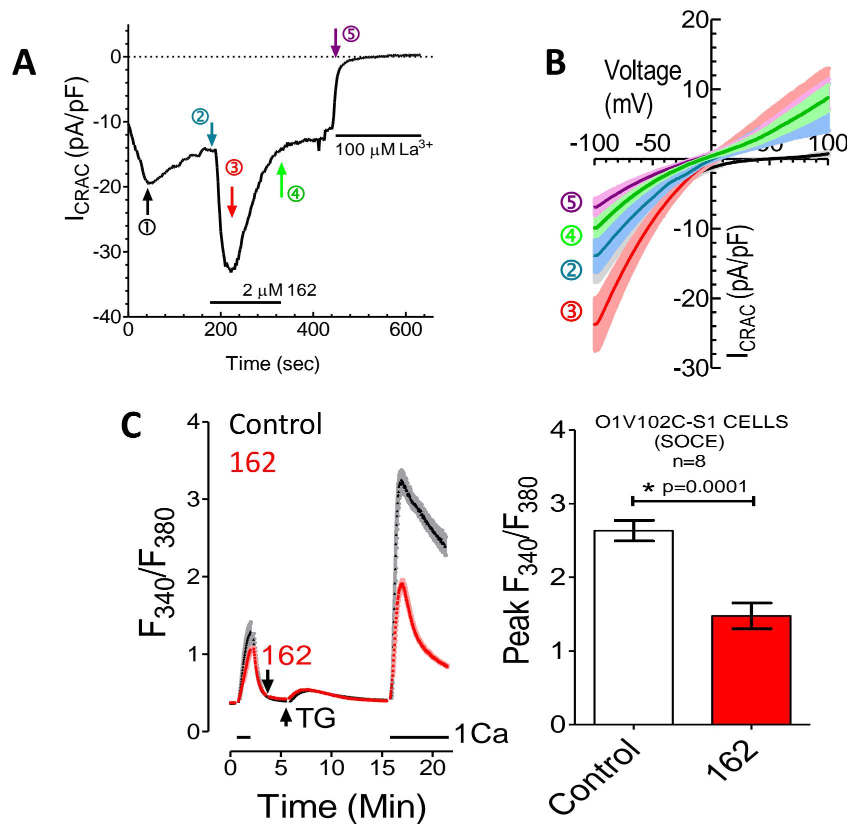


Figure 12: Transient activation and lack of inhibition of Orai1V102C-STIM1 complexes by DPB162-AE

HEK Orai1V102C-STIM1 stable cells. A) Stores were depleted with EGTA in the pipette and currents revealed acute potentiation but no inhibition of the Orai1V102C-STIM1 complex . B) I-V curves at various stages of the current profile revealed a change in reversal potential, thus, channel selectivity upon application of DPB162-AE (marked at 2). C) Ca^{2+} imaging experiments on the effect of DPB162-AE on SOCe, with the small reduction in Ca^{2+} entry like due to the inhibition of endogenous wildtype Orai1-STIM1 channel complexes.

3.6 2-APB and DPB's have differential effect on STIM1 and STIM2

3.6.1 2-APB effects on Ca^{2+} entry via STIM1 vs. STIM2

With some noticeable differences between STIM1 and STIM2, and past evidence of differential effects on the activation of Orai1 by STIM1 and STIM2 (discussed in Chapter 1), it was important to determine differences in the coupling of STIM1 and STIM2 with Orai1. STIM2 was clearly more effective than STIM1 at increasing constitutive Ca^{2+} entry (Fig. 13a) and current (Fig. 13b, c) via Orai1 when stores were full. Understanding differences between 2-APB and DPB's in this regard is important as earlier experiments indicate 2-APB and DPB's have different modes of action.

Constitutive current and Ca^{2+} entry mediated by STIM2 were robustly increased upon addition of 2-APB 50 μM, whereas STIM1 mediated constitutive Ca^{2+} entry was virtually unaffected by 2-APB at the same concentration (Fig. 13a). The 2-APB effected increase in constitutive STIM2 Ca^{2+} entry when stores were full was quite similar to the Ca^{2+} entry mediated by STIM1 when stores were depleted. Yet, for STIM1-Orai1 SOCe, the addition of 2-APB caused a complete inhibition of Ca^{2+} entry after a mild transient activation (Prakriya and Lewis 2001; Soboloff, Spassova et al. 2006; Spassova, Soboloff et al. 2006). The observed activation of STIM2 by 2-APB was further substantiated in DT40 STIM1-STIM2 knockout cells, transiently expressing Orai1-CFP and either STIM1-YFP (Fig. 13d) or STIM2-YFP (Fig. 13e). 2-APB elicited the same potentiation

of STIM2-Orai1 SOCe in cells devoid of endogenous STIM proteins, proving 2-APB induced Ca^{2+} entry is exclusively mediated by STIM2.

To further address the role of 2-APB on STIM2, the entire ER residing N-Terminal domain of STIM1 (Fig. 1) was fused to the cytoplasmic residing C-Terminal domain of STIM2 (STIM2 aa 339-746), forming STIM1-STIM2 chimeric protein. 2-APB activated this chimera, indicating the 2-APB effect on STIM2 was mediated by its C-terminus (Fig 13e), as substitution of the STIM1 N-terminal residues (those proximal to the TM region) onto STIM2ct (distal to the TM region) had no effect on 2-APB potentiation of Ca^{2+} entry. Thus, generation of cytosolic soluble STIM C-terminal domains was deemed necessary to further define the effects of 2-APB, and later DPB's.

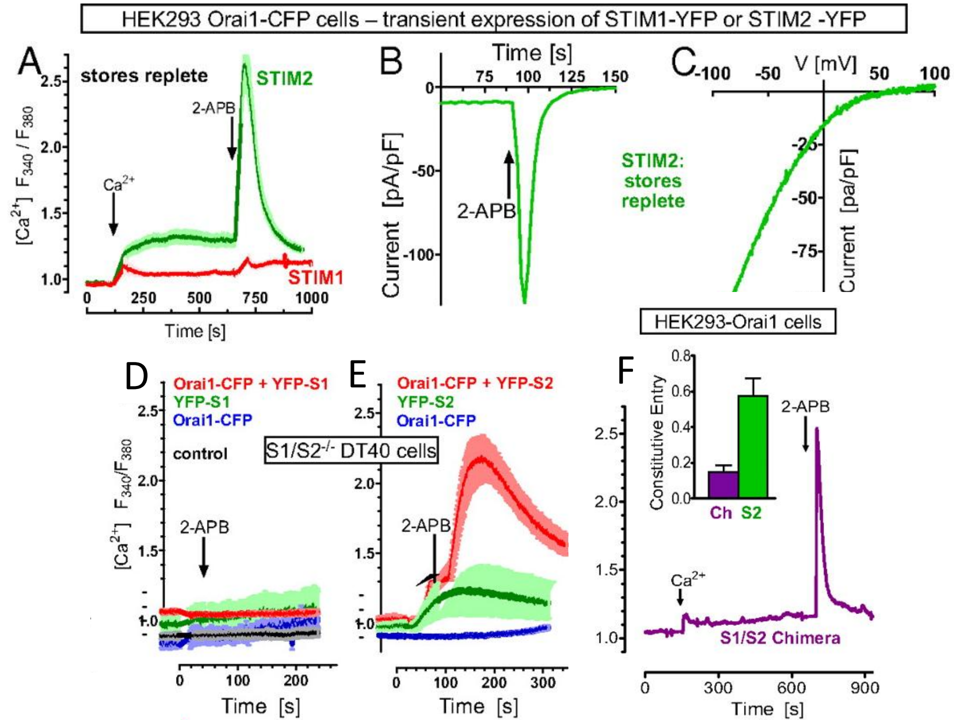


Figure 13: Differential effects of 2-APB on STIM1 and STIM2.

A) STIM2 displays larger constitutive Ca^{2+} entry than STIM1 in Hek Orai1 stable cells, and 2-APB 50 µM effects a large potentiation of that Ca^{2+} entry and current (B, C) when STIM2 is expressed. D) STIM1-STIM2 knockout cells transiently transfected with STIM1 and (E) STIM2 with or without exogenous Orai1. F) STIM1-STIM2 chimera show reduced constitutive Ca^{2+} entry, but the same degree of potentiation of as STIM2 upon application of 2-APB. (Data generated by Youjun Wang)

3.6.2 DPB162-AE and DPB163-AE have differential effects via STIM1 and STIM2

An excellent tool to test functional effects of DPB's on individual STIM proteins has been DT40 STIM KO cells for both STIM1 and STIM2, which allow endogenous expression of STIM1 (STIM2KO) or STIM2 (STIM1KO), or neither (STIM1/2DKO) in a highly proliferative cell line. Based on previous data above, I expected STIM1 and STIM2 would behave differently when treated with DPB’s. In DT40 STIM2 knockout cells (where only endogenous *STIM1* is expressed), SOCe was robustly inhibited by DPB162-AE 2 μM (Fig. 14a, b) supporting the theory of high specificity for Orai1-STIM1 mediated SOCE, and consistent with the effects in HEK293 cells stably expressing STIM1 and Orai1 (Fig. 14c)

In DT40 STIM1KO cells, i.e. those expressing only endogenous *STIM2*, DPB162-AE effected a very minor potentiation of SOCe at 300 nM (Fig. 14d), yet DPB163-AE, in the 100-300 nM, range caused a robust increase in STIM2 mediated SOCe (Fig. 14e), consistent with reported biphasic activity of DPB163-AE (Goto, Suzuki et al. 2010). Surprisingly, DPB163-AE action on STIM2-Orai1 SOCe is not biphasic! Despite the potentiation of STIM2-SOCe in DT40 STIM1 knockout cells by DPB163-AE, there was *never* an inhibitory effect of either DPB162-AE or DPB163-AE over endogenous SOCe in these cells (Fig. 14d, e). This is striking, as it clearly indicates DPB inhibition of SOCE *absolutely requires* STIM1 and Orai1. This is supported by the small degree of DPB162-

AE inhibition in HEK cells over expressing STIM2 and Orai1 (Fig. 14f). These cells have endogenous STIM1, which likely accounts for that reduction in Ca^{2+} entry.

The data confirms that DPB163-AE potentiates Ca^{2+} entry through Orai channels exclusively via its action on STIM2 or the interaction of STIM2 with Orai1, and that DPB inhibitory effects are mediated by STIM1. This suggests the interaction of STIM2 with Orai1 could be slightly different to that of STIM1 with Orai1; that STIM2 confers different properties to the binding site(s) on Orai1 for DPB's, or that STIM2 has a slightly different spatial interaction with itself, or with Orai channels, resulting in an alternative channel state. This alternative state may disrupt or prevent DPB162-AE from eliciting its inhibitory action on Orai1, and may also facilitate the potentiation of STIM2-Orai1 SOCe by DPB163-AE.

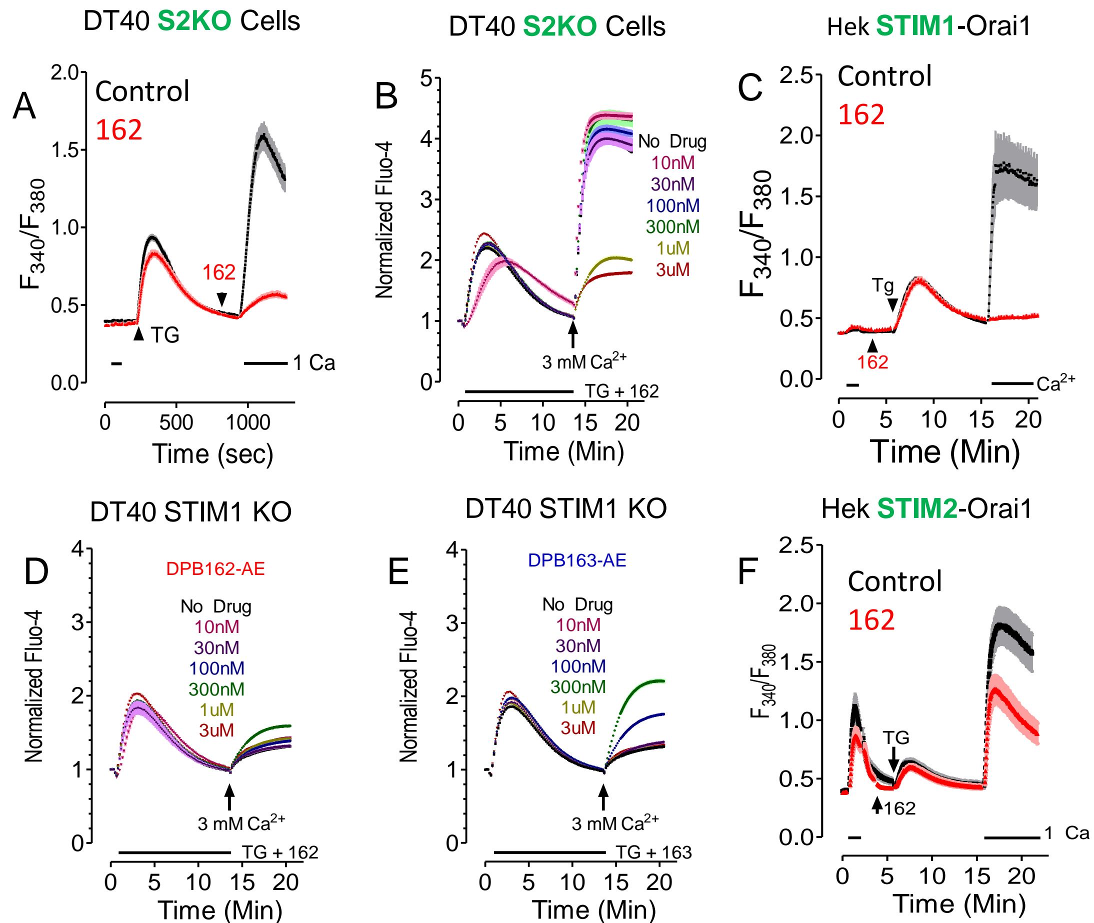


Figure 14: DPB effects on STIM1 and STIM2

A) 2 minute pretreatment with DPB162-AE [2µM] inhibits endogenous SOCe in DT40 STIM2 knockout cells when assessed with FURA-2 and FLUO-4 Ca^{2+} reporter dyes. C) In HEK over-expression systems, there is complete inhibition of STIM1-Orai1 SOCe after 12 minute pretreatment with DPB162-AE . D) STIM1 knockout cells display no inhibitiion by DPB162-AE or DPB163-AE, E), yet DPB163-AE demonstrates robust potentiation of STIM2 mediated SOCe at 100-300 nM. F) STIM1-Orai1 SOCe is partially inhibited by DPB162-AE, likely due to the presence of STIM1 in these cells.

3.6.3 2-APB dramatically alters STIM1ct and STIM2ct function

STIM1 N-terminal regions are unable to interact with Orai1, and with evidence from the STIM1-STIM2 chimera revealing the importance of the C-terminus, we synthesized soluble C-terminal fragments of STIM1 and STIM2, both tagged with YFP; STIM1ct-YFP (amino acids 235-685) and STIM2ct-YFP (amino acids 239-746).

STIM1ct, expressed in HEK Orai1 stable cells, was capable of eliciting constitutive Ca^{2+} entry, with a further rapid increase in Ca^{2+} entry upon addition of 2-APB (Fig. 15a). This was surprising since 2-APB has no effect on whole STIM1 (Wang, Deng et al. 2009) (Fig. 13a). The potentiation effect of 2-APB was also measured as whole cell current in the same cells (Fig 15 b,c), and displayed typical I_{CRAC} characteristics. Further narrowing of STIM1ct region to comprise only the coiled coil domains (STIM1ct-cc; aa 235-505) revealed a similar, though smaller effect of 2-APB to that seen with STIM1ct (aa 235-685) (Fig. 15e). This implies the shorter Stim1ct-cc region, without the downstream variable region and PS & Poly-K domains, to be sufficient for activation of Orai1, as the constitutive component of the Ca^{2+} entry remained the same between the STIM1 C-terminal fragments. Furthermore, constitutive Ca^{2+} entry via STIM1ct, and 2-APB induced entry via STIM1ct are independent events, as STIM1-STIM2 knockout cells transiently expressing STIM1ct had no constitutive Ca^{2+} entry (Fig. 15f). Thus, there is no requirement for endogenous STIM1 or over-expressed Orai1 (Fig. 15g) to facilitate the robust activation of STIM1ct-Orai1 Ca^{2+} entry. Subsequent work from Muallem's lab revealed the SOAR domain (Yuan, Zeng et

al. 2009) (aa 344-442) within STIM1ct-cc to be the minimally required fragment of STIM1 necessary for activation of Orai1.

As well as a robust stimulatory effect on STIM2 constitutive Ca^{2+} entry, and a strong activation of the STIM1-STIM2 chimera, STIM2ct was analyzed to further elucidate 2-APB effects on STIM2 potentiation of Orai1. STIM2ct was expressed in HEK293 cells, though the number of viable cells after transfection was much lower than for STIM1ct. Yet, cells expressing STIM2ct of comparable YFP intensity to STIM1ct, had similar constitutive I_{CRAC}, but a much greater 2-APB induced I_{CRAC} (Fig. 15d) indicating STIM2ct may be more efficiently coupled to Orai1 by 2-APB.

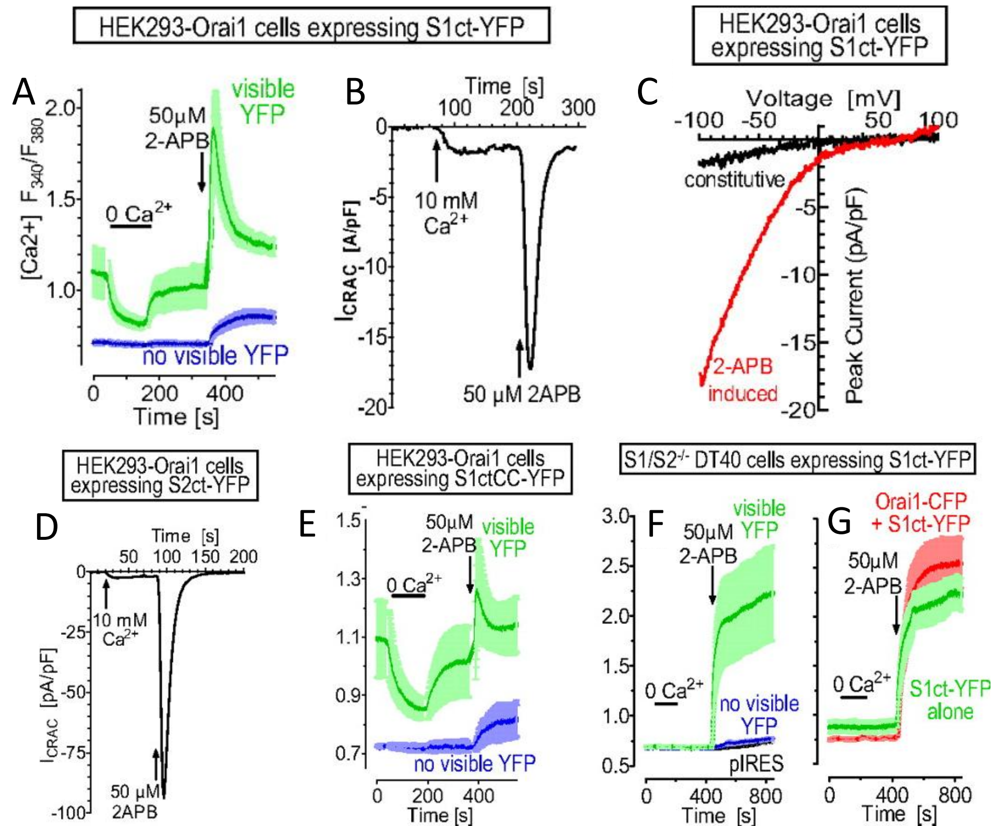


Figure 15: 2-APB enhances Ca2+ entry and I_{CRAC} via STIM1 and STIM2 C-terminal fragments

A) STIM1ct mediated Ca^{2+} entry and B, C) I_{CRAC} are robustly potentiated upon application of 2-APB. D) Constitutive I_{CRAC} mediated by STIM2ct is of a similar magnitude to that of STIM1ct, but 2-APB elicited potentiation of I_{CRAC} is much greater for STIM2ct. E) STIM1ct-CC is constitutively active and still potentiated by 2-APB. F) STIM1ct does not require endogenous STIM1 or over-expressed Orai1 (F) presence for 2-APB to elicit its potentiation effect.

3.6.4 Analysis of STIM1ct and STIM2ct domains with DPB's

Given the noticeable difference in the effects of 2-APB between STIM1ct and STIM2ct (Wang, Deng et al. 2009), DPB's were analyzed to assess differences between the two molecules on STIM2ct. Both DPB162-AE and DPB163-AE were capable of activating Ca^{2+} entry through Orai1 via STIM2ct, similar to the effect of 2-APB, though the rate of Ca^{2+} entry was slower (Fig. 16b). The apparent lack of effect of DPB's on STIM1ct (Fig. 16c) also supports the idea that STIM2 is the means by which potentiation is effected and STIM1 is the inhibitory modulator of DPB's. Furthermore, application of 2-APB to STIM1ct after treatment with DPB162-AE showed no preventative effect on 2-APB activation of STIM1ct (Fig.16c), indicating the possibility of different sites or modes of action for 2-APB and DPB's, despite their intrinsic dimeric similarity (Dobrydneva, Abelt et al. 2006).

Since DPB162-AE was not effective on STIM1ct, I assessed its activity on the constitutively active STIM1ct4EA (Korzeniowski, Manjarres et al. 2010), which has glutamates (E) in the STIM1 inhibitory helix (Fig. 1) mutated to alanine (A), allowing it to freely interact with and activate Orai1. DPB162-AE completely inhibited constitutively active STIM1ct4EA-Orai1 Ca^{2+} entry (Fig. 16d). This result is quite consistent with previous data whereby STIM1-Orai1 SOCe (Figs. 8 & 14c) was inhibited, further supporting the requirement of STIM1 with Orai for inhibition by DPB162-AE.

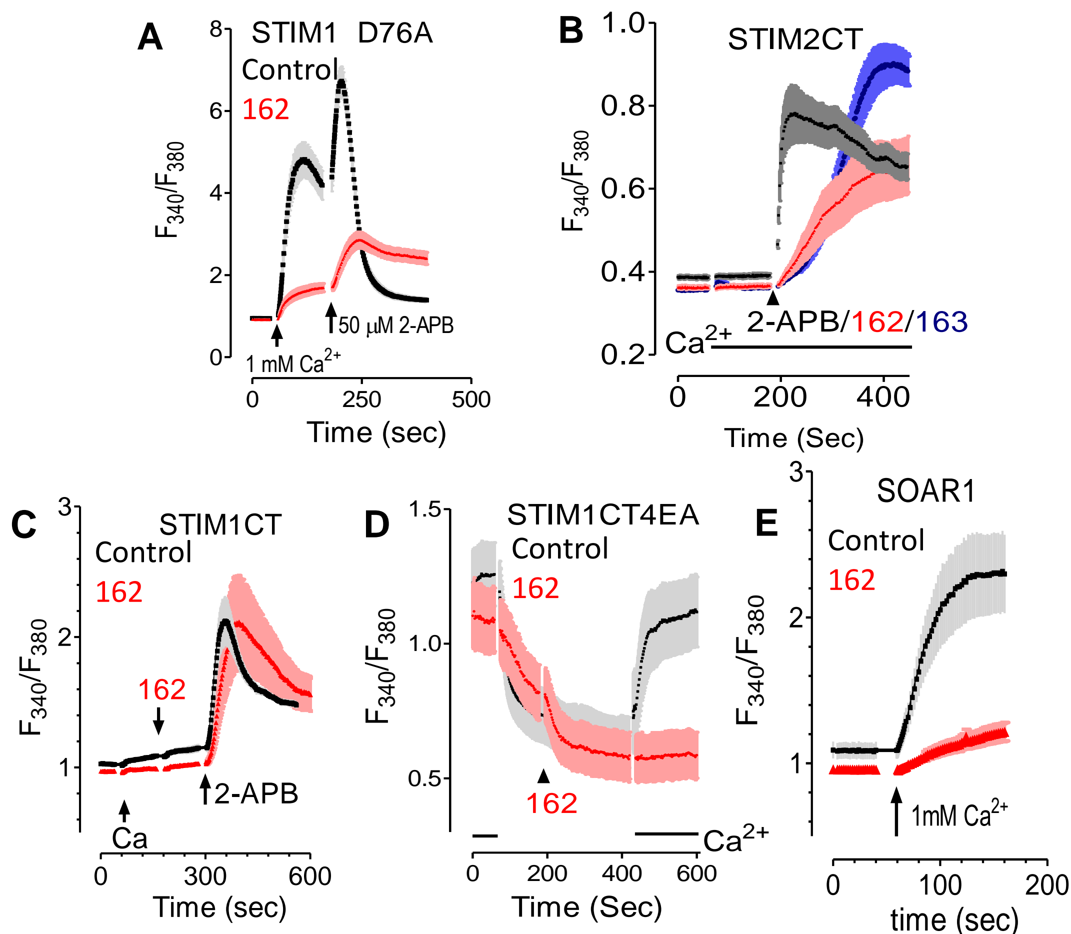


Figure 16: Analysis of STIM1 mutations and truncations.

All experiments were carried out in HEK293-Orai1-CFP stable cells. A) Inhibition of consitutively active STIM1D76A mutant by DPB162-AE. B)DPB's potentiate STIM2ct though at a slower rate than 2-APB (Fig 16c) C) DPB's fail to elicit any effect on STIM1ct, yet do not prevent 2-APB activation of STIM1ct. D) Constitutively active STIM1ct4EA was completely inhibited by DPB162-AE. E) DPB162-AE inhibited constitutive calcium entry mediated by STIM1-SOAR fragment.

3.6.5 DPB's and calcium binding/dissociation at STIM1 EF-hand domains

To ensure DPB's were not controlling Ca^{2+} binding or dissociation with STIM1 N-terminal EF-hand domain, effects of DPB162-AE were analyzed on a constitutively active EF-hand STIM1 mutant. Substitution of aspartate[76] (D76) in the cEF-hand of STIM1 results in abolition of the Ca^{2+} binding site on the ER residing N-terminus. Thus, with Ca^{2+} constantly dissociated from STIM1, it actively associates with Orai channels to initiate SOCe. DPB162-AE robustly blocked constitutive Ca^{2+} entry via STIM1D76A transfected into HEK293 cells stably over-expressing Orai1, when compared to cells not treated with DPB162-AE (Fig. 16a). This indicates no effect of DPB162-AE on the cEF-hand region of STIM1 to prevent Ca^{2+} dissociation, or mimicking Ca^{2+} binding to the EF-hand, which would prevent STIM activation and SOCe activation.

3.6.6 Analysis of Ca^{2+} entry mediated by STIM1-SOAR fragment

To further elucidate the action DPB162-AE on SOCe, I analyzed the smallest active fragment of STIM1, SOAR (Yuan, Zeng et al. 2009). When expressed alone, SOAR is a constitutively active cytosolic protein which can readily bind to Orai1. SOAR-YFP was expressed in HEK Orai1-CFP cells and treated with DPB162-AE, which robustly inhibited constitutive Ca^{2+} entry mediated by SOAR (Fig. 16e). Thus, it appears, while STIM1 is important, the action of DPBs is most likely though its action on Orai1 N-terminus and that STIM1 interaction with the channel is necessary for the inhibitory effect of DPB's.

3.6.7 FRET analysis of STIM and Orai interactions

Since I observed robust inhibition of STIM1-Orai1SOCe by DPB's (Fig. 17a), I sought to determine whether inhibition was due to altered STIM1-Orai1 interactions. Thus, FRET analyses were carried out on a number of different STIM1 (YFP) and Orai1 (CFP) mutants. Neither DPB162-AE nor DPB163-AE had any effect on maximal FRET between STIM1-Orai1 (Fig 17b), despite complete inhibition of Ca^{2+} entry, indicating no effect on the physical coupling between STIM1 and Orai1 C-termini. This cannot rule out an effect on the *functional coupling* between them, whereby DPB's may prevent a conformational change in the Orai structure following STIM1 interaction, or, STIM1 interaction with Orai allows DPB's to access inhibitory sites on the intracellular N-terminus of Orai, accelerating inhibition of the channel. Additionally, DPB's may alter STIM1 gating interactions with Orai1 at the N-Terminal extension region of the channel, while STIM1 still maintains C-terminal 'Orai1-priming' contacts. To further rule out inhibition at the C-Terminus of Orai1, I assessed the DPB162-AE effect on FRET between SOAR-Orai1 (Fig. 17c), finding DPB162-AE had no effect to enhance or diminish FRET between SOAR and Orai1. Neither did DPB162-AE prevent the robust enhancement of FRET caused by 2-APB, further supporting different sites of action for DPB's and 2-APB.

To support the previous FRET finding, I sought to detemine whether altering the Orai1 N-termius would alter FRET between STIM and Orai. To this end, I utilized the mutant Orai1K85E channel (Lis, Zierler et al. 2010) to prevent STIM1-gating

interactions with Orai1 at the N-terminus, providing only Orai1 C-terminus for which STIM1 could interact. 12 minute pretreatment with DPB162-AE did not prevent STIM1-Orai1K85E FRET (Fig. 17d), indicating no effect of DPBs at the STIM1-Orai1 C-terminal interacting sites, and thus no prevention or disruption of interactions which might inhibit 'Orai1 priming' by STIM1.

Finally, mutations which greatly diminish C-Terminal interactions between STIM and Orai and abolish Orai1 activation by STIM were analysed. FRET between Orai1ΔDE (Calloway, Vig et al. 2009) (Fig 2) and STIM1-3KQ (Calloway, Holowka et al. 2010) (Fig.1) was not affected by DPB162-AE (Fig. 17e). Given the effects of the mutations to both proteins, and the lack of inhibition of the remaining weak interactions on Orai1 C-terminal interactions with STIM1, it is likely DPB162-AE mediates its effects via alterations of Orai1 gating, though the exact means by which this occurs remains open.

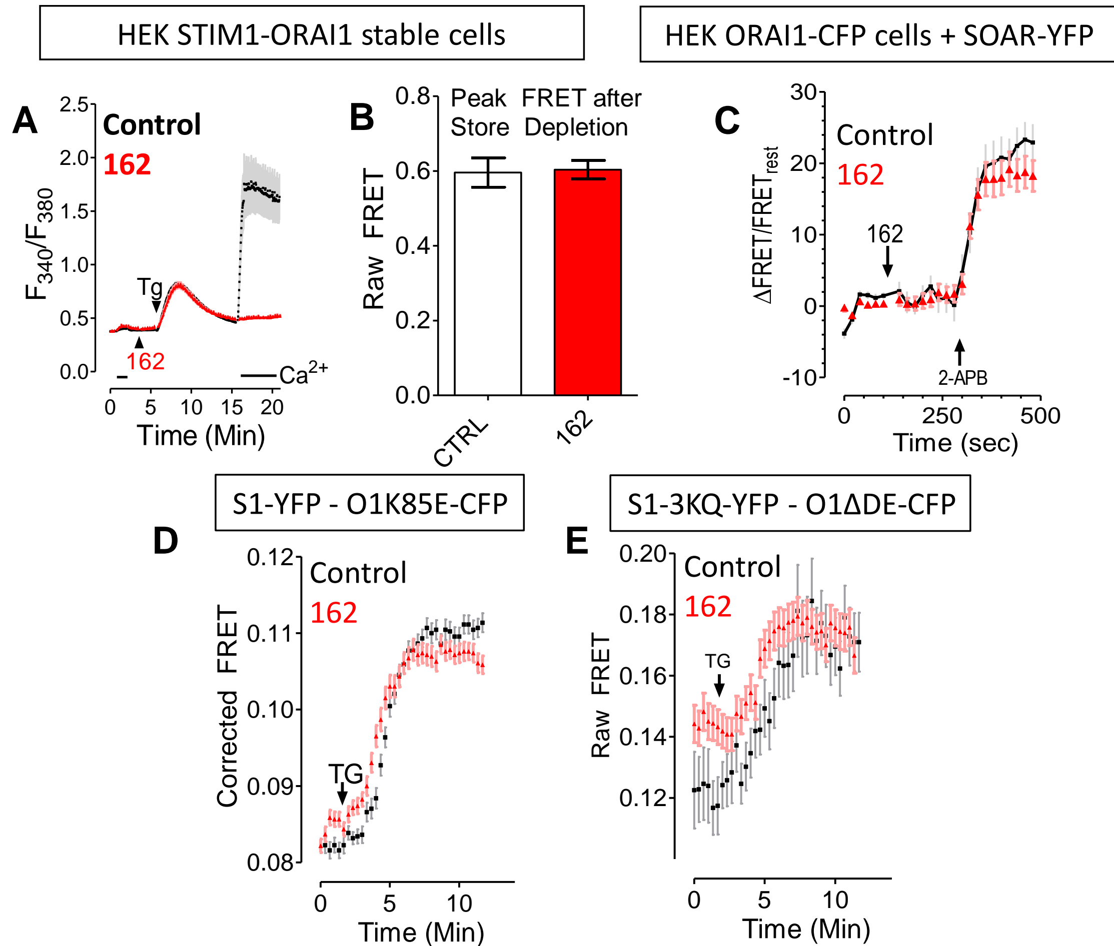


Figure 17: DPB162-AE does not alter STIM1-Orai1 interactions as revealed by FRET

A) DPB162-AE inhibits STIM1-Orai1 SOCe, but not B) STIM1-Orai1 FRET in HEK293 cells stable expressing both proteins. C) HEK293Orai1 stable cells transfected with SOAR revealed no inhibitory effect of DPB162-AE on FRET between SOAR-YFP and Orai1-CFP. D) Similarly, DPB162-AE did not prevent FRET between STIM1 and mutant Orai1K85E, or E) mutant STIM1-3KQ and Orai1ΔDE.

CHAPTER 4

DISCUSSION

Since the discovery of STIM and Orai as the essential components of Store Operated Ca^{2+} Entry, there has been remarkable progress, not only to understand the SOCe process, but also to define the mechanistic events and protein domains which modulate calcium entry though these channels (Muik, Fahrner et al. 2009; Park, Hoover et al. 2009; Yuan, Zeng et al. 2009). Much of the later work has been made possible by, and often carried out in conjunction with, the development of pharmacological agents which modify the STIM-Orai activation process.

Yet, specificity of pharmacological modifiers for STIM and Orai homologues has remained elusive until most recently; the development of two 2-APB analogs DPB162-AE and DPB163-AE (Goto, Suzuki et al. 2010). Previous to that discovery, 2-APB was, and still is, the most commonly used SOCe modifier, despite the fact effects on other cellular channels or processes (Maruyama, Kanaji et al. 1997; Diver, Sage et al. 2001; Missiaen, Callewaert et al. 2001; Bilmen, Wootton et al. 2002; Bootman, Collins et al. 2002) and over a range of concentrations. Despite this, 2-APB has proven useful to help define SOCe , as well as intrinsic properties of Orai homologues, (Ma, Venkatachalam et al. 2002; Soboloff, Spassova et al. 2006; Zhou, Iwasaki et al. 2007; Dehaven, Smyth et al. 2008) and for future development of other compounds, DPB's as case in point. Even more recently, the development of a new compound by Synta – RO2959 (Chen, Panicker et al. 2013) – with IC_{50} less than that for DPB's, is showing the potential for CRAC channel inhibitors that do not contain the highly reactive boron atom, yet work at low nM

concentrations. I analyzed this compound on DT40 Orai knock out cells and found it to be specific for Orai1 over Orai2, but much additional work is required on RO2959 to delineate the exact nature of its inhibition on SOCe as well as its differences in efficacy from DPB's.

4.1 Specific inhibition of SOCe through Orai1 and the essential role of STIM1

This work clearly demonstrates the high degree of specificity of DPBs for Orai1 mediated calcium entry (Fig. 7), over Orai2 (Fig. 8) and Orai3 (Fig. 9). This finding is extremely important as it indicates small and seemingly significant differences between Orai homologues influence channel specificity of SOCe inhibitors. Identifying the essential differences will require a great deal further work, and will be important for developing specific modifiers of Orai2 and Orai3, but the ground work, in terms of potential structures, has been set with DPB162-AE and DPB163-AE, as well as the RO2959 from Synta (Chen, Panicker et al. 2013).

The exact cause of the high selectivity of DPB's for Orai1 is as yet unknown, but the recent publication of *D. melanogaster* Orai1 crystal structure (Hou, Pedi et al. 2012) as well as the STIM1-SOAR structure (Yang, Jin et al. 2012), provides important structural data to help resolve selectivity differences between Orai proteins. It opens the potential for co-crystallization of Orai with STIM to dissect mechanistic processes and determine the nature of STIM-Orai interactions. Furthermore, co-crystallization of the channel complex with some of the many SOCe modifiers to define sites and modes of action of specific pharmacological agents which alter SOCe is also on the horizon with the publication of these crystal structures.

Indications from experiments on Orai1-STIM inhibition (Fig. 8c) and Orai1V102C potentiation (Fig. 11e,f) indicate DPB's mediate their action on the

intracellular side of the channel complex, and most likely via an interaction directly with Orai1 to disrupt functionality of the pore. There are some amino acid differences between the Orai proteins, particularly in the intracellular TM2-TM3 loop, where Orai3 has more polar side chains in that region than Orai1 and Orai2. This may account for the lack of effect of DPB’s on Orai3 mediated SOCe due to less available interacting partners for the partially charged DPB’s (Fig. 5). Of particular interest on Orai3 TM2-TM3 loop are two glutamines Q^{137} and Q^{141} which are conserved glutamates (E) at corresponding positions in Orai1 (E^{162}, E^{166}) and Orai2 (E^{136}, E^{142}) (Fig 16). These differences are intriguing and worth further investigation, by mutation of Q’s to E in Orai3, mutation of the Orai1/2 E’s to Q or mutation of residues to less reactive alanine, to assess whether inhibitory effect of DPB's is altered in the mutant channels when compared with wildtype.

However, these amino acid differences may not account for the reduction in inhibition of Orai2 SOCe when compared to Orai1. Interestingly, there are further amino acid differences between Orai1 and Orai2 in this region, specifically, $Serine^{135}$ ($Lysine^{161}$ in Orai1), $Proline^{134}$ ($Arginine^{160}$ in Orai1) and $Tryptophan^{145}$ ($Histidine^{171}$ in Orai1), which confer a loss of charge (K to S; R to P) and change to a hydrophobic residue (H to Y) respectively in Orai2, all the while the aforementioned Orai1 glutamates are conserved between Orai1 and Orai2 (Fig.16). Thus, the amino acid differences between Orai1 and Orai2 may account for the reduced inhibitory effect seen between Orai1, Orai2 and Orai3, that is assuming DPB's mediate their effects via interactions with the TM2-TM3 loop and TM1 extension to alter channel gating, as described next.

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Orai1 161    KESPHERMHR HIELAWAFST VIGTLLFLAE VVLLCWVKF
Orai2 135    SESPHERMHP YIELAWGFST VLGILLFLAE VVLLCWIKF
Orai3 136    HQSPHQRLHR YVELAWGFST ALGTFLFLAE VVLVGWVKF
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Figure 18: Partial sequence alignment of Orai1, 2 and 3 TM2-TM3 loops

Adapted from Varnai, Hunyady et al. 2009, and depicting some potentially important differences between the TM2-TM3 loops of the three Orai proteins, particularly Q137 and Q141 of Orai3, and S136 & S

The Orai1 crystal structure revealed, not only the hexameric configuration of the Orai subunits, which were previously assumed to be a tetramer, but also the presence of TM 'extension' regions of TM1 and TM4 (Hou, Pedi et al. 2012). Previously assumed as parts of N- and C-terminal tails, these TM extensions provide strong docking points for STIM, and simultaneously, regions with which modifiers of SOCe can potentially interact. DPB's do not affect STIM-Orai interactions, at least at the C-terminal (Fig. 15), as revealed by FRET analysis of whole STIM1, STIM1 mutants and SOAR, as well as with Orai1 mutants. Thus, it is seems plausible DPB's may elicit their effect on Orai channel hexamers by interacting with Orai N-terminal extensions to alter channel gating. With the tight configuration of six Orai1 TM1 domains to form the channel pore (Fig 3), and the close proximity of the intracellular TM2-TM3 loop (with their previously described subtle amino acid differences), it's conceivable DPBs may bind with the loop and TM1 extension to hold the Orai1 gating region in a closed or non-gating position, preventing the pore from opening, despite the interactions of STIM with the N-terminal extension to open the channel. The K85 residue in TM1 extension is in the cytoplasm, directly under TM1, thus, accessible to STIM. The Orai1 mutant, K85E, has STIM-mediated channel gating intrinsically blocked (Lis, Zierler et al. 2010), yet STIM still interacts with Orai, even in the presence of DPBs (Fig 15d). Thus, it's conceivable that with wildtype STIM and Orai, DPBs may hold the N-Terminal extension in the 'channel-closed' configuration, or in an orientation which prevents the gating movement elicited by STIM, yet, still allowing STIM-Orai interactions at the N- and C-termini. Certainly, the direct effect DPB's exhibit on Orai1V102C indicate the molecules can interact with

the Orai1 channel. The visualized increase in Ca^{2+} entry and current, as well as a leftward shift in reversal potential in the Orai1V102C mutant channel, indicates a change in selectivity, most likely due to further widening of the Orai1V102C pore, as discussed below.

4.2 Potentiation of Orai1V102C by DPB's indicates a direct interaction with Orai1 channels

Insights from mutant Orai1V102C channel indicate STIM-Orai1 interactions are essential for DPBs to affect their inhibitory properties on SOCe. Yet, DPB’s are capable of directly potentiating Orai1V102C with or without STIM1 (Figs. 11 & 12), an unusual effect, particularly as DPB162-AE was previously described to be exclusively inhibitory. This direct potentiation by DPB162-AE is obviously a result of the mutation in the pore lining region of Orai-TM1. Clearly, Orai1V102C alters the pore architecture, and potentially shifts the orientation of TM1 extension to increase accessibility of DPB's to their putative, as yet undefined, binding site. Thus, whether or not STIM1 is bound with Orai1, DPB162-AE can elicit an increase Ca^{2+} entry through the channel, perhaps widening the pore further, by an action similar to that of 2-APB on Orai3, but this accessibility may not be possible on wildtype Orai1. Given the potentiation of Orai1V102C is transient, and there is no evidence of inhibition of Orai1V102C, even when STIM1 is interacting with the mutant channel, it appears likely DPB162-AE is causing an alteration of Orai1 gating, as opposed to preventing interactions between STIM1 and Orai1 at the C-terminus (Fig. 15). My perspective is the mutant channel has a different membrane topology to the wildtype channel, i.e. wider at the TM extension, with DPB's making this opening wider still. Despite STIM1 interactions with Orai1V102C, when DPB162-AE is present, there is no reversion back to a highly Ca^{2+} selective state as reported previously (McNally, Somasundaram et al. 2012). However,

the wildtype channel does not have this 'wider pore', therefore, DPB's cannot open the further and only function to inhibit SOCe via STIM1 or potentiate SOCe via STIM2, with STIM2 conferring different properties or membrane topology to Orai1 to facilitate the exclusive potentiation.

While the activation of Orai1 by STIM1 has been proposed to be modular, i.e. with STIM1 binding Orai1ct to 'prime' the channel, and STIM1 binding to Orai1nt to gate the channel, it is more likely a synergistic coupling of STIM1 to both the N and C termini of Orai1, with both interactions stabilizing the channel complex, modulating selectivity and simultaneously 'priming and gating' the channel for Ca^{2+} entry (McNally, Somasundaram et al. 2013). Thus, DPB inhibition of SOCe and I_{CRAC} modifies the synergy by altering gating while not affecting channel selectivity or coupling with STIM1.

Finally, since I observed a sustained inhibition of wildtype Orai1-STIM1 mediated SOCe after washout of DPB162-AE, I sought to discern whether the effect of DPB162-AE on activation of the mutant channel was sustained in a similar manner. In HEK Orai1V102C stable cells, DPB162-AE caused activation of constitutive calcium entry. Following multiple solution changes containing no DPB162-AE, readdition of DPB162-AE was unable to elicit a second potentiation effect (Fig. 11e. left panel). Furthermore, it appears the initial enhancement of calcium entry was sustained, even when DPB162-AE had been washed out, since there was no sharp decrease in calcium entry to the original baseline level. A similar lack of re-potentiation was evident in Ca^{2+} current analysis through the mutant channel (Fig. 11e. right panel). This substantiates the

earlier experiment on STIM1-Orai inhibition, supporting a sustained interaction of DPB162-AE with Orai1, either to inhibit SOCe, as previously shown (Fig. 8c), or to maintain enhanced calcium entry through the mutant channel (Fig.11e), despite washout of the drug.

DPB162-AE and DPB163-AE do not appear to be pore blockers, but instead, are most likely modifiers of Orai1 transmembrane domain structure and orientation, likely via interactions at the TM1 extension perhaps bridging it with the TM2-TM3 intracellular loop. Yet, the exact mechanism of action of the analogs directly on Orai1 requires a great deal of further investigation.

4.3 STIM1 is absolutely required for inhibition by both DPB's

STIM1 and STIM2, while pretty similar in the majority of the important protein domains shared between them, still display remarkable difference in their activation of SOCe and in the effects both DPB's have on SOCe mediated by either protein when expressed in conjunction with Orai1.

Endogenous STIM1-Orai1 SOCe was completely inhibited by DPB's but STIM2-mediated SOCe was not at all inhibited (Fig 14 d,e). While differences between STIM1 and STIM2 have been reported at the N-Terminus (Stathopulos, Zheng et al. 2009; Zhou, Mancarella et al. 2009), it is more likely subtle differences in the SOAR region between STIM1 and STIM2 account for the differential effects of DPB's. For the most part, there is a high degree of identity between SOAR1 and SOAR2, however, one significant amino acid difference in the second α-helix of the SOAR2, whereby SOAR1 has Phenylalanine at position 394 (F394), SOAR2 has Leucine in the corresponding position (L485). The Sα2 helix of SOAR has been proposed to be the gating helix of STIM which interacts with Orai1 N-terminal to gate the channel. Ongoing work in our lab will resolve the importance of this difference between STIM1 and STIM2 in the SOAR region and the implications for STIM1 and STIM2 activation of Orai1 (unpublished). Though the effect of DPB's on a reverse mutation in each protein (STIM1F394L and STIM2L485F) has not yet been investigated, that difference may prove to be the keystone in resolving the difference in inhibitory profiles of DPB's on STIM1 and STIM2. Additionally, coupled with other subtle sequence substitutions in the SOAR regions between STIMs, the amino

acid sequence differences appear important in the function of 2-APB on STIM1 and STIM2 (unpublished). These amino acids may have implications for the lack of inhibitory profile for DPB's on STIM2 vs. STIM1. It is becoming increasingly clear that STIM2 is functionally important, yet much less investigated than STIM1, and that the differences between STIM1 and STIM2 while initially appearing inconsequential, have important physiological implications, as well as provide useful information in the development of inhibitors of SOCe.

4.4 Activation of STIM2 and STIM2ct by DPB163-AE

There is a remarkable difference between the actions of DPB's on the C-terminal STIM mutants. STIM1ct is not at all affected by DPB's, yet STIM2ct is robustly activated to increase Ca^{2+} entry through Orai1 (Fig. 16b). As mentioned in the previous section, there is high sequence identity between STIM1 and STIM2, at least in the N- and C-terminal domains which are conserved between STIM1 and STIM2. Thus, it would seem the differences which are evident within these C-terminal domains, are indeed significant and point to those domains as important for inhibition/activation by DPB's between STIM1 and STIM2. The other alternative is the longer variable region on STIM2ct, which likely affects protein folding and could contribute to slightly different structural orientation and domain accessibility to that seen in STIM1.

The recent publication of the STIM1 SOAR structure (Yang, Jin et al. 2012) from *C. elegans* and the subsequent modeling of human SOAR (Soboloff, Rothberg et al. 2012) on the PDB coordinates from *C. elegans SOAR,* revealed a number of important structural motifs within the SOAR structure. Furthermore, a few potentially significant amino acid differences between STIM1 and STIM2 may have implications for the structure between the two proteins, and indeed may account for the effects of different drugs on SOCe mediated by STIM1 vs. STIM2. Resolving the exact nature of the differences will require a great deal further work, though the exciting publication of a partial crystal structure for SOAR1 reveals the potential for crystallization of SOAR2 and subsequently whole STIM1 and STIM2 proteins. Taken in conjunction with Orai1 crystal

structure, co crystallization of both proteins, as well as with pharmacological agents is essential to define protein interactions, and the influence of subtle amino acid differences on structural differences between STIM1 and STIM2. Whether those differences are sufficient to account for the great disparity in the effect of 2-APB and DPB's on STIM1 vs. STIM2 remains to be seen.

Aside from the lack of effects of DPB's on STIM1ct, the drugs also had no effect on the robust potentiation effect on STIM1ct by 2-APB. Despite the implied dimeric structural similarities between 2-APB (Dobrydneva, Abelt et al. 2006) and DPB's, this result (Fig. 13c) indicates the two groups of compounds are fundamentally different either in how they interact with the channel complex, or that their sites of action on the proteins are different, or both. They also appear to be non competitive; DPB's have sustained effects on inhibition of SOCe (Fig. 8c) and constitutive Ca^{2+} entry though the mutant Orai1 channel (Fig. 11e), implying a long term interaction with the channel complex, even after washout of the drugs. Yet, DPB's did not prevent 2-APB effects on STIM1ct, further supporting a difference in the sites of action of DPB's and 2-APB.

STIM2ct was activated by DPB162-AE, consistent with reports that STIM2ct is easily coerced into interactions with Orai1 (Wang, Deng et al. 2009), though the activation was relatively small when compared to that of DPB163-AE (Fig 14 d,e), and intriguing, given DPB162-AE was previously reported to be exclusively inhibitory (Goto, Suzuki et al. 2010). It is likely this abnormal report was due to the presence of STIM1 in the heterologous testing system used to quantify the effects of DPB's, and indicates the inhibitory effect of DPB162-AE on STIM1 is greater than the excitatory effect STIM2

when the proteins are expressed together. It could also be accounted for by greater expression of STIM1 in that testing system compared to the expression of STIM2. In this report, DPB function was tested STIM1 knockout cells, leaving only STIM2 present in those cells. Therefore, the ability to visualize an effect of DPB162-AE on STIM2 was not clouded by the presence of STIM1. DPB162-AE and DPB163-AE were not inhibitory over STIM2-Orai1 SOCe in DT40 knockout cells (Fig. 14d,e), further indicating a differential role for DPB's over STIM1 vs. STIM2.

The inhibition of STIM1-SOAR mediated entry is fascinating. SOAR is a much smaller fragment than the entire STIM1ct, and does not contain the STIM1 inhibitory helix (Fig 1), Therefore SOAR is can readily bind to and activate Orai1, and that entry is robustly inhibited by DPB162-AE, while the FRET interactions between SOAR and Orai1 are not affected, again indicating a role for STIM1 in DPB162-AE inhibition of SOCe. Thus, DPB's effects on SOCe are likely through N-terminal interactions modifying gating rather than on the C-terminal ‘priming’ interactions between STIM1-Orai1.

4.5 Final Summary

The evidence presented, strongly hints that the DPBs are interacting primarily with Orai1 on the intracellular side of the membrane, likely at the Orai1 N-terminal domain TM1 extension region, to inhibit SOCe. A great deal further work is required to nail down the exact nature of the interactions between STIM1, Orai1 and DPB's.

Analysis of the DPB's on other calcium permeant channels supported the Orai1 specificity proposal. Expressed TRPC3, TRPC6 and $Cav_{1.2}$ were not at all affected by DPB's. In endogenous expression systems (Jurkat, CEM3.71, DT40 and RBL-2H3 cells) SOCe was completely inhibited by both analogs (2 μM).

Overall, I reveal that DPB's are highly potent and specific SOCe modifiers, and likely alter the Orai1 protein rather than STIM, to perturb calcium entry though the Orai1 channel. Inhibition by these drugs is mediated exclusively by STIM1 while potentiation *by both* DPB's is exclusively via STIM2.

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