

# Characterisation of store-operated calcium entry in a vascular endothelial cell line and impact on the production of nitric oxide

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This thesis is dedicated to my mother

**Philippa Jane Batchelor**

1957-2013

*You cannot direct the wind*

*but you can adjust the sails*

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## Abstract

Store-operated  $\text{Ca}^{2+}$  entry (SOCE) is a principal mechanism for extracellular  $\text{Ca}^{2+}$  entry in non-excitabile cell types, and is primarily facilitated by the  $\text{Ca}^{2+}$ -release activated  $\text{Ca}^{2+}$  (CRAC) channel; itself comprised of the pore-forming Orai-1 and  $\text{Ca}^{2+}$ -sensing Stromal interaction molecule (STIM)-1 proteins. Depletion of endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  stores initiates STIM-1 translocation to defined ER-plasma membrane puncta, and subsequent Orai-STIM interaction and opening of Orai. The importance of this mechanism in  $\text{Ca}^{2+}$  signalling in diverse tissue types is becoming increasingly clear. The vascular endothelium is a dynamic tissue, involved in the maintenance of vascular homeostasis and haemostasis. Many endothelium-derived bioactive agents, such as endothelin-1, prostaglandins, and the potent vasodilator nitric oxide (NO), are known to be produced via  $\text{Ca}^{2+}$ -dependent mechanisms. However, the role of the CRAC channel in the vascular endothelium is poorly defined with little known about downstream targets of  $\text{Ca}^{2+}$  influx through CRAC channels.

The dysregulation of NO production by endothelial nitric oxide synthase (eNOS) is a major contributory factor in many vascular disease states, yet the  $\text{Ca}^{2+}$  channel responsible for eNOS activation has yet to be identified. Within this thesis, I establish the endothelial cell line sEnd.1 as a new model system for studying CRAC channel signalling in the vascular endothelium, defining sEnd.1 SOCE as being CRAC channel-dependent. Inhibition of CRAC channels with an array of inhibitors, and knock-down of STIM-1, both reduced ATP- and TG-induced SOCE. The sEnd.1 model system was subsequently used to identify  $\text{Ca}^{2+}$  entry through

the CRAC channel as the elusive activation mechanism for eNOS. Through real-time imaging with the fluorescent NO dye DAF-2-DA, we established that NO production is non-linear, with a slow initial increase preceding a faster NO production phase. These kinetics, with a characteristic delay before fast production have, to our knowledge, not previously been reported. The time taken to reach the fast phase of NO production could be manipulated through changes in both local and bulk  $\text{Ca}^{2+}$  rises, which indicated roles for both elements of  $\text{Ca}^{2+}$  signalling in eNOS activation. eNOS regulation by  $\text{Ca}^{2+}$  is complex, occurring not only through direct binding of  $\text{Ca}^{2+}$ -calmodulin, but additionally through changing post-translational modifications, which in turn regulate the  $\text{Ca}^{2+}$ -dependency of eNOS, such as phosphorylation of Ser1177. We propose that the delay in fast production of NO is due to the time taken to alter eNOS post-translational modifications, which thus remove inhibition on eNOS. Activation of CRAC channels increased phosphorylation of residue Ser1177 via  $\text{Ca}^{2+}$ -calmodulin kinase II (CaMKII), with a similar time course to that required to reach the fast phase of NO production. Inhibition of CaMKII increased the time taken to reach fast activation.

In conclusion this thesis presents a new model system for investigation of CRAC channel signalling in the endothelium. Furthermore, we clearly identify a critical endothelial pathway as being regulated by CRAC channels, by demonstrating the production of NO in response to both ATP and TG, which stimulate  $\text{Ca}^{2+}$  entry through CRAC channels.

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# Nomenclature

$I_{\text{CRAC}}$   $\text{Ca}^{2+}$ -release activated  $\text{Ca}^{2+}$  current

$\text{ONOO}^-$  peroxynitrite

ACE angiotensin-converting enzyme

ACh acetylcholine

AMPK 5'-adenosine monophosphate-activated protein kinase

APT-1 acyl-protein thioesterase-1

ATP adenosine triphosphate

B biopterin

BAEC bovine aortic endothelial cell

BAPTA 1,2-bis(2-aminophenoxy)ethane-N-N-N'-N'-tetraacetic acid

$\text{BH}_2$  7',8'-dihydrobiopterin

$\text{BH}_4$  tetrahydrobiopterin

BHQ 2,5-di-*t*-butyl-1,4-benzohydroquinone

BK bradykinin

BSA bovine serum albumin

CAD CRAC-activating domain

CaM calmodulin

CaMKII calmodulin kinase II

cAMP 3'-5'-cyclic adenosine monophosphate

cAMP cyclic adenosine 3',5'-monophosphate

CMD CRAC modulatory domain

CPA cyclopiazonic acid

CPAE cow pulmonary artery endothelial cell

CRAC  $\text{Ca}^{2+}$ -release activated  $\text{Ca}^{2+}$

DAF-2 4,5-dimamiofluroscein

DAF-2-T DAF-2-triazole

DAG diacylglycerol

DAPI 4',6-diamidino-2-phenylindole

DHE dihydroethidium

DHFR dihydrofolate reductase

DVF divalent free solution

EDRF endothelium-derived relaxation factor

EGTA ethylene glycol tetraacetic acid

eNOS endothelial nitric oxide synthase

ER endoplasmic reticulum

ERM ezrin-radixin-moesin domain

ET-1 endothelin-1

FAD flavin adenine dinucleotide

FCCP trifluorocarbonylcyanide phenylhydrazone

FMN flavin mononucleotide

GPCR G-protein coupled receptor

GSNO s-nitrosogluthione

HDMECs human dermal microvascular endothelial cells

HEK human embryonic kidney cell

HPLC high performance liquid chromatography

HSP-90 heat-shock protein-90

HUVEC human umbilical vein endothelial cell

I<sub>SOC</sub> store-operated Ca<sup>2+</sup> current

iNOS inducible nitric oxide synthase

IP<sub>3</sub> inositol 1,4,5-triphosphate

KN-62 1-[N,O-bis(1,5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenyl-piperazine



MCU mitochondrial  $\text{Ca}^{2+}$  uniporter

MgGTP magnesium guanosine 5'-triphosphate

MLCK myosin light chain kinase

mRNA messenger ribonucleic acid

NADPH nicotinamide dinucleotide phosphate

NCX sodium calcium exchanger

NF $\kappa$ B nuclear factor- $\kappa$ B

nNOS neuronal nitric oxide synthase

NO nitric oxide

NOHA N-hydroxy-L-arginine

Nor-NOHA N $^{\omega}$ -hydroxy-nor-Arginine

NOS nitric oxide synthase

NSC non-selective channel

PBS phosphate-buffered saline

PBS-T phosphate-buffered saline with Tween

PI3K phosphatidylinositol 3-kinase

PIP<sub>2</sub> phosphatidylinositol 4,5-bisphosphate

PKA protein kinase A

PKC protein kinase C

PKG cGMP-dependent protein kinase

PLC phospholipase C

PMCA plasma membrane  $\text{Ca}^{2+}$ -ATPase

PMSF phenylmethylsulfonyl fluoride

PP1 protein phosphatase 1

RBL-1 rat basophilic leukemia cell

ROC receptor-operated channel

ROS reactive oxygen species

RT room temperature

SAM steile-alpha motif

SCID severe combined immunodeficiency

SERCA sarcoendoplasmic reticulum ATPase

sGC soluble guanylyl cyclase

siRNA short interfering ribonucleic acid

siRNA short-interfering ribonucleic acid

SNO s-nitrosothiols

SNP sodium nitroprusside

SOAR STIM-1 Orai-1 activating region

SOC store-operated channel

---

SOCE store-operated  $\text{Ca}^{2+}$  entry

STIM stromal interaction molecule

TBS Tris-buffered saline

TBS-T Tris-buffered saline with Tween

TG thapsigargin

TRPC canonical transient receptor potential channel

UT untreated

VEGF vascular endothelial growth factor

VOC voltage-operated channel

VSMC vascular smooth muscle cell

vWf von Willibrand factor

WT wild type

# Chapter 1

## Introduction

$\text{Ca}^{2+}$  is a prolific intracellular signal, which elicits responses ranging from exocytosis to gene expression, and controls vital processes including fertilisation, cell differentiation and proliferation, and apoptosis. Specificity of  $\text{Ca}^{2+}$  signalling is controlled by the amplitude of cytosolic  $\text{Ca}^{2+}$  rise, the spatio-temporal profile of the signal, and the speed with which sensors bind  $\text{Ca}^{2+}$ . Two processes control the intracellular  $\text{Ca}^{2+}$  profile: 1) the increase in cytosolic  $\text{Ca}^{2+}$  levels, and 2) the removal of intracellular  $\text{Ca}^{2+}$ , which are each controlled by numerous mechanisms and pathways, ensuring a malleable and diverse signalling system [26].

The release of intracellular stores, and the entry of external  $\text{Ca}^{2+}$  are responsible for rises in cytosolic  $\text{Ca}^{2+}$  levels. These processes are not mutually exclusive [26]. Cells contain many intracellular  $\text{Ca}^{2+}$  stores: the endoplasmic reticulum (ER), mitochondria, and the acidic stores, such as endosomes, lysosomes, and the Golgi body [261]. Release of store  $\text{Ca}^{2+}$  can initiate  $\text{Ca}^{2+}$  entry by a process termed store-operated  $\text{Ca}^{2+}$  entry (SOCE), and conversely external  $\text{Ca}^{2+}$  entry can cause

store release through  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release.

$\text{Ca}^{2+}$  entry into cells can be facilitated by many different channel types, including the store-operated channels (SOCs), receptor-operated channels (ROCs), second-messenger-operated channels (SMOCs), voltage-operated channels (VOCs), and non-selective channels (NSCs). Store-operated  $\text{Ca}^{2+}$  currents are of particular interest in the  $\text{Ca}^{2+}$  signalling mechanisms of non-excitabile cells.

The removal of excess cytosolic  $\text{Ca}^{2+}$  is essential to attenuate the  $\text{Ca}^{2+}$  signal and prevent  $\text{Ca}^{2+}$ -dependent toxicity.  $\text{Ca}^{2+}$  handling encompasses both the uptake of  $\text{Ca}^{2+}$  into intracellular  $\text{Ca}^{2+}$  stores and extrusion across the plasma membrane.  $\text{Ca}^{2+}$  extrusion is principally controlled by the plasma membrane  $\text{Ca}^{2+}$ -ATPase (PMCA) and the  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger (NCX). PMCA is a  $\text{Ca}^{2+}$ -calmodulin activated  $\text{Ca}^{2+}$  removal pump; saturated at low  $\text{Ca}^{2+}$  concentrations due to a high affinity for  $\text{Ca}^{2+}$ . It is therefore involved in handling small rises in intracellular  $\text{Ca}^{2+}$ . The NCX pump conversely has a high  $\text{Ca}^{2+}$  transport rate and low  $\text{Ca}^{2+}$  affinity; NCX is adapted to respond rapidly to large increases in the cytosolic  $\text{Ca}^{2+}$  concentration [300].

$\text{Ca}^{2+}$  removal is supported by the re-uptake of  $\text{Ca}^{2+}$  into stores, which can act as a feedback mechanism to halt further  $\text{Ca}^{2+}$  influx in certain entry mechanisms. Furthermore, the mitochondria also play a critical role by providing the adenosine triphosphate (ATP) required for the active transport of  $\text{Ca}^{2+}$ , and additionally acting as a  $\text{Ca}^{2+}$  buffer. Large increases in cytosolic  $\text{Ca}^{2+}$  results in increased uptake by the mitochondrial uniporter MCU and other mitochondrial uptake channels such as Letm.1 [18,68,174]. This is driven by the mitochondrial membrane potential that is set up by the electron transport chain. The increased mitochondrial

$\text{Ca}^{2+}$  is re-released into the cytoplasm over a prolonged period, from where it can then be extruded from the cell. Mitochondrial  $\text{Ca}^{2+}$  release is facilitated by the mitochondrial NCLX [253, 256, 295]. Abrogation of the mitochondria limits the sustained  $\text{Ca}^{2+}$  entry of SOCE [144, 145].

This thesis will focus on the role of the  $\text{Ca}^{2+}$ -release activated  $\text{Ca}^{2+}$  (CRAC) channel in the store-operated  $\text{Ca}^{2+}$  fluxes of the vascular endothelium and its importance in activation of the production of the vasodilator nitric oxide (NO).

## 1.1 Orai, STIM, and store-operated $\text{Ca}^{2+}$ entry

### 1.1.1 Store-operated $\text{Ca}^{2+}$ entry

Store-operated  $\text{Ca}^{2+}$  entry can be described by a two-step mechanism proposed by Putney in 1986 [276]. Membrane receptor activation stimulates phospholipase C (PLC) production of inositol 1,4,5-triphosphate ( $\text{IP}_3$ ) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ ). The generation of  $\text{IP}_3$  activates  $\text{IP}_3$  receptors ( $\text{IP}_3\text{R}$ ) on the ER causing  $\text{Ca}^{2+}$  store release [24, 42, 43, 320]. The subsequent store depletion opens membrane store-operated  $\text{Ca}^{2+}$  channels to allow the influx of external  $\text{Ca}^{2+}$ . Entering  $\text{Ca}^{2+}$  fulfills both a signalling role, as well as providing negative feedback on the open channels; being sequestered and refilling ER stores by re-uptake facilitated by the sarcoendoplasmic reticulum ATPase (SERCA) (Fig.1.1) [233, 276, 326, 332].

Electrophysiological studies of store-operated currents indicated that SOCE was mediated by a single population of cell surface channels. The current, termed the

$\text{Ca}^{2+}$ -release activating  $\text{Ca}^{2+}$  current ( $I_{\text{CRAC}}$ ), was first demonstrated in mast cells and is carried by the  $\text{Ca}^{2+}$ -release activated  $\text{Ca}^{2+}$  (CRAC) channel [142, 379].

### 1.1.2 The CRAC channel

The CRAC channel has a single channel conductance of 9fS in 2mM external  $\text{Ca}^{2+}$  and a unitary current of  $\sim 6\text{fA}$  [272].  $I_{\text{CRAC}}$  is a strongly inwardly rectifying current with a positive reversal potential and is characterised by a high selectivity towards  $\text{Ca}^{2+}$ , although CRAC channels are permeable to other divalent ions including  $\text{Sr}^{2+}$  and to a lesser extent  $\text{Ba}^{2+}$ . It is, however, impermeable to monovalent ions, including  $\text{Na}^+$  ( $P_{\text{Ca}}/P_{\text{Na}} > 1000$ ) and  $\text{K}^+$ , distinguishing it from alternative  $\text{Ca}^{2+}$  entry channels including the TRP ion channel family [142, 143]. Only with the removal of all extracellular divalent ions will a monovalent current be supported [143].

Subsequent to the concept of the SOCE mechanism, the molecular components of the CRAC channel took over 20 years to identify. The first elements to be discovered of the CRAC channel were the stromal-interaction molecules (STIM): STIM-1 and -2. The STIM proteins were discovered through multiple siRNA screens in *Drosophila* and human, and had previously been identified as cell adhesion molecules, acting as tumour suppressors [200, 250, 287, 290]. A classical experiment to demonstrate SOCE utilises a  $\text{Ca}^{2+}$ -sensitive fluorophore to visualise increases in intracellular  $\text{Ca}^{2+}$  concentration. Depletion of stores in the absence of extracellular  $\text{Ca}^{2+}$ , followed by readmission of external  $\text{Ca}^{2+}$  dissects the contribution of internal store releases from extracellular  $\text{Ca}^{2+}$  influx. Demonstration of a robust knockdown of  $\text{Ca}^{2+}$  entry using this classic experiment in STIM- defi-

cient cells showed the mechanistic importance of STIM, and led to their proposal as the  $\text{Ca}^{2+}$ -sensing elements of the CRAC channel [200]. STIM-1 and -2 are located in the plasma membrane and, critically, in the ER [213, 358]. Thapsigargin (TG) is a potent inhibitor of SERCA [332]. Within seconds of depletion of stores with TG, or physiological agonist histamine, STIM-1 translocates from a dispersed conformation on the ER to defined puncta 10-17nm from the plasma membrane [200, 360, 372].

The EF-hand of STIM-1 binds  $\text{Ca}^{2+}$  with an affinity in 0.6mM, releasing  $\text{Ca}^{2+}$  upon store depletion [317]. Mutation of  $\text{Ca}^{2+}$ -binding residues, such as D76A, in the EF-hand, causes STIM to form puncta even when stores are filled, constitutively activating CRAC channels [200, 372]. Cumulatively the evidence overwhelmingly suggests a  $\text{Ca}^{2+}$ -sensing role for the STIM proteins in SOCE. Although both STIM-1 and STIM-2 can act as  $\text{Ca}^{2+}$  sensors, their physiological roles differ, as will be discussed later within this chapter.

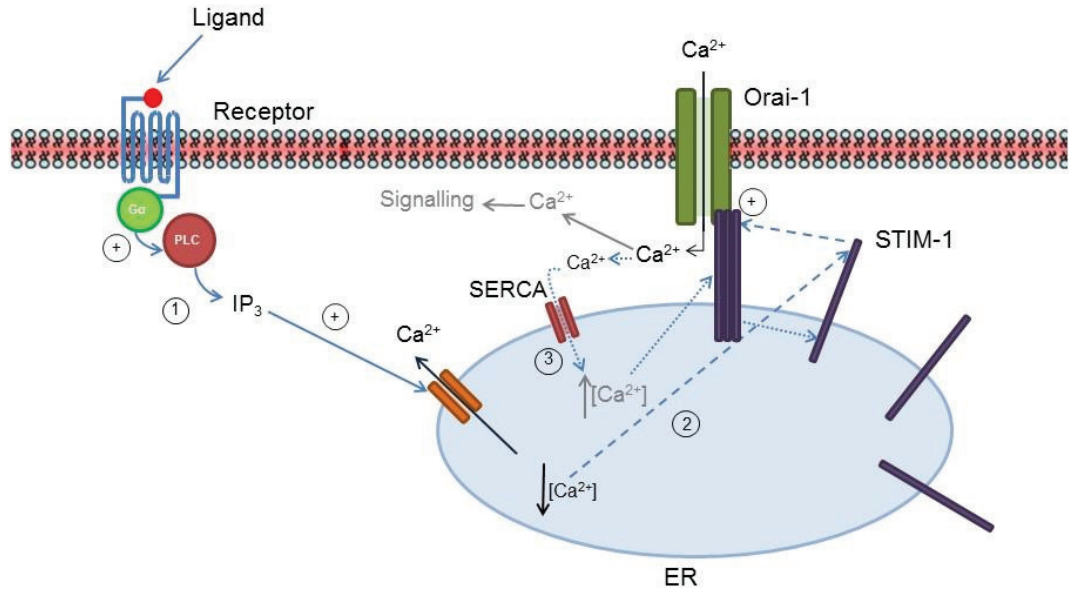
Shortly after the discovery of STIM, the pore-forming subunit of CRAC channels was elucidated. The immune cells of severe combined immunodeficiency patients (SCID) had previously been shown to have impaired SOCE. A combination of linkage analysis of SCID patients and a *Drosophila* RNA<sub>i</sub> screen implicated the Orai protein family in SOCE. The migration of transcription factor NFAT to the nucleus is SOCE-dependent. A whole genome RNA<sub>i</sub> screen of *Drosophila* tracked NFAT-GFP migration in response to TG stimulation, showing abrogated translocation with knockdown of dOrai and dSTIM. Further to this analysis of SCID patients identified a single missense point mutation in the protein Orai-1. Overexpression of wild type Orai-1 in T-cells from these patients rescued the SOCE phenotype [95].



This finding was corroborated in subsequent RNA<sub>i</sub> studies [347,371]. With STIM-1 being the Ca<sup>2+</sup>-sensor, Orai-1 was proposed to be the pore forming component of the CRAC channel; the ability to multimerise and being plasma membrane protein added weight to this theory [346].

Overexpression of STIM-1 and Orai-1 together increases the size of I<sub>CRAC</sub> in expression systems where the current is difficult to detect [222,263]. Co-immunoprecipitation experiments indicated the physical interaction of Orai-1 and STIM-1, and is supported by Foerster-resonance energy transfer (FRET) experiments between STIM-1-YFP and Orai-1-CFP [237,346]. The oligomerisation of STIM-1 occurs rapidly upon store depletion, making migration of oligomers to the ER-PM junctions the rate limiting process in activation of I<sub>CRAC</sub> [199].

Subsequent to the identification of the protein components of CRAC channels, their importance in a wide range of tissues and diseases has now been established. They have been found to be particularly prevalent in non-excitable cells. Orai and STIM mutations show prominent phenotypes in immune cells; this is manifested in the generation of allergic responses, in particular nasal polyposis, and allergic asthma. In mast cells derived from nasal polyps a positive feed-forward reaction is created. Ca<sup>2+</sup> influx through CRAC channels initiates production and release of leukotriene C4 which, in turn, activates CRAC channels in neighbouring mast cells through the receptor cysLT1 [77]. More recent work has indicated that CRAC channels are important in anti-tumour immune responses [353]. Orai and STIM have additionally been linked to congenital myopathies, anhydrosis, dental defects, pancreatitis and kidney issues [94,217,265].



**Figure 1.1 The CRAC channel signalling mechanism:** (1) Activation of PLC-coupled receptor by external stimuli, e.g. histamine, activates intracellular PLC to cleave  $\text{PIP}_2$  generating  $\text{IP}_3$  and DAG.  $\text{IP}_3$  activates ER localised  $\text{IP}_3$  receptors promoting  $\text{Ca}^{2+}$  release from ER stores [276]. (2) ER store release depletes store  $\text{Ca}^{2+}$ , releasing  $\text{Ca}^{2+}$  from the EF-hand of STIM-1, initiating STIM-1 migration to ER plasma membrane junctions [199,200,287,372]. Here, STIM-1 interacts with Orai-1 to form an active CRAC channel, allowing extracellular  $\text{Ca}^{2+}$  influx and a cytosolic rise in  $\text{Ca}^{2+}$  concentration [259]. (3) Extracellular  $\text{Ca}^{2+}$  entry replenishes stores and generates signals within the cell [228]. Rise in ER  $\text{Ca}^{2+}$  concentration allows STIM-1  $\text{Ca}^{2+}$  binding, and dissociation of STIM from Orai closing the CRAC channel.

### 1.1.3 Orai

The Orai ion channel family, consisting of Orai-1,-2, and -3, shows little resemblance to other known ion channels [95]. Orai isoforms are all tetraspanning 33kDa membrane proteins which form homohexameric teardrop-shaped structures [146,215,346]. The Orai pore is formed of a transmembrane (TM)-1 domain lining the pore, TM-2 and -3 domains creating a middle ring around the pore, and TM-4 constituting an outer ring [146,219,377].

Orais -1, -2, and -3 all show similar  $\text{Ca}^{2+}$  selectivity, however Orai-1 is the dominant isoform in SOCE [72]. Overexpression of Orai-1 and -2 with STIM-1 results in enhanced  $\text{Ca}^{2+}$  influx. Orai-3 overexpression, although able to rescue Orai-1 deficiency, does not display increased  $I_{\text{CRAC}}$  when overexpressed with STIM-1 [222]. Contrary to the general belief that Orais only form homomultimers, an Orai-1 and -3 pentameric arrangement has been suggested to form a novel  $\text{Ca}^{2+}$ -conducting ion channel activated by arachidonic acid, known as the archadionate-regulated  $\text{Ca}^{2+}$ -selective (ARC) channel [226]. The function of this channel however is unclear.

#### The Orai pore

The Orai pore environment is created by the side chains of pore-lining residues of the TM-1 region which oppose each other along the entire pore length [219]. The narrow pore diameter of 0.39nm is, in part, responsible for the specificity of CRAC channels, conferring exclusion of larger ions from the pore [11]. The pore can be divided into distinct sections: a hydrophobic region proximal to the extra-

cellular mouth of the pore, a basic region located towards the cytoplasmic side of the channel, and a further cytoplasmic pore extension. The hydrophobic region is well-conserved; it forms a narrow rigid structure, which is likely to contribute to the structural integrity of the pore, as well as contribute to the pore's discrimination of larger divalent ions [146, 219, 377]. The hydrophobic and basic regions are connected by a conserved flexible linker, which opens the cytoplasmic basic region upon channel activation. This reduces electrostatic hindrance to ion conductance imposed in the closed state by the charged basic region [146, 288, 373].

The extracellular portion of the pore is formed of the TM-1/TM-2 extracellular loop, which creates a channelled entrance into the pore mouth. The TM-1/TM-2 loop contains three aspartate residues (D110, D112 and D114) which coordinate and facilitate  $\text{Ca}^{2+}$  ion entry [219, 346].

The pore-lining residue E106 forms a glutamate ring, which confers  $\text{Ca}^{2+}$  selectivity to Orai and is conserved in all three Orai isoforms from *Drosophila* to humans [146, 219, 346, 365, 377]. The glutamate ring is predicted to be just 6 Å across. The narrow structure of the glutamate ring predicts that  $\text{Ca}^{2+}$  would directly interact with the  $\text{O}_2$  component of the glutamate residues when permeating the pore [288].

The mutation R91W is seen in human SCID patients, which reduces the  $\text{Ca}^{2+}$  conductance of Orai. R91 is a TM-1 residue found in a narrow section of the pore close to the cytoplasmic mouth [373]. Mutation of R91 to an amino acid with a larger side chain can create a steric occlusion of Orai [140, 219]. This is supported by the R91C mutant maintaining normal activation, including STIM interaction, oligomerisation, and  $\text{Ca}^{2+}$  conductance. Reduction of the cysteine residues to form disulphide bonds, however abrogates  $\text{Ca}^{2+}$  entry through Orai.

Together this demonstrates that R91 isn't required to facilitate  $\text{Ca}^{2+}$  permeation, but the presence of a steric hindrance at this narrow section of the pore is capable of reducing  $\text{Ca}^{2+}$  entry [373].

The pore structure can be altered through mutations of non-pore residues, such as TM-3 E190. Mutation of E190 alters selectivity through allosteric changes in the tertiary structure, which sterically occludes the pore [219, 272, 346]. Mutation of E190 reduces pore diameter, decreasing  $\text{Ba}^{2+}$  permeation thus altering selectivity [219, 346].

#### 1.1.4 Stromal-interaction molecule

STIM-1 and -2 are ubiquitously expressed, single span membrane proteins which transduce the  $\text{Ca}^{2+}$  level of the ER stores to plasma membrane-embedded Orai [213, 357, 360]. The cytosolic C-terminus interacts with Orai, instigating channel opening, whilst the ER-located N-terminus senses  $\text{Ca}^{2+}$  and controls oligomerisation [357].

STIM-1 and -2 are closely related, sharing multiple homologous regions, mainly varying in their length and C-terminal regions. The functional domains in both the N- and C-termini are highly conserved between STIM-1 and -2, however STIM-1 is dominant in the generation of SOCE [357].

STIM-2 is expressed at a lower level than its homologue, STIM-1, activating a smaller fraction of Orai-1; thus, not generating a detectable  $I_{\text{CRAC}}$  current [205, 247]. STIM-2 has been reported not to redistribute and form puncta in response to store depletion; however it is found close to the plasma membrane prior to store

depletion [260]. Nevertheless, STIM-2 positively regulates  $\text{Ca}^{2+}$  influx and can support NFAT migration to the nucleus in the absence of STIM-1. It can also partially rescue SOCE when STIM-1 is deficient [247].

The key role of STIM-2 is thought to be in  $\text{Ca}^{2+}$  homeostasis within the cell. STIM-2 binds  $\text{Ca}^{2+}$  with lower affinity than STIM-1 and, therefore, requires less store depletion to induce a  $\text{Ca}^{2+}$  influx. It is thought that under resting conditions a significant pool of STIM-2 may already be activated and coupled to Orai-1; maintaining basal cellular  $\text{Ca}^{2+}$  levels and ensuring stores are not depleted through passive leak [28, 33]. It has been suggested that STIM-2 is able to signal both dependently and independently of store depletion, with a population of STIM-2 remaining constitutively active [124, 260].

### **The $\text{Ca}^{2+}$ -sensing mechanism of STIM-1**

The typical resting ER luminal  $\text{Ca}^{2+}$  content is between 600-800 $\mu\text{M}$  in non-excitabile cells. Upon agonist-induced store depletion, local  $\text{Ca}^{2+}$  content in the ER stores falls to between 200-400 $\mu\text{M}$  [25]. STIM-1, although exhibiting a variable temperature-dependent  $K_d$  of  $\text{Ca}^{2+}$ -binding, will bind  $\text{Ca}^{2+}$  with a  $K_d$  of  $\sim 500\mu\text{M}$  at physiological temperatures [148]. STIM-1 is, therefore, well suited to being a sensor of  $\text{Ca}^{2+}$  store content after stimulation.

The N-terminus of STIM-1 contains the principle elements of  $\text{Ca}^{2+}$ -sensing: the canonical and hidden EF-hands, and the sterile-alpha motif (SAM) domain [318, 319].

The EF-hand is a conserved  $\text{Ca}^{2+}$ -binding motif found in a plethora of  $\text{Ca}^{2+}$ -

binding proteins [363]. The hidden EF-hand of STIM-1 stabilises the canonical EF-hand  $\text{Ca}^{2+}$ -binding region [319]. Only the canonical EF-hand binds  $\text{Ca}^{2+}$ ; each STIM-1 monomer is, therefore, saturated with the addition of just one  $\text{Ca}^{2+}$  ion [317].

The EF-hand structure is linked to the SAM domain via a short  $\alpha$ -helix [319]. SAM domains are common structural elements found in over 1000 proteins, and are important in invoking intermolecular interactions, through association with other SAM domains, as well as non-SAM elements [169].

The EF-SAM domain has a primarily negative electrostatic surface under physiological conditions. Depletion of  $\text{Ca}^{2+}$  and release from the EF-hand increases the hydrophobicity and decreases the stability of STIM monomers as they move from an 'open' to a 'closed' conformation- demonstrating a change from a tight structure to an elongated state. Exposure of hydrophobic residues promotes oligomerisation, which is facilitated by the SAM domain [319].

The C-terminus of STIM-1 is required for the formation of stable resting dimers, as well as the higher order multimers observed upon store-depletion. C-terminal deletion mutants of STIM still aggregate due to the clustering abilities of the N-terminal SAM domains but display reduced aggregate stability, which indicates a stabilisation role of the C-terminus. It is also required for STIM-Orai interactions [56].

The C-terminus of STIM-1 is a polybasic region comprised of three coiled-coil domains, a CRAC modulatory domain (CMD), a serine-proline rich region, and a C-terminal lysine-rich region [140, 147].

The first coiled-coil is required for stabilisation of STIM-1 oligomers in both resting and activated states [56]. The second and third coiled-coil domains, with a small C-terminal sequence extension, form the minimal activating sequence of STIM-1, known as both the CRAC-activating domain (CAD) and STIM-1 Orai-1 activating region (SOAR) [259, 367]. The CAD/SOAR region stretches 107 amino acids between amino acids 342-448 [259]. CAD/SOAR directly interacts with the C- and N-termini of Orai-1 [237, 259]. Expression of a truncated STIM protein consisting of the CAD/SOAR domain is sufficient to activate Orai-1 constitutively without store depletion [235]. The CAD/SOAR domain therefore represents the minimal activating domain of STIM.

### 1.1.5 Orai-STIM interaction

STIM-1 oligomerisation upon  $\text{Ca}^{2+}$  depletion is the activating switch for CRAC channel activity, however it is not sufficient to open Orai channels [205, 259, 367]. Orai-1 extends 10nm into the cytoplasm, and when combined with the C-terminus of STIM-1, the proteins are able to cumulatively span the 14-17nm distance between the ER and PM, allowing their direct interaction and coupling [376, 377]. Electrostatic coupling between the CAD/SOAR domain of STIM and the N- and C-termini of Orai-1 clusters Orai in the ER-PM puncta [259]. Both termini play a role in STIM activation of Orai. Truncation of the N-terminus of Orai-1 still supports STIM-1 binding but prevents  $\text{Ca}^{2+}$  flux through Orai-1, whereas mutation or truncation of the C-terminus prevents STIM-1 interaction and, thus, prevents STIM activation of Orai-1 [374]. Weak electrostatic interaction of the N-terminus with STIM modulates the shape of the pore increasing Orai permeability [75].



The C-terminal TM-4 cytoplasmic extension is proposed to robustly interact with STIM-1 by electrostatic interactions ensuring the physical coupling of Orai and STIM [107, 237]. Upon interaction with Orai-1, the STIM-1 C-terminal switches from a closed to an elongated open conformation [236]. A hexameric structure for Orai-1 has recently been proposed, with it recently being established the 2 STIM-1 and required per Orai monomer, the stoichiometry of the CRAC channel has now been established as 12 STIM proteins to 1 Orai pore [141, 146].

## 1.2 The physiological role of the vascular endothelium

$\text{Ca}^{2+}$  signalling is critical in the endothelium, with store-operated  $\text{Ca}^{2+}$  entry playing a major role in these non-excitabile cells.

The vascular endothelium was once thought of as an inert barrier layer, separating and protecting the underlying vascular smooth muscle from the shear stress exerted by blood flow. Indeed the endothelium does act as a dividing layer, albeit a semi-permeable membrane, however the endothelium is additionally a highly complex and dynamic tissue.

In 1981 the regulatory function of the endothelium was discovered by Furchgott and Zawadzki, who observed vasoconstriction in response to acetylcholine (ACh) in the absence of endothelial cells but, conversely, vasodilation in their presence [112]. They concluded that ACh must cause the release of a relaxation-inducing compound from the endothelium; they named this compound endothelium-derived

relaxation factor (EDRF) , now known to be nitric oxide (NO) [252].

Since this time, the endothelium has been recognised to facilitate a wide range of homeo- and haemostatic mechanisms including transfer of nutrients and  $O_2$  into tissues, mediation of white blood cell migration, control of thrombus formation, regulation of vascular tone and smooth muscle proliferation, and initiation of angiogenesis [69]. The endothelium fulfills these wide roles through the production and release or externalisation of many bioactive agents which include, but are not limited to, prostaglandins, endothelin-1, NO, and angiotensin-converting enzyme (ACE) (see Fig.1.2 for overview).

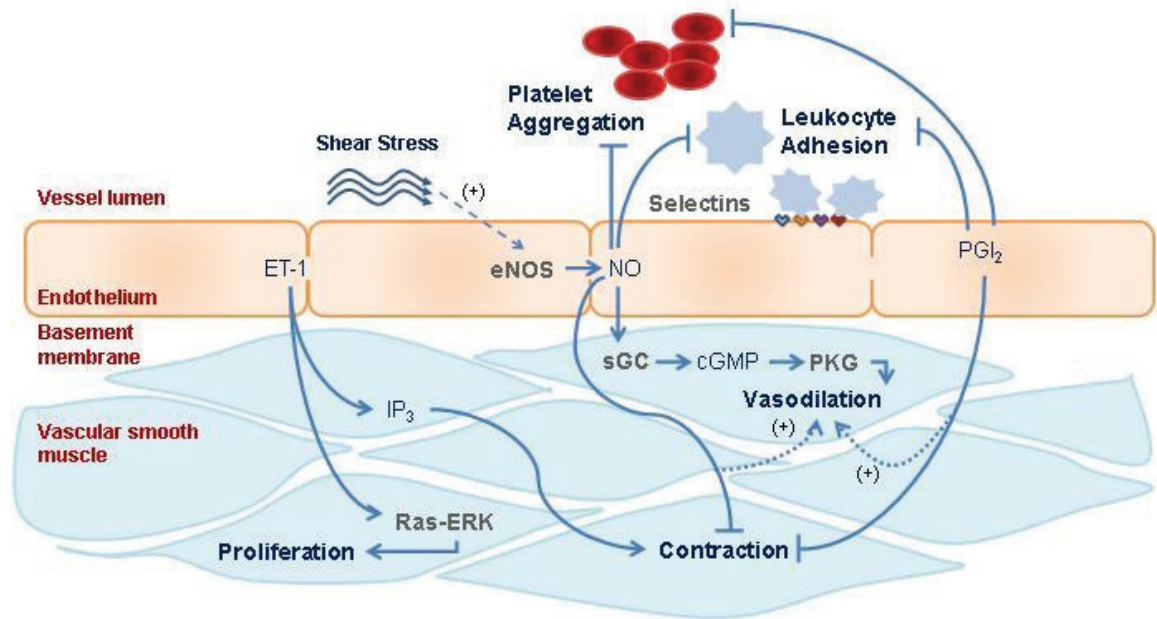


Figure 1.2 A scheme demonstrating the haemo- and homeostatic effects of endothelium-derived bioactive agents

Regulation of vascular tone is a key role of the endothelium, and is managed by the release of many interacting bioactive agents which alter the balance between constriction and dilatation (Fig.1.2). Vascular contraction is mediated by an increase in intracellular  $\text{Ca}^{2+}$  in vascular smooth muscle cells, which results in the formation of  $\text{Ca}^{2+}$ -calmodulin (CaM).  $\text{Ca}^{2+}$ -CaM activates myosin light chain kinase (MLCK), which subsequently phosphorylates myosin. Phosphorylation activates myosin, and allowing its interaction with the actin filament, thus facilitating contraction by crossbridge cycling. Many vasodilators act on this pathway to prevent contraction.

Prostaglandins, produced from arachidonic acid by cyclooxygenase (COX)-1 and -2, are important inflammatory mediators. Prostaglandins signal through  $\text{G}_q$ -coupled protein receptors, activating adenylyl cyclase and therefore increasing 3'-5'-cyclic adenosine monophosphate (cAMP) levels [209]. Increase in cAMP activates protein kinase A (PKA), which phosphorylates MLCK, inactivating the enzyme, thus preventing vasoconstriction [129,284]. Prostaglandins have a variable effect on vasodilation across vessel beds, and modulates local, rather than systemic changes, in blood pressure [209]. Furthermore, prostaglandins can additionally be converted into thromboxane by thromboxane-A-synthase in platelets which, conversely, is a potent vasoconstrictor and pro-thrombotic agent. The balance between thromboxane and  $\text{PGI}_2$  helps maintain vascular homeo- and haemostasis [209]. In the endothelium,  $\text{PGI}_2$  also demonstrates anti-thrombotic effects through inhibition of platelet aggregation [229,232,309].

Angiotensin II regulates cell growth and apoptosis by stimulation of growth factor production. It is a potent vasoconstrictor, further inducing production of alter-

native vasoconstrictors. It regulates plasma volume and thirst, and plays a role in sympathetic nervous system signalling [334]. Angiotensin II is produced by a two-stage process. Angiotensinogen is cleaved by renin to form angiotensin I, which is then converted to the more potent angiotensin II by ACE on vascular endothelial cells [297]. ACE and angiotensin II are significant in the pathology of clinical hypertension. Treatment of a hypertensive patient with ACE inhibitors, e.g. perindopril and captopril, significantly alleviates the rise in blood pressure, whereas very limited changes are observed in healthy individuals.

Endothelin-1 (ET-1) is produced by the endothelium, macrophages, leukocytes, cardiomyocytes, and vascular smooth muscle cells (VSMC) [206]. There are 2 known ET receptors  $ET_A$  and  $ET_B$ , which exert opposing effects upon ET-1 binding.  $ET_B$  receptors, found primarily in the endothelium, activate NO production and vasodilation. Activation of  $ET_A$  results in VSMC PLC activation and a subsequent increase of cytosolic  $Ca^{2+}$  and  $IP_3$ , generating an extended and potent vasoconstriction [173, 206]. ET-1 increases constriction in atherosclerotic arteries and stenoses [173]. It is a major contributor to many pathologies in the cardiovascular system, in particular in pulmonary hypertension, and heart failure, where it is a predictor of prognosis [206].

The free radical NO is the final major contributor to vascular tone, and will be discussed in detail later in this chapter.

## 1.3 $\text{Ca}^{2+}$ signalling in the vascular endothelium

The vascular endothelium is a dynamic tissue required to adapt to its changing environment. Exposure to external factors such as cytokines, pH, and shear stress, result in the activation of signalling cascades within individual endothelial cells and further transmission of signals through the endothelial monolayer.

As a major signalling messenger, a rise in intracellular  $\text{Ca}^{2+}$  concentration above the basal level of  $\sim 100\text{nM}$  is known to control many essential processes [25]. These include the synthesis of NO, prostaglandin, and von Willibrand factor (vWf), modulating vascular permeability, cell proliferation and angiogenesis [92, 243, 351].

The  $\text{Ca}^{2+}$  entry pathways controlling signalling in the endothelium are complex, in large part because  $\text{Ca}^{2+}$  entry channels and  $\text{Ca}^{2+}$  handling mechanisms differ between vascular beds, creating heterogeneity across the tissue. Examples of this have been seen in the variation of SOCE signals between different endothelial models [298].

All of the major  $\text{Ca}^{2+}$  entry pathways have been identified in endothelial cells including, surprisingly for a non-excitable cell, VOCs.  $\text{Ca}^{2+}$  entry in endothelial cells is mainly governed by NSCs and SOC, with CRAC and TRPC channels prevailing across vascular beds [348, 352].

### 1.3.1 SOCE in the vascular endothelium

The presence of SOCE in the endothelium has long been established. Early work by Rajan in 1992 tested the SERCA blockers TG, cyclopiazonic acid (CPA),

and 2,5-di-*t*-butyl-1,4-benzohydroquinone (BHQ) in bovine aortic endothelial cells (BAEC) and cow pulmonary artery endothelial cells (CPAE). They demonstrated SERCA block could induce the release of  $\text{Ca}^{2+}$  from bradykinin (BK)-sensitive internal  $\text{Ca}^{2+}$  stores. The  $\text{Ca}^{2+}$  response was not maintained in the absence of extracellular  $\text{Ca}^{2+}$  and rapidly declined. However, in the presence of external  $\text{Ca}^{2+}$ , a biphasic response was generated, indicating the requirement of both  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  entry.  $\text{Ca}^{2+}$  influx in CPAE was blocked by the lanthanide  $\text{La}^{3+}$  [298].

SOCE was independently confirmed electrophysiologically by two groups, using physiological agonists (ATP and BK), SERCA blockers TG and BHQ, and inclusion of  $\text{IP}_3$  in the patch pipette [249,342]. Currents induced by BHQ, BK, and ATP showed some inward rectification, moderate  $\text{Ca}^{2+}$  selectivity ( $P_{\text{Ca}}/P_{\text{Na}} > 10$ ), a positive reversal potential, and a 11pS conductance [342]. Although the endothelial store-operated currents was described to be similar to the prototypic  $I_{\text{CRAC}}$  seen in mast cells, the lower  $\text{Ca}^{2+}$  selectivity and large unitary conductance show that the currents were rather different [249]. The classic store-operated current of  $I_{\text{CRAC}}$  has more recently been identified in endothelia by the Trebak group, although under conditions where the conduction of  $\text{Na}^+$  was measured [1]. The main contributors to SOCE in endothelium are not well understood, and have been strongly debated, however evidence points to a major role for STIM and Orai proteins.

### 1.3.2 TRPCs and SOCE in the Vascular Endothelium

Prior to the discovery of the molecular components of the CRAC channel, most interest was garnered around the canonical transient receptor potential channels (TRPC) as the primary mechanism of SOCE in all cell types. However, contri-

bution of TRPCs was always contentious, with many TRPCs suggested to play contradictory roles. The confusion surrounding TRPCs and SOCE was debated in many reviews at the time [258,312]. The discovery of CRAC channel proteins, Orai and STIM, however demonstrated the sought after components of the SOC channel, with the RNA<sub>i</sub> study, which identified STIM, finding no role for *Drosophila* TRPs in SOCE [287]. It was subsequently suggested that Orai/STIM interacted with TRPC channels to mediate SOCE [147,203]. However, a study from the Putney group showed that knock-down of TRPC-1,-3,-5,-6, and -7 had no effect on store-operated current in human embryonic kidney (HEK)-293 cells [70]. A further argument against TRPCs in SOCE comes from the pharmacological profile, showing different sensitivities than CRAC channels to many non-specific Ca<sup>2+</sup> channel blockers. In endothelial cells as in other cell types, however, the notion of TRPC channels in SOCE continues to persist despite the large amount of conflicting data, and strong indications of no role for the channels. The TRPC family in humans consists of 6 proteins. Overexpression of TRPC isoforms in model systems has suggested them to be Ca<sup>2+</sup> permeable non-selective channels, although the permeability ratio between Ca<sup>2+</sup> and Na<sup>+</sup> seems to vary greatly between studies [245]. It has been demonstrated that TRPC proteins are capable of forming homo- and heteromultimers, with the relative expression levels of isoforms in cells generating cell-type specific TRPC combinations that display different ion channel properties [139]. Two defined groups are thought to heteromultimerise: TRPC-1,-4, and -5, and TRPC-3,-6, and -7 [2,245].

Like the CRAC channel, TRPC channel activation is thought to be downstream of PLC, however the physiological mechanisms responsible for activation of mam-



malian TRPCs is still unclear. Earlier work indicated activation mechanisms through DAG and IP<sub>3</sub>, with TRPC-3 and -6 shown to be directly activated by DAG [137, 344]. TRPC-7 was suggested to be alternatively regulated by SERCA independently of store depletion [70], and TRPC-1, -4, and -5 were all shown to be insensitive to DAG, potentially regulated by IP<sub>3</sub> [137, 138]. However, in these studies grossly non-physiological concentrations of messengers were used, and so the relevance of these findings in physiological systems is uncertain. It has more recently been proposed that in PIP<sub>2</sub> and other phosphoinositides may be regulating TRPC activity. TRPC-6 has been shown to be positively regulated by PIP<sub>2</sub> and phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>), by preventing the binding of inhibitory Ca<sup>2+</sup>-CaM [182]. PIP<sub>2</sub> activation has also been shown with TRPC-1 and -7, although TRPC-5 and -3 have been found to be insensitive to this mode of regulation [182, 190]. Conversely, interaction of other TRPCs with PIP<sub>2</sub> inhibits the ion channel activity, and a decrease in membrane PIP<sub>2</sub> is associated with channel activation. This is observed with TRPC-4 [251]. Although contributing to regulation, phosphoinositides are thought to be part of a complex multifaceted activation of TRPC channels [182, 286].

Although the activating mechanisms of TRPCs appear diverse and unlikely to be mediated by store-operated Ca<sup>2+</sup> entry [286], TRPC-1 and TRPC-4 have been suggested to sustain store-operated currents.

### **TRPC-1 and -4**

TRPC-1 and -4 have been proposed to form homo- and heteromeric channels generating a somewhat Ca<sup>2+</sup>-selective current, termed the store-operated Ca<sup>2+</sup>

current ( $I_{\text{SOC}}$ ) [53, 139]. This has been suggested to be important in endothelial cells, where reports of reduced  $\text{Ca}^{2+}$  selectivity in endothelial store-operated  $\text{Ca}^{2+}$  currents lent support to  $I_{\text{SOC}}$ , rather than the classical  $I_{\text{CRAC}}$ , being the main store-operated current. This notion is supported by the impairment of endothelial SOCE in TRPC-4(-/-) mice [323].

It has also been proposed that interactions of TRPC1/4 with Orai-1 in endothelial cells regulate  $\text{Ca}^{2+}$ -selectivity of the CRAC channel, or alternatively act as a coupler between the  $\text{Ca}^{2+}$ -sensing STIM-1 and the TRPC1/4 channel [53, 194]. Co-immunoprecipitation has been used to indicate association between STIM-1 and TRPC-1, with interaction increasing with store depletion [147, 369]. Further to this, STIM-1 has also been proposed to gate TRPC-1 through electrostatic interactions [369], and TRPC-4 [323].

The role for TRPC1/4 channels in the generation of a  $\text{Ca}^{2+}$ -selective store-operated current and particularly its requirement for the components of the CRAC channel in order to function, remains controversial. The Trebak group has been instrumental in refuting the role of TRPCs as the primary endothelial SOCE [1, 270, 337]. Patch clamp experiments showed  $K_d$  of STIM-1 and Orai-1 binding in human umbilical vein endothelial cells (HUVECs) resulted in loss of  $I_{\text{CRAC}}$ . Conversely, knockdown of TRPC-1 and TRPC-4 failed to affect store-operated  $\text{Ca}^{2+}$  entry [1].

### 1.3.3 Orai, STIM, and the CRAC channel in endothelial cells

It has been conclusively shown in other cell types that overexpression of Orai and STIM together is sufficient to significantly exceed native currents, strongly indicating that STIM and Orai are the only limiting factors of  $I_{\text{CRAC}}$  development [263].

A substantial body of evidence demonstrates the presence of Orai, STIM, and  $I_{\text{CRAC}}$  in endothelia. The presence of Orai-1 mRNA and protein has been detected in numerous types of endothelial cell including: Ea.hy926 cells, human lung microvascular cells, immortalised mouse lung endothelial cells, HUVECs and endothelium isolated from diabetic mice [1, 4, 88, 192, 323]. Agonist-induced activation of the CRAC channel has been demonstrated, and can be inhibited by the relatively specific Orai-1 channel blocker Synta-66 [192, 193]. Knockdown of Orai-1 in endothelia using short-interfering ribonucleic acid (siRNA) modulates endothelial physiology, being involved in endothelial cell migration, and inhibiting proliferation, as well as remodelling events, such as tube formation in angiogenesis [1, 192, 338]. It has recently been reported that CRAC channels are required in endothelia to mediate histamine-induced translocation of NFAT to the nucleus and is, therefore, of importance in vascular inflammation [375].

Detection of SOCE currents in endothelial cells is notoriously difficult due to the flat cell shape, intracellular junctions, and the activation of large  $\text{Cl}^-$  currents when cell shape or volume is interrupted [248]. The Trebak group have recently reported the first recording of a definitive  $I_{\text{CRAC}}$ , mediated by Orai-1 and STIM-1

in endothelial cells utilising human dermal microvascular endothelial cells (HD-MECs). They demonstrated an inward rectifying current, with positive reversal potential, which developed in divalent free solution (DVF) in the presence of 20mM 1,2-bis(2-aminophenoxy)ethane-N-N-N'-N'-tetraacetic acid (BAPTA). The current could be blocked with the lanthanide  $Gd^{3+}$ , and was additionally abrogated by siRNA knockdown of either STIM-1 or Orai-1 [307].

## 1.4 Nitric oxide in the vascular endothelium

### 1.4.1 The properties of nitric oxide in vascular physiology

NO is a rapidly diffusible and highly reactive free radical with a short half life *in vivo* which has been reported in literature to be between 1-30 seconds [46,167]. NO is both hydro- and lipophilic allowing free diffusion through and between cells [20].

The production of NO in physiology has generated an exciting and diverse field of interest. However, approaches to study the effects of NO are often inherently flawed. With difficulties in measuring NO directly on a real-time basis, and the reactivity of NO in both *in vitro* and *in vivo* settings, a wealth of data has been generated which is complex to effectively analyse and determine the contribution of NO. An example of this is the use of NO donors, such as sodium nitroprusside (SNP). SNP releases a nitrosyl group spontaneously at physiological pH [152]. Although it is clear that SNP can be used clinically to reduce blood pressure, its use in experimental studies may indicate roles of NO which could be attributed to other aspects of SNP, such as cyanide release [20,152]. Indeed interpretation of

NO experimental data should be approached with caution.

NO reacts rapidly with other free radicals, and transition metals [20]. Certain NO reactions dominate *in vivo* due to the rapid rate of reactions, their availability, and the quick diffusion of NO. The reaction of NO can confer a stable downstream signal through the formation of s-nitrosothiols, that modulate target protein activity.

### **Formation of s-nitrosothiols**

The reaction of NO with the thiol-groups of cysteine residues on proteins has emerged as the major mechanism of NO signal transduction and a ubiquitous post-translational protein modification [134]. S-nitrosothiols (SNO) are perfectly placed as communicators of signals, being fully reversible modifications which are restricted both spatially and temporally. Nitrosylation of cysteines is very specific, with often only one cysteine in a protein permitting a SNO modification [73]. SNO-proteins are diverse and include ion channels and pumps, signalling receptors, structural proteins, and enzymes [134]. SNO are present *in vivo* at concentrations greater than 1 $\mu$ M and demonstrate a half-life in serum proteins of over 40 minutes—a significant increase on the half-life of NO *in vivo* [20]. Disruption of SNO signalling pathways has been implicated in disease and cellular dysregulation [106]. Interestingly, activation of Ca<sup>2+</sup> signalling through eNOS agonists, such as ACh, can also induce release of SNO, and may be important in the early stage of NO increases after NOS stimulation [52].

S-nitrosylated proteins and dinitrosyl-non-haem iron complexes can act as intra-

cellular NO stores. The first identified of these was SNO-albumin found in the circulation. With the inhibition of endogenous NOS activity, concentration of SNO-albumin decreased, but was mirrored by an increase in free SNO, which was capable of mediating a vasodilatory response [316]. The low molecular weight thiol s-nitrosogluthione (GSNO) is another source of stored NO. Release of NO is regulated by GSNO reductase, which increases the metabolism of GSNO to release NO and return glutathione. The release of these 'NO stores' can explain the lack of complete inhibition of NO-mediated vasodilation in the presence of inhibitors of NO production [91].

NO can be donated to form SNO by NO oxides, metal-NO complexes, other nitrosothiols, as well as direct reaction of NO itself [134]. Reactions require an electron acceptor, commonly in the form of transition metals and nitrogen dioxide [20]. Protein-bound transition metals such as  $\text{Cu}^{2+}$  and haem are able to catalyse SNO formation when in a close locality to the target cysteine residue [134].  $\text{Ca}^{2+}$  has also been implicated in SNO formation, such as on endothelial transglutaminase [183].

### **Peroxynitrite formation**

The nitric oxide synthase (NOS) family of proteins produce NO. Under certain conditions, often during oxidative stress, NOS can also produce superoxide ( $\text{O}_2^-$ ). The contemporaneous production of NO and  $\text{O}_2^-$  production facilitates their irreversible reaction to form peroxynitrite ( $\text{ONOO}^-$ ) a highly reactive oxidant which reacts in particular with iron/sulphur centres, zinc fingers and protein thiols. Superoxide dismutase generally controls the levels of  $\text{O}_2^-$  *in vivo*, however in cells such as endothelial cells, where large quantities of NO and  $\text{O}_2^-$  are produced together,

formation of  $\text{ONOO}^-$  can become a problem. As the concentration of intracellular NO rises, having not reacted with  $\text{O}_2^-$ , NO competes with superoxide dismutase for the  $\text{O}_2^-$  pool. This results in a steady level of  $\text{ONOO}^-$  being produced; a problem in *in vitro* experiments. *In vivo* concentrations of NO do not reach the concentrations where competition is a scenario due to diffusion into the blood [20].

### Nitric oxide and haemoglobin

NO reacts with deoxy- and oxyhaemoglobin to form iron-nitrosyl-haemoglobin and methemoglobin respectively, in what is considered the sink for NO. Reaction with haemoglobin prevents the build-up of NO which would result in the formation of oxidants such as nitrogen dioxide [20, 120]. A second reaction of NO with haemoglobin has been more recently identified. SNO-haemoglobin, created via NO transfer from iron-nitrosyl haemoglobin, can act as a transport mechanism [120, 134, 158]. SNO preferentially forms in larger oxygenated vessels, and releases in smaller vessels, where the release of  $\text{O}_2$  from haemoglobin triggers NO release. This triggers NO-mediated vasodilation in areas of low  $\text{O}_2$  availability [127]. Experiments have been undertaken *in vitro* and *in vivo* under highly non-physiological conditions, making the physiological worth of this pathway as yet unknown, however is an appealingly elegant mechanism [120].

### **1.4.2 The function of endothelium-derived nitric oxide in vascular biology**

NO has a wide range of actions as a signalling molecule with involvement in vasodilation, host defence and immunological functions as well as control of platelet aggregation and heart contractility. In the vasculature, endothelium-derived NO is responsible for the maintenance of vascular haemostasis and homeostasis. NO controls initiation of vasodilation, inhibition of vasoconstriction and VSMC proliferation, as well as prevention of platelet aggregation and inhibition of leukocyte adhesion and migration into the vessel wall. In the absence of NO, the endothelium develops a dysfunctional state which is defined by the attenuation of vasodilation in response to ACh and shear stress. A dysfunctional endothelium releases reduced levels of bioactive agents, such as NO and prostacyclin. Reduction of NO and progression into endothelial dysfunction is a prerequisite for many cardiovascular disease states, particularly atherosclerosis [333].

#### **Promotion of vasodilation**

The production of NO in both the vascular endothelium and non-adrenergic-non-cholinergic neurons induces vasorelaxation via soluble guanylyl cyclase (sGC) [230,231]. Nitrosylation of the sGC haem in target cells, increases the conversion of magnesium guanosine 5'-triphosphate (MgGTP) to cGMP by 50- to 200-fold, creating a widespread second messenger effect [151]. Critically, cGMP activates cGMP-dependent protein kinase (PKG), initiating multiple downstream phosphorylation events, which are prolific in inhibiting VSMC contraction. PKG activ-



ity reduces intracellular  $\text{Ca}^{2+}$  both by increasing  $\text{Ca}^{2+}$  removal from the cytosol by SERCA, and inhibition of  $\text{Ca}^{2+}$  release and entry, through phosphorylation of  $\text{IP}_3\text{R}$ , preventing store release, and by potentiation of the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels known as the BK channels respectively. Activation of BK channels results in hyperpolarisation of the plasma membrane, which reduces the driving force for  $\text{Ca}^{2+}$  entry through voltage-gated channels. PKG also acts to regulate the phosphorylation of the myosin protein, a critical step in cross-bridge cycling, and therefore contraction. PKG activity activates myosin light chain phosphatase activity, and therefore negatively regulates phosphorylation of myosin [198].

### **Inhibition of platelet aggregation**

Endothelial NO contributes to the anti-thrombotic nature of the healthy endothelium by preventing platelet aggregation, promoting disaggregation of platelets, and suppressing platelet adhesion to the endothelium [278, 279]. NO release increases intracellular cGMP via activation of platelet guanylyl cyclase [280, 350]. NO works synergistically with prostaglandins which upregulate cAMP production [278–280]. Together, the increase in cyclic nucleotides inhibits the action of PLC and production of  $\text{IP}_3$  and DAG- messengers which begin the cascades of platelet activation. Human platelets have been found to synthesise their own NO in response to activation, thus exhibiting autoregulation [280]. NO-cGMP upregulation also activates G kinase phosphorylation of certain aggregation-associated proteins, such as the thromboxane  $\text{TxA}_2$  receptor, which suppresses their pro-aggregation actions [350].

**Inhibition of leukocyte adhesion and activation**

Entry into the vessel wall by leukocytes, after endothelial dysfunction, is an early event in atherogenesis. Endothelial production of NO inhibits leukocyte adhesion to the endothelium, and prevents leukocyte chemotaxis to sites of injury [15, 180]. Inhibition of NO production in whole vessel preparations led to a 15-fold increase in leukocyte adhesion [180]. Multiple mechanisms are thought to control this process, although they are still not fully defined [188].

NO controls leukocyte adhesion by two means: altering the leukocyte-binding ability to some endothelial adhesion molecules and modulating the level of adhesion molecule expression [15, 66, 180]. Control of protein expression is, in part, mediated by tonic inhibition of NF $\kappa$ B- the transcription factor responsible for initiating expression of vascular cell adhesion molecule-1, E-selectin, and interleukins-6 and -8. NO synthesis both induces synthesis of an NF $\kappa$ B inhibitor, I $\kappa$ B $\alpha$ ; increasing messenger ribonucleic acid (mRNA) levels; and stabilises I $\kappa$ B $\alpha$ , preventing breakdown. Cumulatively these events result in suppression of endothelial cell adhesion molecules and therefore limit leukocyte adhesion [264].

Decreased NO production or the reaction of NO with O $_2^-$  to form ONOO $^-$  reduces NO bioavailability and alleviates the inhibition of NO on leukocyte adhesion. In endothelial dysfunction, where NO availability is reduced progression into further disease states such as atherosclerosis is, therefore, promoted [180].

**Inhibition of vascular smooth muscle cell proliferation**

Proliferation of the vascular smooth muscle and migration into the intima occurs in response to vascular injury and is a hallmark of atherosclerosis [84]. The increase in reactive oxygen species (ROS) seen in vascular disease states promotes this process. The first evidence of this was provided by Berk in 1992, who, recognising the effect of immune cells and endothelial cells on VSMC proliferation, implicated the production of  $H_2O_2$  in this process. Activation of xanthine/xanthine oxidase-dependent DNA synthesis increased proliferation in VSMC [282]. This was later found to also be dependent on cyclophilin A and regulation of ERK1/2 signalling [159]. VSMC presence in the intima initially stabilises the fibrous cap of atherosclerotic plaques by collagen production. However, the prolonged increase in ROS leads to VSMC apoptosis, and plaque destabilisation [195].

Shortly after the discovery of NO as the primary EDRF, it was seen that NO suppressed the proliferation and mitogenesis of VSMC [115]. This is mediated by both a cGMP-dependent and -independent processes [115, 153]. cGMP-independent inhibition occurs through nitrosylation of ornithine decarboxylase- a principle enzyme in the formation of arginine and other polyamines. Polyamine deficiency is proposed to limit proliferation, as has been previously observed in other cell types [153].

**Inhibition of vasoconstriction**

Constriction of blood vessels is controlled by the sympathetic nervous system via norepinephrine release and  $\alpha$ -adrenergic receptor activation on VSMCs. NO re-

lease not only mediates vasodilation through VSMC relaxation, but additionally inhibits sympathetic signalling in the periphery, reducing vasotone. Neuronal-derived NO in the sympathetic stellate ganglion reduces noradrenaline release via a cGMP-dependent mechanism, which limits tachycardia and reduces vasotone [132]. Furthermore, a role for endothelium-dependent NO release in the brain stem has recently been proposed to increase  $\gamma$ -amino butyric acid (GABA) in the sympathetic nervous system via NMDA receptors, which inhibits sympathetic-induced vasoconstriction [175].

### **1.4.3 Activation of NO production in the vascular endothelium**

The NOS family consists of three isoforms: inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS). The principle roles for NO produced by these enzymes is in inflammatory response, neurotransmission, and vasodilation respectively.

The primary isoform found in the endothelium, as the name suggests, is eNOS. eNOS is mainly localised to lipid rafts and caveolae in the plasma membrane, and to the perinuclear Golgi body [122, 302, 305].

eNOS requires an elevation of  $\text{Ca}^{2+}$  above basal cytosolic levels (40-60nM) in order to be activated [281], which is achieved by a SOCE-dependent rise in cytosolic  $\text{Ca}^{2+}$  [197]. The induction of SOCE can be achieved through the binding of numerous agonists, including ATP, histamine, thrombin, and vascular endothelial growth factor (VEGF), to membrane-embedded G-protein coupled receptors (GPCR).

Utilisation of different GPCRs allows exquisite control of eNOS regulation by different agonists, which is mediated by extracellular  $\text{Ca}^{2+}$  entry [45,204,310]. Indeed, the initial  $\text{Ca}^{2+}$  rise due to  $\text{Ca}^{2+}$  store-release does not affect eNOS activity; the secondary sustained rise of entry is the activating switch for eNOS [197]. Despite it being known that SOCE is required for eNOS activation, the molecular basis of this SOCE current has not been identified.

ATP and histamine are well characterised agonists both in terms of SOCE signalling, and in eNOS activation. They will be used within the experimental portion of this thesis.

## ATP

ATP concentration is raised in the vasculature under conditions of disease and stress. Principle releasers include red blood cells, platelets, and endothelial cells. The release of ATP by red blood cells occurs in response to changes in pH, physical deformation, and hypoxia, with high levels liberated upon red blood cell lysis [313]. Platelets have a non-metabolically active store of ATP in dense granules, which is released upon degranulation and aggregation events; aberrant ATP release is associated with bleeding disorders [64]. The endothelium itself also releases ATP, which creates an autocrine signalling loop, in response to shear stress and hypoxia [44]. This extracellular ATP, derived from many cells under stress conditions, activates  $\text{P}_2\text{Y}_1$  and  $\text{P}_2\text{Y}_2$  receptors on the endothelial cell surface, inducing a SOCE-dependent rise in  $\text{Ca}^{2+}$  [44,342]. SOCE, in turn, activates eNOS via  $\text{Ca}^{2+}$ -CaM [44]. ATP was one of the earliest agonists shown to relax the vascular smooth muscle in an endothelium-dependent manner, which is now known to be through

eNOS activation [67].

ATP-initiation of SOCE has been suggested to be TRPC-3 dependent, however ATP activation of store-operated currents is consistent with  $I_{CRAC}$  [31, 342].

## Histamine

Histamine is a known vasodilator, readily released in inflammatory events and allergic responses by immune cells, in particular basophils and eosinophils. It can, however, be produced by many other cells in the vasculature including platelets. Histamine stimulates store-operated  $Ca^{2+}$  entry as demonstrated through  $Mn^{2+}$  quenching experiments utilising the  $Ca^{2+}$ -sensitive ratiometric fluorescent dye, Fura-2; it binds the histamine receptor  $H_1$ , which links to the  $G_q$ -protein coupled receptor, inducing PLC activation and thus SOCE entry [36, 156, 168].

Histamine has also been shown to upregulate eNOS gene expression in endothelial cells via a Calmodulin kinase II (CaMKII) pathway, demonstrating histamine regulation of NO at multiple levels [191].

### 1.4.4 Production of nitric oxide by eNOS

All NOS convert L-arginine to L-citrulline and NO using the co-substrates  $O_2$  and nicotinamide dinucleotide phosphate (NADPH), [105, 230, 277]. NOS exist as homodimers. Each monomer consists of an N-terminal oxygenase domain, a central  $Ca^{2+}$ -CaM binding region, and a C-terminal reductase domain. Monomers align in opposite orientations to allow interactions between the oxygenase and reductase domains of the other monomer [321]. Dimerisation is thought to be

stabilised by the presence of the co-factor tetrahydrobiopterin ( $\text{BH}_4$ ) [23, 61, 339]. Zinc interacts with NOS thiol residues to perform a structural role by stabilising the active site [105].

### **L-arginine**

NOS substrate L-arginine is one of the most common naturally-occurring amino acids, and is considered conditionally essential. The kidney cortex is the principle source of circulating L-arginine, and generates L-arginine with the enzymes arginosuccinate synthase and arginosuccinate lyase [54, 76]. L-arginine exists in blood at a concentration of  $\sim 100\text{-}200\mu\text{M}$ , and intracellularly at  $\sim 840\mu\text{M}$  [19, 359]. The strong concentration gradient is facilitated by active uptake into cells by  $y^+$  and  $y^+L$ , namely heterodimeric amino acid- and cationic amino acid- transporters. L-arginine uptake is promoted by accumulated intracellular amino acids in a process known as trans-stimulation [54]. Additionally, endothelial cells have been shown to produce L-arginine from a protease-sensitive pool and by conversion of L-citrulline as in the kidney [131]. It should be noted, however, that presence of L-glutamine will inhibit L-citrulline conversion in cultured endothelial cells due to their heightened glutaminolysis activity [303]. Interestingly, despite the high intracellular concentration, eNOS binds L-arginine at concentrations of  $2\text{-}3\mu\text{M}$ , meaning L-arginine is present physiologically at saturating levels [216].

## NO reaction

Conversion of L-arginine to NO is a two-stage process, involving consecutive atypical monooxygenation reactions generating an intermediate of N-hydroxy-L-arginine (NOHA) [63]. The overall reaction requires the donation of 3 electrons by 1.5 molecules of NADPH. Donation of electrons by NADPH in the form of hydride ions ( $\text{H}^-$ ) to the reductase domain initiates the catalytic conversion of L-arginine to NO. Electrons are passed through the reductase domain via the co-factors flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN).  $\text{Ca}^{2+}$ -CaM facilitates the exchange of electrons to the oxygenase domain of the opposite monomer, reducing the bound haem, allowing the binding of  $\text{O}_2$  and generation of a ferric-haem-superoxy species [105, 321, 322]. This complex requires an additional electron, which is donated by redox cycling of the co-factor  $\text{BH}_4$  forming a transient  $\text{BH}_3^-$  radical. Absence of  $\text{BH}_4$  at this stage results in  $\text{O}_2^-$  release, as discussed later [322]. The addition of the donated electron allows the conversion of L-arginine into NOHA. A further electron from the transport chain and a second molecular oxygen allow conversion of the NOHA intermediate into L-citrulline and NO. In order to release the product NO, the  $\text{BH}_3^-$  radical reclaims the donated electron from the flavins thus completing the cycle [246, 322].

### 1.4.5 Regulation of eNOS

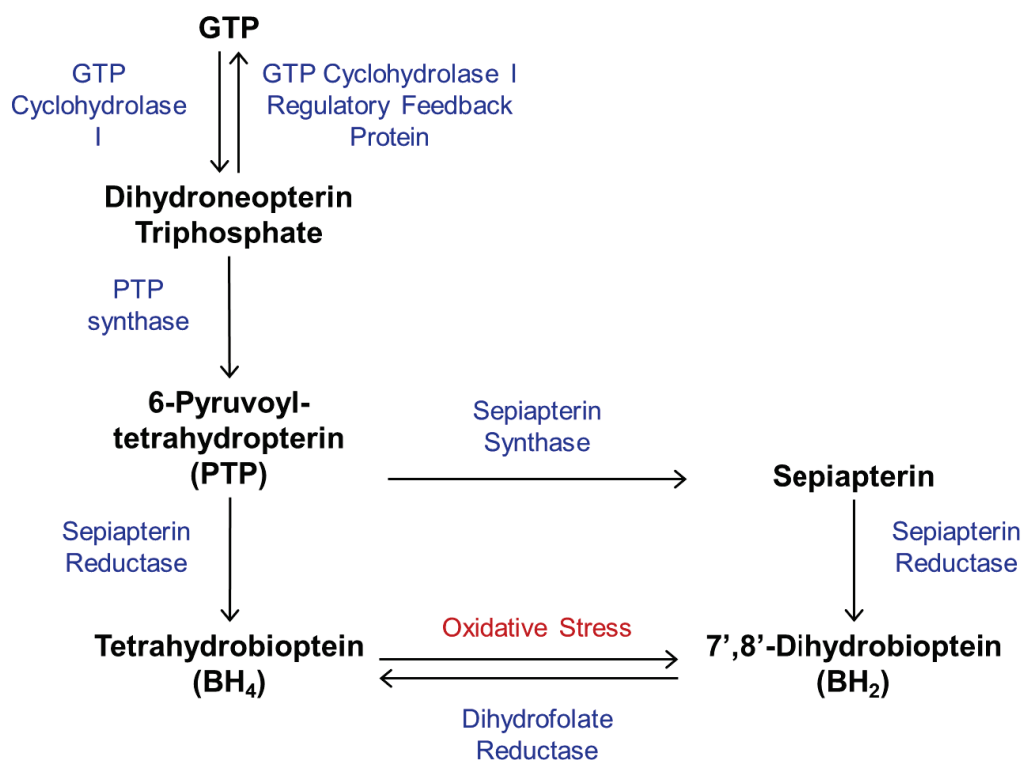
Regulation of eNOS is complex; co-factor binding, phosphorylation, nitrosylation, eNOS binding proteins, subcellular localisation, dimerisation, and autoregulation by an auto-inhibitory loop and a proposed flexible arm, all contribute to the control



of eNOS and whether NO or  $O_2^-$  is produced. In this section I will discuss the major  $Ca^{2+}$  dependent regulatory mechanisms of eNOS, as well as the critical  $BH_4$  pathway, which ensures NO, rather than  $O_2^-$ , is produced by eNOS.

### **Tetrahydrobiopterin and the ‘coupling’ states of NOS**

$BH_4$  can be oxidised to form 7',8'-dihydrobiopterin ( $BH_2$ ) and then again to form biopterin (B).  $BH_4$  can be synthesised *in vivo* through a *de novo* pathway from GTP or, alternatively, from the salvage pathway by conversion of  $BH_2$  by dihydrofolate reductase (DHFR) (Fig.1.4.5). Intracellular levels of  $BH_2$  and B increase with cellular oxidative stress. Critically, both  $BH_4$  and  $BH_2$  can bind to the NOS oxygenase domain with the same efficiency, although  $BH_2$  is inactive as a NOS co-factor. NOS binding to  $BH_2$  leads to an ‘uncoupled’ state where the electron transferred through the reductase domain is no longer passed to the oxygenase domain. This results in the transfer of the electron to  $O_2$  creating the superoxide free radical ( $O_2^-$ ) [343]. The  $BH_4:BH_2$  ratio is a key determinant of the ‘coupled’ status of NOS. It is controlled by the balance of oxidation of  $BH_4$  to  $BH_2$  which is determined by the oxidative stress of the cells, and the conversion of  $BH_2$  back to  $BH_4$  by DHFR [58, 60]. Increased production of  $O_2^-$  not only initiates self-propagating uncoupling, reducing the  $BH_4:BH_2$  ratio further, but additionally reacts rapidly with NO to form peroxynitrite ( $ONOO^-$ ), further limiting the pool of available NO [20]. The availability of  $BH_4$  is, therefore, of great importance when considering the catalytic activity of eNOS.



**Figure 1.3** *De novo* and salvage pathways of BH<sub>4</sub> synthesis

The balance between BH<sub>4</sub> and BH<sub>2</sub> is essential in order to maintain enzymatic coupling of eNOS. BH<sub>4</sub> can be synthesised *de novo* from GTP via two intermediates catalysed by the enzymes: GTP cyclohydrolase I, 6-pyruvoyl tetrahydrobiopterin (PTP) synthase, and sepiapterin reductase. It is readily degraded by free radicals generated during oxidative stress, to form its inactive and competitive counterpart BH<sub>2</sub>. BH<sub>2</sub> ‘uncoupling’ of eNOS exacerbates the degradation of BH<sub>4</sub> by generating O<sub>2</sub><sup>-</sup>. The enzyme dihydrofolate reductase is critical in maintaining the BH<sub>4</sub>:BH<sub>2</sub> balance and maintaining eNOS in a coupled (NO-producing) state [60].

### 1.4.6 Caveolin-1

Caveolin-1 is a myristoylated protein, which is a major component of the plasma membrane cholesterol-rich lipid rafts, the caveolae. Caveolin-1 tonically inhibits membrane-localised eNOS through interaction with a specific caveolin-binding do-

main on eNOS [336]. Increased intracellular  $\text{Ca}^{2+}$ -CaM levels after agonist stimulation, facilitated by eNOS-binding partner heat shock protein 90 (HSP-90), act to displace caveolin-1 binding, thus removing inhibition [93,126]. Cessation of the  $\text{Ca}^{2+}$  signal allows re-association of caveolin-1 to eNOS, which is potentiated by eNOS targeting to the caveolae by the reversible modification palmitoylation [93]. The release of caveolin-1 from eNOS is recognised as a major factor in eNOS activation.

### **$\text{Ca}^{2+}$ -Calmodulin control of eNOS activation**

CaM is a cytosolic  $\text{Ca}^{2+}$ -sensor protein which acts as a signal transducer of intracellular  $\text{Ca}^{2+}$  rises.  $\text{Ca}^{2+}$ -CaM binds a plethora of proteins conferring activity or inhibition. CaM consists of two globular domains, each containing two  $\text{Ca}^{2+}$ -binding EF-hands, linked via a loop structure [220,221,363]. EF-hands consist of a helix-loop-helix structure.  $\text{Ca}^{2+}$  binds to the linking loop, and forces a conformational change within the EF-hand [363]. Cooperative binding of  $\text{Ca}^{2+}$  in each pair of EF-hands causes a decrease in the angle between CaM helices, and opens up the central  $\alpha$ -helix region forming the ‘open’ conformation [179,370]. This acts to expose hydrophobic residues, which interact with target proteins. The central linker region has a helix structure, which provides flexibility and altered length to the CaM protein, allowing interaction with target protein motifs of different sizes [154,220,221].

$\text{Ca}^{2+}$ -CaM is essential for all NOS isoforms, binding the central linker region between the NOS oxygenase and reductase domains at the CaM binding motif. However, only eNOS and nNOS are  $\text{Ca}^{2+}$ -sensitive. Due to an increase in covalent

bonding in the iNOS binding site,  $\text{Ca}^{2+}$ -CaM remains bound even with cytosolic  $\text{Ca}^{2+}$  concentrations in the range of 40nM; iNOS is therefore  $\text{Ca}^{2+}$ -insensitive at physiological  $\text{Ca}^{2+}$  concentrations [105, 314]. iNOS additionally lacks an autoinhibitory loop present in the other NOS isoforms, which acts to destabilise CaM binding at low cytosolic  $\text{Ca}^{2+}$  [292]. In eNOS and nNOS, however, the binding of  $\text{Ca}^{2+}$ -CaM is a mechanism of activation. CaM binding to eNOS in basal conditions is minimal [103].  $\text{Ca}^{2+}$  must be bound to all 4 EF-hands, in order to activate NOS proteins [314].

The electrostatic interactions between CaM and all NOS proteins was determined to be important in both binding and increasing NOS activity. CaM residues 84 and 87 directly interact with eNOS CaM binding motif residues. Mutation of these residues interrupts CaM binding and reduces eNOS-dependent NO production by 74% compared to wild-type (WT) CaM [315]. It still remains unclear as to exactly how CaM increases eNOS activity [266]. Two mechanisms, likely to work cumulatively, are thought to promote NOS electron flux. It is known that binding of CaM to nNOS alters the conformation of the NOS reductase domain, increasing the accessibility of FMN to FAD. This increases the rate of electron transfer in the reductase domain thus, increasing the catalytic activity of NOS [355]. CaM has been suggested to be a mediator of electron transfer from the reductase to the oxygenase domain, or from FMN to haem [311]. It appears through mutation of the arginine (Arg) 752 in eNOS FMN domain and glutamic acid (Glu) 47 in CaM, that a bridging mechanism was abrogated which allowed CaM-mediated increases in electron flux. Electron transfer when interaction was prevented resulted in a 90% decrease in FMN-haem electron transfer [331].

**Regulation of eNOS  $\text{Ca}^{2+}$  dependency**

The  $\text{Ca}^{2+}$  sensitivity of eNOS can be modulated by phosphorylation of specific serine, threonine, and tyrosine residues [101]. Combinations of phosphorylation alter the  $\text{Ca}^{2+}$  dependency of eNOS, with phosphorylation of Serine 1177 (Ser1177) and Serine 615 (Ser615) allowing 50% activation at 100nM and 100% activation at 300nM  $\text{Ca}^{2+}$  [335]. Although the effect of the majority of phosphorylations on eNOS activity are poorly defined, Ser1177, and threonine (Thr)495 are well characterised.

**Threonine 495** found within the CaM-binding domain, is constitutively phosphorylated on eNOS by 5'adenosine monophosphate-activated protein kinase (AMPK) and protein kinase C (PKC) [50, 103]. Phosphorylation decreases eNOS activity through interference with  $\text{Ca}^{2+}$ -CaM binding [50, 103]. Recent biophysical studies indicate that phosphorylated Thr495 reduces  $\alpha$ -helix formation and establishes electrostatic repulsion between the CaM-binding site and CaM. When Thr495 is phosphorylated, CaM binding to CaM-binding motif is minimal at physiological levels of elevated  $\text{Ca}^{2+}$  ( $\sim 225\text{nM}$ ). Saturating levels supports full CaM binding even with Thr495 phosphorylation [266]. Upon agonist-induced extracellular  $\text{Ca}^{2+}$  entry Thr495 is likely dephosphorylated by protein phosphatase 1 (PP1). Dephosphorylation of Thr495 has been observed both in response to rises in  $\text{Ca}^{2+}$ , with BK, histamine,  $\text{Ca}^{2+}$  ionophore stimulation and shear stress [14, 103].

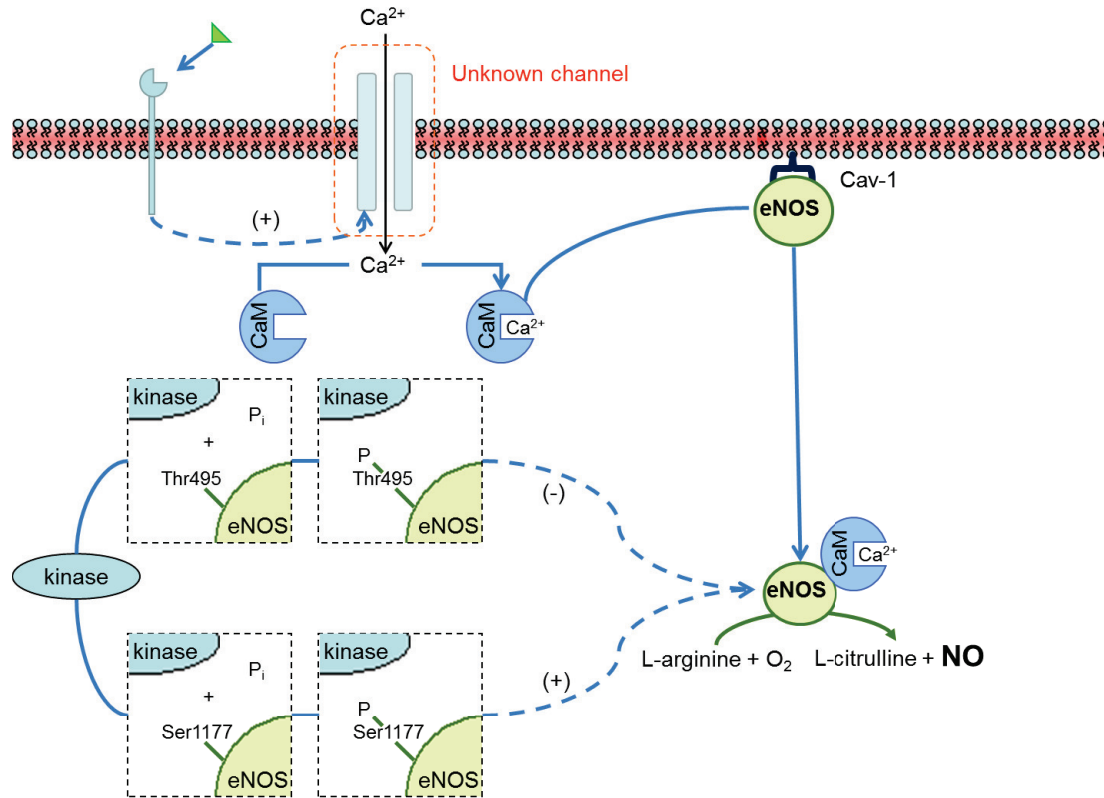
**Serine 1177** is minimally phosphorylated under basal conditions [103]. However, with eNOS activation by shear stress, and agonists including oestrogen,

VEGF, insulin, and BK, increased phosphorylation of Ser1177 can be seen [81, 102]. Ser1177 phosphorylation is mediated by the activation of multiple different kinases, dependent on the stimulatory mechanism, and these include Akt, AMPK, PKA and CaM kinase II (CaMKII) [50, 102, 111]. Use of Ser1177 phosphomimetics demonstrated an enhancing role for phospho-Ser1177, in direct contrast to phospho-Thr495. Phosphorylation of Ser1177 reduces the  $\text{Ca}^{2+}$  dependency of eNOS, allowing maximal eNOS activity at reduced  $\text{Ca}^{2+}$  concentrations [81]. AMPK phosphorylation of eNOS depends on the intracellular  $\text{Ca}^{2+}$  concentration. The presence of ethylene glycol tetraacetic acid (EGTA) reduces basal cytosolic  $\text{Ca}^{2+}$  levels and favours Thr495 phosphorylation, however when overcome with exogenous  $\text{Ca}^{2+}$ -CaM, AMPK favours the phosphorylation of Ser1177 on eNOS [50].

Ser1177 phosphorylation increases the flow of electrons through the reductase domain by 2- to 4-fold above the dephosphorylated state [216]. The effect of phosphorylation is thought to be due to the negative charge inferred on the Ser1177, as its effect can be replicated upon serine to aspartate mutation [81, 216]. Phosphorylation of Ser1177 has been suggested to relieve autoinhibition by the autoinhibitory loop in nNOS and eNOS [50, 111]. Indeed, Ser1177 phosphorylation by Akt has been shown to be necessary for NO-dependent endothelial activities, such as endothelial migration as induced by VEGF [81].

Shear stress is thought to induce vasorelaxation via increased NO production independently of a sustained  $\text{Ca}^{2+}$  rise [8]. Activation of phosphatidylinositol 3-kinase (PI3K) and thus Akt and PKA, increases Ser1177 phosphorylation; lowering the  $\text{Ca}^{2+}$  threshold for eNOS activation [81, 100]. Although differential phosphoryla-

tion induced by shear stress alters  $\text{Ca}^{2+}$  sensitivity, like iNOS, lowering of cytosolic  $\text{Ca}^{2+}$  renders shear-stress-stimulated eNOS inactive [81]



**Figure 1.4 The  $\text{Ca}^{2+}$ -dependent regulation of eNOS:** eNOS constitutively produces a low basal level of NO. In its resting inactive state, eNOS is tonically inhibited by binding to the plasma-membrane embedded protein caveolin-1. Upon  $\text{Ca}^{2+}$  entry into cells,  $\text{Ca}^{2+}$  binds to CaM, and displaces caveolin, thus activating eNOS [126, 223]. eNOS  $\text{Ca}^{2+}$  sensitivity is further modulated by the phosphorylation state of Thr-495 and Ser-1177. Phosphorylation of eNOS by AMPK is differentially regulated by cytosolic  $\text{Ca}^{2+}$  levels. Raised  $\text{Ca}^{2+}$  results in preferential phosphorylation of Ser1177; reducing the  $\text{Ca}^{2+}$  requirement of eNOS [81]. In turn, Thr495 phosphorylation is favoured by AMPK at resting  $\text{Ca}^{2+}$  levels. Thr495 increases the  $\text{Ca}^{2+}$  requirement of eNOS through interfering with  $\text{Ca}^{2+}$ -CaM binding. The rise in  $\text{Ca}^{2+}$  reduces the levels of inhibiting phospho-Thr495, whilst raising phospho-Ser1177. Actions of phosphatases, such as PP1, further exacerbate the reduction in phospho-Thr495 [103].

## 1.5 Conclusion

The vascular endothelium is a dynamic tissue, which has been shown to be critically important in the maintenance of a healthy cardiovascular system. Since the proposal of the SOCE mechanism, a role for store-operated  $\text{Ca}^{2+}$  channels in endothelial cells has been clear. However, the principle store-operated channel, CRAC, has been poorly characterised in endothelia, and the function of the CRAC channel in the endothelium has only been addressed in a handful of studies.

The production of NO is one of the major processes attributable to the endothelium. However, the source of the activating  $\text{Ca}^{2+}$  remains largely unknown. Evidence indicates a role for SOCE in eNOS activation, but the role of CRAC channels in NO production has never been directly investigated. This is important because identification of the  $\text{Ca}^{2+}$  channel that activates eNOS will identify a potential therapeutic target for managing cardiovascular disorders, such as hypertension.

This thesis aims to both further characterise the CRAC channel in the endothelial model system sEnd.1, and directly address whether the channels can activate eNOS and, thus, NO production.



# Chapter 2

## Materials and Methods

### 2.1 Cell culture

sEnd.1 cells were gifted by Prof. Keith Channon, Department of Cardiovascular Medicine, University of Oxford. sEnd.1 cells were frozen at passage 1 in freezing medium, and stored in liquid nitrogen. sEnd.1 cells were cultured up until passage 8, after which point a new aliquot was thawed. Freshly thawed cells were passaged once prior to experimental use. Rat basophilic leukemia (RBL)-1 cells were bought from ATCC. All cells were maintained in high glucose L-glutamine (4mM) Dulbeccos modified Eagles medium (DMEM) supplemented with 10% heat inactivated serum, 200units/ml penicillin and 200 $\mu$ g/ml streptomycin. Cells were incubated at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere.

## 2.2 Cytosolic $\text{Ca}^{2+}$ imaging

Cells were plated on glass coverslips and used 24-48 hours after plating at ~low60% confluency. Cells were loaded with Fura-2AM (2 $\mu\text{M}$ ), dissolved in standard external solution, for 40 minutes in the dark, at room temperature, before being washed 3 times in standard external solution. Cells were kept in standard external solution for a further 15 minutes to allow for further deesterification.

Standard external solution composition (in mM): NaCl 145, KCl 2.8,  $\text{CaCl}_2$  2,  $\text{MgCl}_2$  2, D-glucose 10, HEPES 10, pH 7.4 with NaOH.  $\text{CaCl}_2$  was replaced by 0.1mM EGTA in standard  $\text{Ca}^{2+}$ - free external solution [164]. Low  $\text{Na}^+$  external solution composition (in mM): NaCl 10, Tris-base 145, KCl 2.8, HEPES 10,  $\text{MgCl}_2$  2. EGTA is known to chelate  $\text{La}^{3+}$ . Experiments using  $\text{La}^{3+}$  in  $\text{Ca}^{2+}$ -free solution were therefore carried out in the absence of EGTA.

Imaging experiments utilised the Till Photonics IMAGO CCD camera-based system with TillVision software, as previously described [241]. Cells were excited at 356nm and 380nm, with 20ms exposures; 0.5Hz. Emmission was measured at 510nm. Plots are shown as change in ratio between 356:380. Rate of divalent ion entry was calculated through differentiation of individual waves, and normalised to control [164].

When imaged at physiological temperature cells were maintained within the range between 36°C and 37.9°C, with an TC-10 npi heated chamber system. Cells were maintained at 37°C prior to imaging.

## 2.3 Intracellular NO imaging

sEnd.1 cells were plated on glass coverslips 24-48 hours prior to imaging, and imaged at 60% confluency. Standard external solution used for all NO experiments was supplemented with 100 $\mu$ M L-arginine, to ensure sufficient substrate for eNOS activity. Cells were loaded with NO sensitive fluorescent dye DAF-2-DA (10 $\mu$ M) for 30 minutes at 37°C in standard NO external solution in the dark. Cells were washed 3 times with standard external solution, with L-arginine (standard NO external solution), before and after loading. Cells were maintained at 37°C prior to imaging, and maintained at physiological temperature during experiments as previously described.

Imaging experiments utilised the same Till Photonics system as described previously. Cells were excited at 490nm, with 30ms exposures, every 10seconds.

Waves are shown as normalised to baseline cell fluorescence ( $F/F_o$ ). Baseline fluorescence calculated as the mean of fluorescence measured over 100secs prior to stimulation. Time to reach 10% total peak was defined as 10% of the difference between calculated baseline and peak height following a rapid increase in NO production.

## 2.4 Western blot

Western blot was carried out as previously described [48]. Briefly, cells were lysed in lysis buffer; RIPA buffer (Sigma) with protease inhibitor cocktail (Sigma), 10mM Na<sub>3</sub>VO<sub>4</sub> (Santa Cruz) and 1mM phenylmethylsulfonyl fluoride (PMSF)

(Santa Cruz). Protein content was assessed using a modified-Lowry assay (Bio-rad). 50 $\mu$ g protein samples were denatured at 70°C for 10mins. Samples were separated by SDS - PAGE on a 10% gel, and transferred to nitrocellulose membranes (Amersham Biosciences) by semi-dry transfer. Membranes were blocked at 4°C overnight in 5% non-fat dry milk in phosphate buffered saline (PBS) with 0.1% Tween 20 (PBS-T) buffer. Membranes were washed 3 times in PBS-T and incubated 2 hours in primary antibody at room temperature. Antibodies against STIM-1 and STIM-2 (Cell Signalling) were used at 1:3000.  $\alpha$ -tubulin (Santa Cruz) was used as a loading control at 1:1000. Cells were washed 6 times at 10min intervals with PBS-T. Secondary antibody; HRP-conjugated goat IgG raised against rabbit and rabbit anti-mouse HRP-conjugated (Amersham Biosciences) were both used at 1:5000 in 5% milk PBS-T, and incubated for 2hrs at room temperature. Membranes were washed as previously described, prior to visualisation by enhanced chemiluminescence (ECL)-plus Western blotting detection system (Amersham Biosciences).

Phosphorylation blots were carried out using the same lysis buffer as above, with the addition of a phosphatase inhibitor cocktail (Pierce). Cells were treated at 37°C and put on ice immediately at the end of stimulation. Cells were washed 3 times with ice cold Tris-buffered saline (TBS) prior to lysis. Samples were denatured, run, and transferred onto nitrocellulose membranes as per standard Western blots. Membranes were blocked in 5% bovine serum albumin (BSA) (Sigma) in TBS with 0.1% Tween (TBS-T).

Anti-phospho Ser1177 antibody was used 1:1000 (New England Biolabs) in 5% BSA TBS-T and incubated overnight at 4°C. Washes were performed with TBS-T

as per standard Western blots.

Membranes were stripped with buffer containing: Tris-Hcl (pH6.7) 56mM, SDS 2%, and beta-mercaptoethanol 0.7% made up in ultra-pure water, for 30 minutes at room temperature and re-blocked in 5% milk PBS-T prior to incubation with second antibody.

## **2.5 Reverse transcriptase polymerase chain reaction**

Total cell RNA was extracted by RNeasy Mini Kit (Qiagen) with QiaShredders (Qiagen) to remove cell debris as per manufacturer's protocol. RNA quantity was determined by absorbance at 260nm using a Nanodrop Lite spectrophotometer (Thermo scientific). 1 $\mu$ g RNA was reverse transcribed to cDNA using iScript cDNA synthesis kit (Biorad) as previously described [48]. cDNA was amplified using GoTaq Green master mix (Promega) as per manufacturers protocol. PCR products were separated by electrophoresis on an agarose gel, and visualised with ethidium bromide staining.

RT-PCR was performed with the following primers:

**Table 2.1 RT-PCR primers**

| Gene             | Primer- sense (5'-3')    | Primer- antisense (5'-3') | Amplicon length |
|------------------|--------------------------|---------------------------|-----------------|
| Orai-1           | AGTCCTCACCTTCCCACTGG     | GCCTCTTCCTTCCACACTCTG     | 133             |
| Orai-2           | ACGCATCACAAGAGCCACAG     | TGCGAGGACAGGAGTATGGAG     | 82              |
| Orai-3           | GCAACATCCACAATCTCAACTCTG | ACCCAGCCCACCAAAACAAAC     | 92              |
| $\beta$ -actinin | TTGTAACCAACTGGGACGATATG  | GATCTTGATCTTCATGGTGCTAGG  | 764             |

**Table 2.2 RT-PCR cycling conditions**

| Gene             | Cycling Step         | Temperature (°C) | Time (secs) | No. cycles |
|------------------|----------------------|------------------|-------------|------------|
| All              | Initial Denaturation | 95               | 180         | 1          |
| Orai-1 & Orai-3  | Denaturation         | 95               | 30          | 32         |
| Orai-2           |                      | 95               | 45          | 32         |
| $\beta$ -actinin |                      | 95               | 60          | 27         |
| Orai-1 & Orai-3  | Annealing            | 55               | 30          | 32         |
| Orai-2           |                      | 54               | 30          | 32         |
| $\beta$ -actinin |                      | 56               | 30          | 27         |
| Orai-1 & Orai-3  | Extension            | 72               | 30          | 32         |
| Orai-2           |                      | 72               | 30          | 32         |
| $\beta$ -actinin |                      | 72               | 45          | 27         |
| All              | Final Extension      | 72               | 300         | 1          |
| All              | Hold                 | 4                | Forever     | 1          |

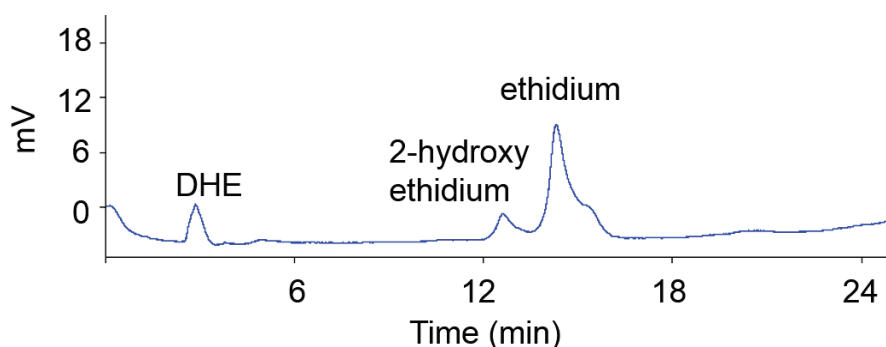
## 2.6 Dihydroethidium assay

The conversion of dihydroethidium (DHE) to 2-hydroxyethidium was analysed by high performance liquid chromatography (HPLC) which reflects the production of superoxide as previously described [57]. Cells were plated at  $1 \times 10^5$  in 12-well plates 24 hours prior to experiments. Cells were pre-incubated for 5mins in the presence or absence of 1mM L-NAME in standard external 2mM  $\text{Ca}^{2+}$  solution

before spiking with agonists. Cells were incubated at 37°C post-stimulation for 15mins. 50µM DHE (Invitrogen) was added and cells incubated for a further 20mins at 37°C. Cells were harvested in ice cold PBS, spun down 3,000rpm, 5mins. Pellet was resuspended in ice cold methanol and 0.1M HCl. Suspensions were spun at 13,000rpm, 10mins to remove cell debris.

Levels of 2-hydroxyethidium were determined using HPLC (Jasco, UK) separation with an ODS3 reverse phase column (250mm,4.5mm) (Hichrom UK), with mobile phases of mobile phase A 0.1% trifluoroacetic acid and mobile phase B 0.085% trifluoroacetic acid in acetonitrile. Fluorescence detector was set at excitation: 510nm and emission: 595nm.

Data was analysed using Azure Software (v4.02) (Datalys).



**Figure 2.1 Dihydroethidium assay peak identification.**

Representative HPLC trace of untreated sEnd.1 cells. Three peaks are visible in the chromatographs separated by elution times: DHE (4mins), 2-hydroxyethidium (13mins) and ethidium (16mins). 2-hydroxyethidium is considered specific for  $O_2^-$  production.

## 2.7 L-arginine to citrulline assay

Conversion of L-citrulline to L-arginine as a method of quantifying NO production was carried out as previously described [65].

sEnd.1 cells were plated in 12-well plates at  $1 \times 10^5$  and incubated for 24hrs prior to experiment. Culture medium was removed and cells washed 3 times in standard external solution without L-arginine.

Standard solution additionally containing arginase inhibitor N<sup>ω</sup>-hydroxy-nor-Arginine (Nor-NOHA) (10mM) and <sup>14</sup>C-L-arginine (1.3875Bq/ml) was applied to cells (500μl/well) and pre-incubated at room temperature for 5mins, with 1mM L-NAME and 50μM La<sup>3+</sup> where appropriate. Cells were either left untreated or were stimulated with TG (2μM) or Ca<sup>2+</sup> ionophore ionomycin (2μM). Cells were incubated at 37°C for a further 1.5-2hrs. Supernatant was kept. Cells were lysed in water by 3 times freeze-thaw on dry ice, and lysates were deproteinated with 10% trichloroacetic acid. Supernatant and lysates were combined and spun at 13,200rpm for 5mins. Supernatants were run on HPLC to assay <sup>14</sup>C-L-arginine conversion to citrulline.

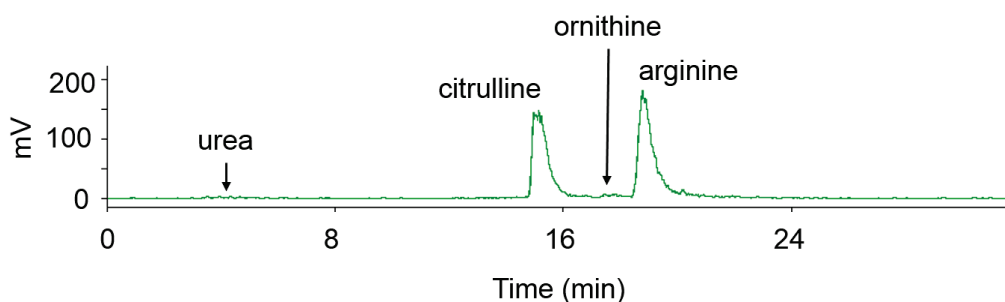
<sup>14</sup>C-L-arginine reaction products were resolved by HPLC on a Supelco Supelcosil LC-SCX 5mCM (250x4.6mm) column with a Supelco Supelguard LC-SCX (200mm) guard column (both Jasco). Samples were loaded onto the HPLC apparatus using a Jasco AS-2057 Plus (Jasco) cooled autosampler. Buffer A, distilled water (0.01% sodium azide), was run for 5 minutes, before a linear gradient over 10mins of buffer A + buffer B was applied. 100% buffer B, 200mM sodium citrate (pH 3.0) (0.01% sodium azide) was run for 15mins. Column was washed with 100% distilled water for 3mins in between samples. Buffers were applied and



mixed using a Jasco DG-980-50 degasser, 2x Jasco PU-2080 Plus pumps, and a Jasco MX-2080-32 dynamic mixer. Scintillant fluid (LabLogic) was mixed after elution from the column at 0.5:1 ratio, and then passed through scintillation detector. Compounds were detected using a Lab Logic  $\beta$ -RAM Model 3 (LabLogic Systems Ltd.) scintillation detector.

External solutions containing  $^{14}\text{C}$ -L-arginine were used as standards for total  $^{14}\text{C}$ -L-arginine.  $^{14}\text{C}$ -L-arginine (Amersham) and  $^{14}\text{C}$ -L-citrulline (NEN life science) were used as elution time standards.

Data was analysed using Azure Software (v4.02) (Datalys).



**Figure 2.2**  $^{14}\text{C}$ -L-arginine to L-citrulline conversion assay peak identification.

Representative HPLC trace. Four peaks are visible in the chromatographs separated by elution times. The elution times of detectable products are: urea (5mins), ornithine (19mins), citrulline (15.5mins) and remaining arginine (21mins).

## 2.8 Transfections

The method of transient transfections of sEnd.1 cells has not been previously reported in the literature. Successful transfection of sEnd.1 was more difficult than initially anticipated. Numerous transfection techniques were trialled, in order to

ensure the best transfection was achieved. Although, this process was time consuming, NanoJuice was identified as the most effective transfection method. Data from NanoJuice transfections will be presented in chapter 4, and other transfection methods will be discussed. Here, transfection protocols of all methods trialled are briefly described.

### **2.8.1 Lipofectamine**

Lipofectamine (Invitrogen) was carried out as per manufacturer's recommendations. Briefly, cells were plated 24hrs prior to transfection and transfected at  $\sim 70\%$  confluency. Lipofectamine 2000 (Invitrogen) was diluted in Opti-mem (Invitrogen) in a 1:12.5 ratio. Separately DNA/siRNA was also diluted in Opti-mem as per correct final concentration. Both solutions were incubated for 5mins at room temperature prior to combining in a 1:1 ratio, and incubating for a further 20mins. siRNA/DNA-Lipofectamine solutions were applied to cells in antibiotic-free media and incubated for 1hr. Transfection solution was removed and replaced with fresh antibiotic-free media. Cells were further incubated for 36-48hrs at  $37^{\circ}\text{C}$ .

### **2.8.2 Amaxa**

Amaxa cell line nucleofactor kit V (Lonza) was used with accompanying electroporation machinery. Briefly, sEnd.1 cells at  $\sim 80\%$  confluency were trypsinised and spun down for 3mins, 1000rpm. The cell pellet was resuspended in 100 $\mu\text{l}$  nucleofactor, and siRNA/DNA was added for the appropriate final concentration. Suspension was transferred to Amaxa cuvette and electroporated using pre-programmed

protocol G-30.

Cells were plated in standard antibiotic cell medium and incubated for 36-48hrs hours at 37°C. Medium was changed after 24hrs.

### 2.8.3 Neon

The Neon electroporation system (Invitrogen) was optimised as per manufacturer's protocol. Briefly, cells were grown up to ~80% confluency. Trypsinised cells were washed in PBS in absence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{+}$ .  $1 \times 10^5$  were used per transfection. DNA/siRNA were used within the recommended ranges. Cells were plated in antibiotic-free medium. Cells were incubated for between 36-48hrs. Medium was changed after 24hrs.

### 2.8.4 FuGene 6 and HD

Cells were plated 24hrs prior to transfection, and were transfected at *sim*60% confluency. FuGene 6 and FuGene HD (Promega) was used as per manufacturer's protocol. Briefly, FuGene was used in a 3:1 ratio with siRNA/DNA. siRNA/DNA was added to fresh cold DMEM and incubated at room temperature for 5mins. FuGene reagent was added and further incubated for 15mins at room temperature. Transfection mix was applied dropwise onto cells with fresh standard antibiotic medium. Cells were incubated for 36-48hrs at 37°C. Medium was changed after 24hrs.

### 2.8.5 NanoJuice

Cells were plated 24hrs prior to transfection, and were transfected at  $\sim 60\%$  confluency. Nanojuice was optimised as per manufacturers protocol. Transfection core reagent and booster were diluted in a 1:40 and 1:80 ratio in Opti-mem respectively, and incubated for 5mins at room temperature. siRNA/DNA was added as per appropriate final concentration. Transfection reactions were incubated for a further 15mins at room temperature, before being added dropwise to cells in standard antibiotic cell medium. Cells were incubated for 36-48hrs at  $37^{\circ}\text{C}$ . Medium was changed after 24hrs.

## 2.9 STIM-1 knockdown

STIM-1 knockdown used a pre-designed STIM-1 silencer select siRNA (Invitrogen) ID: S74488.

## 2.10 Quantification of gels

Gel films and photographs were scanned on a flatbed scanner. Relative band intensities were analysed using ImageJ. Bands were normalised to  $\alpha$ - and  $\beta$ -tubulin controls before comparison.

## 2.11 Statistical analysis

Number of experiments on separate days is defined as N. Number of cells/total repeats is defined as n. All experiments were performed on at least 2 separate occasions. Data-set normality was assessed using the Shapiro-Wilks test. Normal and log-normal data is presented as mean $\pm$ SEM, and significance was determined using a Student's T-test, or for multiple comparisons a one-way ANOVA was performed with Tukey's post test. Non-normal data has been assessed for significant differences in distribution of data using the Kolmogorov-Smirnov test. Data is presented in cumulative percentile plots. Significance is considered as  $P < 0.05$ . Outliers of non-normal data were determined through John Tukey analysis and were removed from analysis. Correlation was determined as a Spearman's rank coefficient of correlation.

| Product                                                              | Supplier          |
|----------------------------------------------------------------------|-------------------|
| DMEM                                                                 | Gibco             |
| Foetal Bovine Serum                                                  | Gibco             |
| Penicillin-streptomycin                                              | Gibco             |
| Trypsin                                                              | Gibco             |
| Cell freezing medium                                                 | Gibco             |
| 13mm glass coverslips                                                | VWR               |
| Petri dish small                                                     | Corning           |
| Petri dish medium                                                    | Corning           |
| Petri dish large                                                     | Corning           |
| D-(+)-Glucose                                                        | Sigma             |
| CaCl <sub>2</sub>                                                    | Sigma             |
| NaCl                                                                 | Sigma             |
| HEPES                                                                | Sigma             |
| KCl                                                                  | Sigma             |
| MgCl <sub>2</sub>                                                    | Sigma             |
| Ethylenediaminetetra-acetic acid (EDTA)                              | Fluka             |
| Ethylene-glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA)  | Fluka             |
| MeOH                                                                 | Sigma             |
| EtOH                                                                 | Sigma             |
| NaOH                                                                 | Fluka             |
| HCl                                                                  | Sigma             |
| Dimethylsulphoxide                                                   | Sigma             |
| L-arginine                                                           | Sigma             |
| Sodium nitroprusside dihydrate                                       | Sigma             |
| Thapsigargin                                                         | Calbiochem        |
| Fura-2-AM                                                            | Life Technologies |
| DAF-2-DA                                                             | Calbiochem        |
| N <sup>ω</sup> -nitro-L-arginine methyl ester hydrochloride (L-NAME) | Sigma             |
| 2-APB                                                                | Tocris            |

| <b>Product</b>                              | <b>Supplier</b> |
|---------------------------------------------|-----------------|
| FCCP                                        | Tocris          |
| LaCl <sub>2</sub>                           | Sigma           |
| Mg-ATP                                      | Sigma           |
| Synta-66                                    | GSK             |
| Ionomycin                                   | Tocris          |
| Qiagen mini Rneasy kit                      | Qiagen          |
| Qiashredders                                | Qiagen          |
| iScript cDNA synthesis kit                  | Bio-Rad         |
| GoTag Green Master Mix                      | Promega         |
| Universal Negative control                  | Invitrogen      |
| RNase Zap                                   | Sigma           |
| Agarose                                     | Sigma           |
| Hyperladder IV                              | Bioline         |
| Qiagen mini Rneasy kit                      | Qiagen          |
| Qiashredders                                | Qiagen          |
| iScript cDNA synthesis kit                  | Bio-Rad         |
| GoTag Green Master Mix                      | Promega         |
| Universal Negative control                  | Invitrogen      |
| RNase Zap                                   | Sigma           |
| Agarose                                     | Sigma           |
| Hyperladder IV                              | Bioline         |
| RIPA buffer                                 | Sigma           |
| Phosphatase Inhibitor Cocktail              | Pierce          |
| Protease Inhibitor Cocktail                 | Sigma           |
| Na <sub>3</sub> VO <sub>4</sub>             | Santa Cruz      |
| PMSF                                        | Santa Cruz      |
| Bovine serum albumin protein assay standard | Bio-Rad         |
| Tris/Glycine/SDS buffer (10x)               | Bio-Rad         |
| Resolving Gel Buffer                        | Bio-Rad         |

| Product                                           | Supplier          |
|---------------------------------------------------|-------------------|
| Sodium-n-Dodecyl Sulphate 20% (w/v) solution      | Calbiochem        |
| Ammonium persulphate                              | Sigma             |
| TEMED                                             | Sigma             |
| Sample buffer                                     | Sigma             |
| PageRuler molecular weight marker                 | Thermo Scientific |
| 40% Acrylamide/Bis solution 37.5:1                | Bio-Rad           |
| Dc-protein assay                                  | Bio-Rad           |
| Protran<br>Premium 0.45uM Nitrocellulose membrane | Amersham          |
| ECL<br>Prime Western Blotting Detection Reagent   | Amersham          |
| High<br>performance chemiluminescence film        | Amersham          |
| Extra thick blotting paper                        | Bio-Rad           |
| Bovine<br>serum albumin                           | Sigma             |
| Skimmed<br>milk powder                            | Fluka             |
| Tris-buffered<br>solution (10x)                   | Bio-Rad           |
| Phosphate-buffered<br>solution (10x)              | Bio-Rad           |
| 2-mercaptoethanol                                 | Sigma             |
| Tween-20                                          | Sigma             |
| Dihydroethidium                                   | Invitrogen        |
| Water with 0.1% Trifluoroacetic acid              | Fluka             |
| 0.085%<br>Trifluoroacetic acid in acetonitrile    | Fluka             |
| Nor-NOHA                                          | Cayman Chemical   |
| Trichloroacetic<br>acid                           | Fluka             |
| Sodium citrate                                    | Sigma             |
| <sup>14</sup> C-L-arginine                        | Amersham          |
| <sup>14</sup> C-L-citrulline                      | NEN life science  |
| Citric<br>acid                                    | Fluka             |



# Chapter 3

## Characterisation of the CRAC channel in sEnd.1

### 3.1 Introduction

The role of SOC channels in the vascular endothelium is still poorly defined in terms of downstream functions, despite it being clear that SOCE is crucial for the maintenance of many major endothelial functions (previously discussed in chapter 1). Research into endothelial SOCE largely focused, prior to the discovery of Orai and STIM on the complex field of TRPC channels and their formation of homo- and heteromultimers to support SOCE. This wealth of data has limited the research into the role of CRAC channels in endothelia [53, 139, 323].

Although the relevance of  $I_{\text{CRAC}}$  in immune cells is readily accepted, such acceptance in the endothelial field has been far slower. The Trebak group first demon-

strated endothelial  $I_{\text{CRAC}}$  in 2008; robustly showing a role for Orai and STIM in HUVECs and HPAECs. They utilised both patch clamp and  $\text{Ca}^{2+}$  imaging to ensure rigorous identification of CRAC channels. Pharmacological profiles and knockdowns of Orai-1 and STIM-1 demonstrated significant reduction in  $I_{\text{CRAC}}$  and  $\text{Ca}^{2+}$  signals in imaging experiments [1]. However, this data was questioned in part due to previous TRPC data, which created a dialogue to establish the presence and significance of Orai/STIM-dependent SOCE [21, 192, 337]. This has slowed research into downstream functions of CRAC channel signalling in endothelial cells. Subsequently, CRAC channels and  $I_{\text{CRAC}}$  have been identified in a multitude of endothelial cells including: Ea.hy926, endothelial progenitor cells, human saphenous vein endothelial cells, and HUVECs. These studies have also addressed the role of TRPC channels in endothelial SOCE, and ultimately determined SOCE to be dependent upon Orai/STIM rather than a TRPC channel [4, 192].

Few downstream functions of endothelial SOCE have yet been identified. The field has primarily focused on CRAC channel involvement in endothelial cell proliferation and migration in angiogenesis [1, 83, 192, 202]. Recent work has also linked STIM-1 to mediation of thrombin regulation of endothelial vascular permeability. Intriguingly, this function has been shown to be independent of Orai signalling, indicating an additional role for STIM-1 in endothelia [307].

The importance of CRAC channels in inflammatory vascular diseases is starting to become clear [88, 113]. SOCE has been linked to activation of eNOS, and production of NO, a critical endothelial function [197]. The regulation of eNOS is of great interest in vascular health and disease, with reduced vascular NO being linked to many vascular disease states. Despite this, the eNOS-activating SOC

channel has never been identified. Within this thesis I aim to determine whether CRAC channels play a role in this critical pathway.

The sEnd.1 cell line was isolated from a murine endothelioma transformed by polyoma virus middle T antigen [356]. Although not a commonly used endothelial cell model, the sEnd.1 cell line is advantageous when investigating mechanisms relating to eNOS regulation and downstream functions. sEnd.1 exhibits high eNOS expression, and strong  $\text{BH}_4$  production, ensuring ‘coupled’ NOS and thus strong NO production [118, 330, 349]. sEnd.1 cells therefore provide an excellent model system for characterising the  $\text{Ca}^{2+}$  regulation of eNOS.

$\text{Ca}^{2+}$  signalling has not previously been investigated in sEnd.1 . Here, I examine the profile of store-operated  $\text{Ca}^{2+}$  signalling in sEnd.1 employing the  $\text{Ca}^{2+}$ -sensitive fluorescent dye Fura-2-AM with live-cell imaging. I define SOCE in sEnd.1 cells as being mediated by the CRAC channel, through both direct pharmacological depletion of stores, and with physiological agonists. Further to this I describe the  $\text{Ca}^{2+}$  handling mechanisms and extrusion pathways, which modulate the duration of  $\text{Ca}^{2+}$  signals in sEnd.1 .

## 3.2 Results

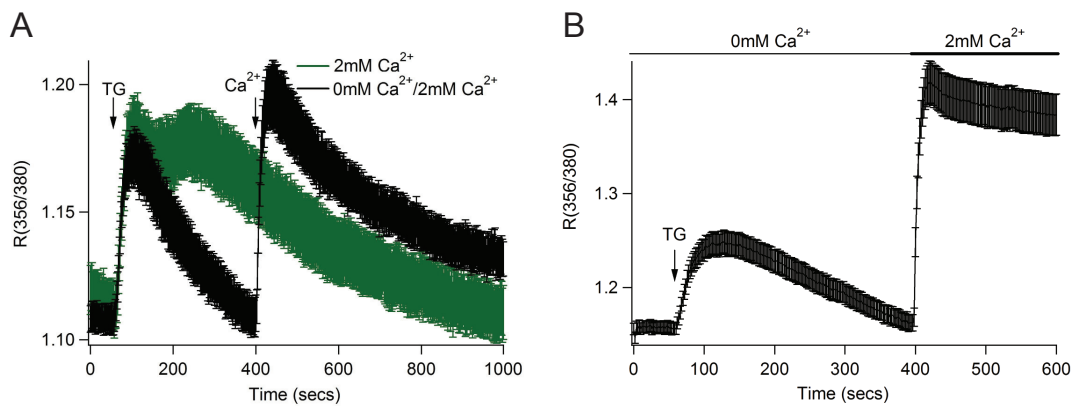
### 3.2.1 Induction of store-operated $\text{Ca}^{2+}$ entry in sEnd.1 cells

The ER  $\text{Ca}^{2+}$  store is inherently leaky, due to the presence of ill-defined  $\text{Ca}^{2+}$  transporters. Maintenance of a high  $\text{Ca}^{2+}$  concentration within the stores is mediated by the continual uptake of cytosolic  $\text{Ca}^{2+}$  by the sarco-endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) pump. Inhibition of SERCA with thapsigargin (TG) is a broadly recognised method of inducing store-operated  $\text{Ca}^{2+}$  entry, by passively depleting the endoplasmic reticulum  $\text{Ca}^{2+}$  store, with SERCA blocked,  $\text{Ca}^{2+}$  leakage is no longer compensated for by re-uptake, leading to store depletion. The  $\text{Ca}^{2+}$  entry subsequent to TG depletion of stores is strongly associated with the CRAC channel [27,326]. Store depletion in presence of extracellular  $\text{Ca}^{2+}$  shows a biphasic response associated with store-release followed by  $\text{Ca}^{2+}$  influx (Fig.3.1A). Although this demonstrates a sustained  $\text{Ca}^{2+}$  rise with store depletion, it is difficult to separate store release from  $\text{Ca}^{2+}$  entry in the continual presence of external  $\text{Ca}^{2+}$ . Store depletion with TG in the absence of external  $\text{Ca}^{2+}$ , followed by re-admittance of external  $\text{Ca}^{2+}$  allows these processes to be dissected. Following, TG treatment of sEnd.1 cells in the absence of external  $\text{Ca}^{2+}$  a slow increase in cytoplasmic  $\text{Ca}^{2+}$  occurs reflecting the store leak. The increase is transient, with  $\text{Ca}^{2+}$  extruded from the cell by mechanisms, which may include PMCA, NCX, and mitochondrial  $\text{Ca}^{2+}$  buffering (Fig.3.1A). The  $\text{Ca}^{2+}$  transient is complete within  $\sim 340$ secs, within which time it returns to basal cytosolic  $\text{Ca}^{2+}$  levels. Subsequent add-back of external  $\text{Ca}^{2+}$  (2mM) results in a rapid and sustained increase in intra-

cellular  $\text{Ca}^{2+}$ , which is associated with the influx of  $\text{Ca}^{2+}$  through SOC channels. Dissection of store release and  $\text{Ca}^{2+}$  entry in this manner allows manipulation of each process independently. Sustained rises after TG stimulation require extracellular  $\text{Ca}^{2+}$ . This was further confirmed with the addition of the fast  $\text{Ca}^{2+}$  chelator BAPTA (2.1mM) after  $\text{Ca}^{2+}$  add-back protocol. Chelation of external  $\text{Ca}^{2+}$  rapidly reduced intracellular  $\text{Ca}^{2+}$  (data not shown) ( $n=17$ ,  $N=2$ ).

The RBL-1 cell line is a common model system for investigation of CRAC channels and SOCE. Use of the same  $\text{Ca}^{2+}$  add-back protocol in these cells demonstrates a similar profile to that seen in sEnd.1 (Fig.3.1B). It should be noted however, the difference in the strength of the signals, and additionally the lower basal  $\text{Ca}^{2+}$  level in sEnd.1 cells. The extent of  $\text{Ca}^{2+}$  entry is distinctly lower in sEnd.1 cells when compared to RBL-1 (Fig.3.1A-B). Both a low resting  $\text{Ca}^{2+}$  level and small SOCE signals is consistent with the literature on endothelia, with the signal amplitudes observed in sEnd.1 being additionally seen in Ea.hy926, primary HUVECs and HPAECs [1, 4, 6, 90]. Despite the small signals, it can therefore be concluded that sEnd.1 are capable of supporting store-operated  $\text{Ca}^{2+}$  entry.

It was also noted that  $\text{Ca}^{2+}$  signals decay quickly in sEnd.1 cells (Fig.3.1A). This will be discussed in more depth later within this chapter.



**Figure 3.1** Store-operated  $\text{Ca}^{2+}$  entry can be induced in sEnd.1

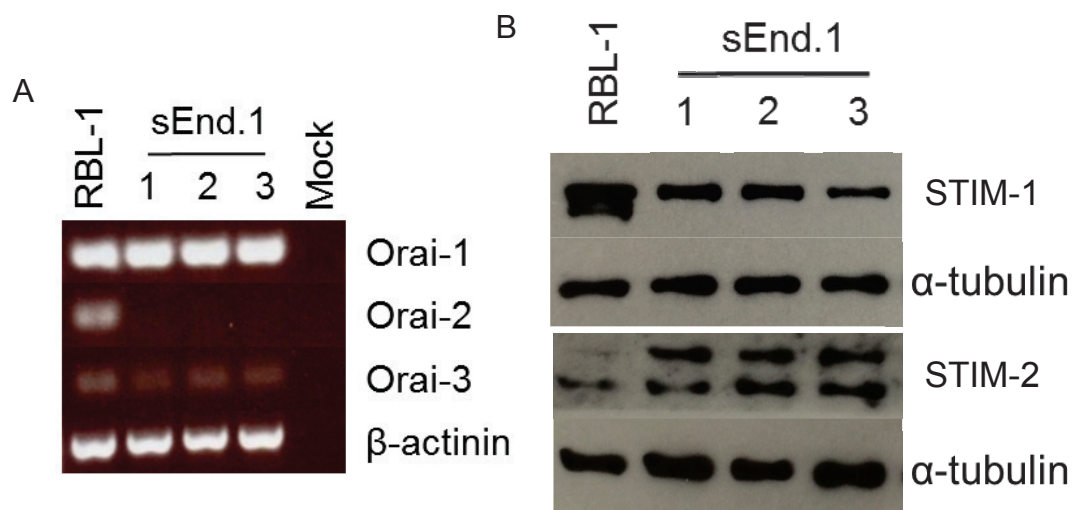
(A) Passive store depletion of stores with TG in the presence of external  $\text{Ca}^{2+}$  results in the classic biphasic rise in intracellular  $\text{Ca}^{2+}$  (green wave). The signal can be dissected; depleting stores in the absence of  $\text{Ca}^{2+}$ , and adding  $2\text{mM}$  external  $\text{Ca}^{2+}$  after  $\text{Ca}^{2+}$  recovery post-store depletion, allows a robust store-dependent  $\text{Ca}^{2+}$  entry to be observed (black wave). (B) Dissection of store depletion and  $\text{Ca}^{2+}$  entry in RBL-1 cells.

### 3.2.2 Presence of CRAC channel components Orai and STIM in sEnd.1

CRAC channel components have not previously been identified in sEnd.1 cells. The expression levels of Orai-1, -2, and -3 were established by RT-PCR, and compared to RBL-1. Both Orai-1 and -3 were robustly expressed within sEnd.1 to a similar extent as observed in RBL-1. Orai-2 expression, however, was absent (Fig.3.2A). Orai-2 has not been shown to mediate endogenous  $\text{Ca}^{2+}$  current in any vascular cell, and indeed recently has been shown not to contribute to TG- or histamine-induced SOCE signalling in HUVEC [375]. Furthermore, absence of Orai-2 in endothelia has previously been reported in microvascular endothelial cells and isolated lung endothelial cells [323]. Although this is not universal over

all endothelial cell types, with robust expression reported in HUVECs [1]. sEnd.1 cells originate from the microvasculature, lack of Orai-2 is therefore not surprising, with the absence of Orai-2 potentially being a hallmark of microvascular cells.

The protein levels of STIM-1 and -2 were also investigated by Western blot, with both STIM-1 and -2 expressed in sEnd.1 (Fig.3.2B). Expression of both components is to be expected, with STIM-1 controlling SOCE, and STIM-2 important in maintenance of basal ER store filling [28,33]. A double band was observed with STIM-2, with the larger band representing the highly phosphorylated form of the protein [357]. The expression of STIM-1, but not STIM-2, was markedly lower in sEnd.1 compared with RBL-1. Low STIM-1 expression has previously been reported, and suggested as a limiting factor to endothelial  $I_{CRAC}$  [1].



**Figure 3.2 Presence of CRAC channel components in sEnd.1**

(A) RT-PCR demonstrating the expression of Orai-1 and -3 in sEnd.1 cells. Expression levels of three separate sEnd.1 samples were assessed against an RBL-1 positive control (B) STIM-1 and -2 expression was identified through Western blot. Three separate samples were assessed against RBL-1.

### 3.2.3 sEnd.1 SOCE is dependent on CRAC channel activity

The molecular identity of channels supporting endothelial SOCE is still contended as previously discussed (section 1.3.1). With the identification of the principle CRAC channel proteins Orai-1 and STIM-1 in sEnd.1 I have aimed to determine the role of CRAC channels in generating sEnd.1 SOCE signals.

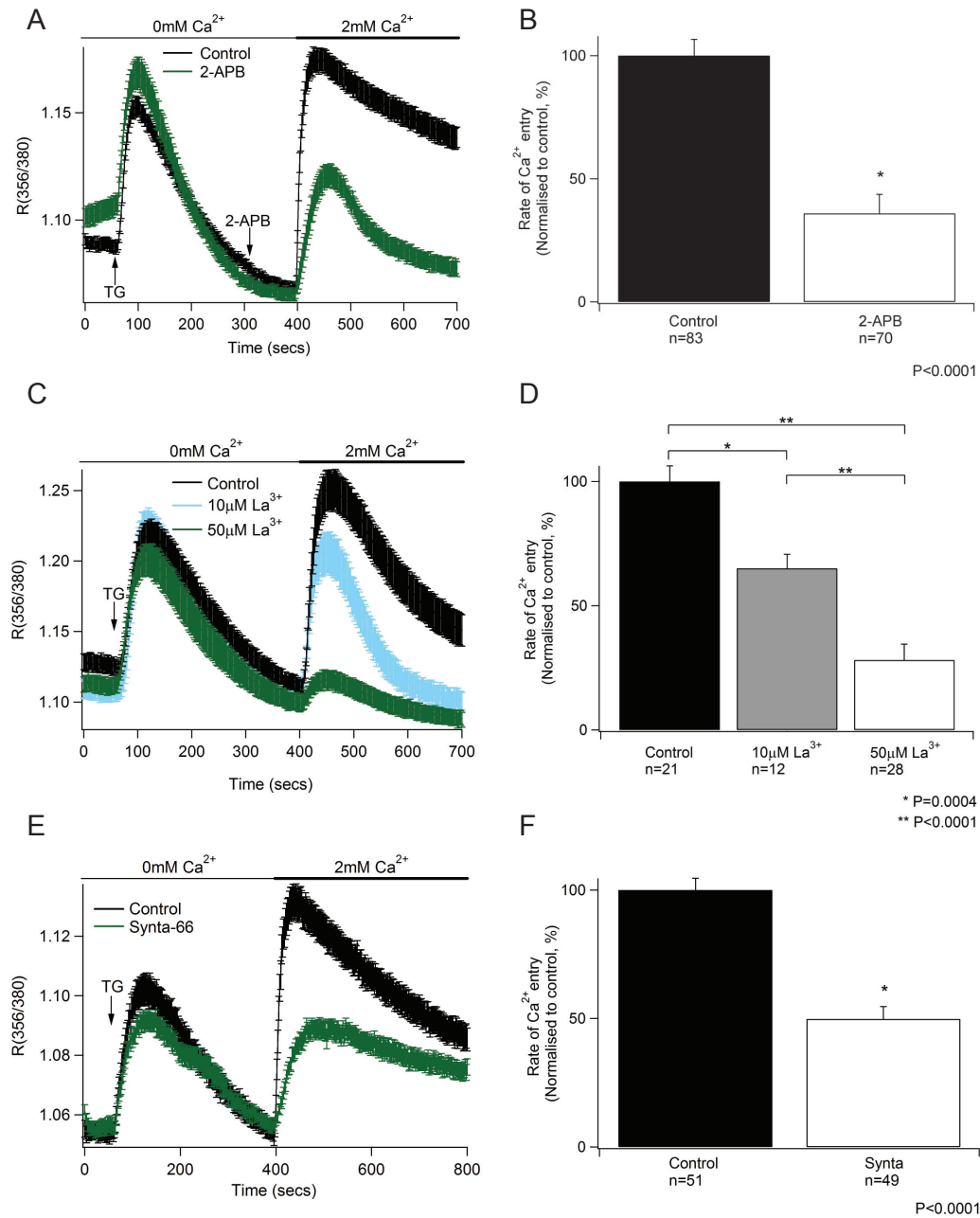
**Inhibition of SOCE with CRAC channel inhibitors** Various pharmacological agents can be used to inhibit CRAC channel activity with varying levels of specificity, acting both directly and indirectly. Due to the lack of a truly specific CRAC channel inhibitor at present, I have employed three different pharmacological agents:  $\text{La}^{3+}$ , 2-APB and Synta-66, to demonstrate that sEnd.1 SOCE requires the CRAC channel.

2-APB has a complex profile in activating and inhibiting CRAC channels, both potentiating and inhibiting channels comprised of Orai-1 and -2, and activating Orai-3 channels. Potentiation of Orai-1 is weak and occurs at low  $\mu\text{M}$  ranges of 2-APB ( $\text{EC}_{50}=4\mu\text{M}$ ) [262]. 2-APB block of Orai-1-mediated  $\text{Ca}^{2+}$  currents has an  $\text{IC}_{50}$  of  $8\mu\text{M}$  and has been shown to inhibit by up to 95% at concentrations  $>10\mu\text{M}$ . The mechanism of 2-APB block of Orai-1  $\text{Ca}^{2+}$  entry occurs by both STIM-1-dependent and -independent mechanisms [71, 262, 273]. It has further been speculated that 2-APB inhibits CRAC channels by binding and blocking an extracellular region of Orai-1 and -2 [9]. 2-APB is also suggested however to inhibit TRPC channel-dependent SOCE [196, 208]. The application of 2-APB ( $20\mu\text{M}$ ) post-TG store depletion reduced rate of  $\text{Ca}^{2+}$  entry by  $64\%\pm 8\%$  (Fig.3.3A-B).



$\text{La}^{3+}$ , and other lanthanides, can block CRAC channels in the low  $\mu\text{M}$  range ( $<100\mu\text{M}$ ) by binding to the glutamate ring within the Orai pore [146, 275]. In addition to this, lanthanides are also able to block other suggested store-operated channels, including the TRPC channels. Crucially however, TRPC inhibition requires concentrations  $>100\mu\text{M}$  [275]. I constructed a  $\text{La}^{3+}$  dose response curve (data not shown) which yielded a  $\text{IC}_{50}$  of  $8\mu\text{M}$ , and substantial block of  $\text{Ca}^{2+}$  entry at  $50\mu\text{M}$  (here shown as  $10\mu\text{M}$  and  $50\mu\text{M}$ ) (Fig.3.3C-D).  $10\mu\text{M}$   $\text{La}^{3+}$  and  $50\mu\text{M}$   $\text{La}^{3+}$  reduced rate of  $\text{Ca}^{2+}$  entry by  $34\%\pm 6\%$ , and  $72\%\pm 6\%$  respectively. Reduction of SOCE at these concentrations strongly implies the role of CRAC channels.

Synta-66 (GSK1349571A) has been defined as a CRAC channel inhibitor, with varying potency across cell types with  $\text{IC}_{50}$  values ranging from  $26\text{nM}$  to  $3\mu\text{M}$  [193, 240]. It can be considered a more specific CRAC channel inhibitor having, to this date, not been described as inhibiting any other  $\text{Ca}^{2+}$  entry process, and has been shown specifically not to effect TRPC-1, -5, -6 or TRPV4 [192, 193]. Its mechanism of action is unclear, although it is independent of the formation of STIM-1 puncta [193]. Pre-incubation (8mins) of sEnd.1 cells with Synta-66 ( $10\mu\text{M}$ ), before stimulation with TG, resulted in a  $50\%\pm 5\%$  reduction in the rate of  $\text{Ca}^{2+}$  entry (Fig.3.3E-F).



**Figure 3.3 Known CRAC channel inhibitors can reduce SOCE in sEnd.1**  
 (A-B) 20 $\mu$ M 2-APB applied 100secs prior to  $Ca^{2+}$  add-back reduced rate of entry by 64%. (C-D) A graded inhibition of SOCE could be achieved by varying  $La^{3+}$  concentrations. Pre-incubation (5mins) with 10 $\mu$ M and 50 $\mu$ M  $La^{3+}$  induced a 34% and 72% reduction in the rate of  $Ca^{2+}$  entry respectively. (E-F) Pre-incubation (8mins) with CRAC channel inhibitor Synta-66 (10 $\mu$ M) reduced rate of entry by 50%.

**Ca<sup>2+</sup> selectivity of sEnd.1 SOCE** CRAC channels exhibit a high specificity for divalent Ca<sup>2+</sup> ions, over monovalent ions, and can also notably distinguish between different cationic divalent ions with channel permeability having been reported as Ca<sup>2+</sup> >Sr<sup>2+</sup> >Ba<sup>2+</sup> [142, 379]. Specificity can be used as a defining feature of CRAC channel SOCE and further confirm the identity of the sEnd.1 store-operated Ca<sup>2+</sup> channel as the CRAC channel. TRPCs do not exhibit the same level of selectivity as CRAC channels [21].

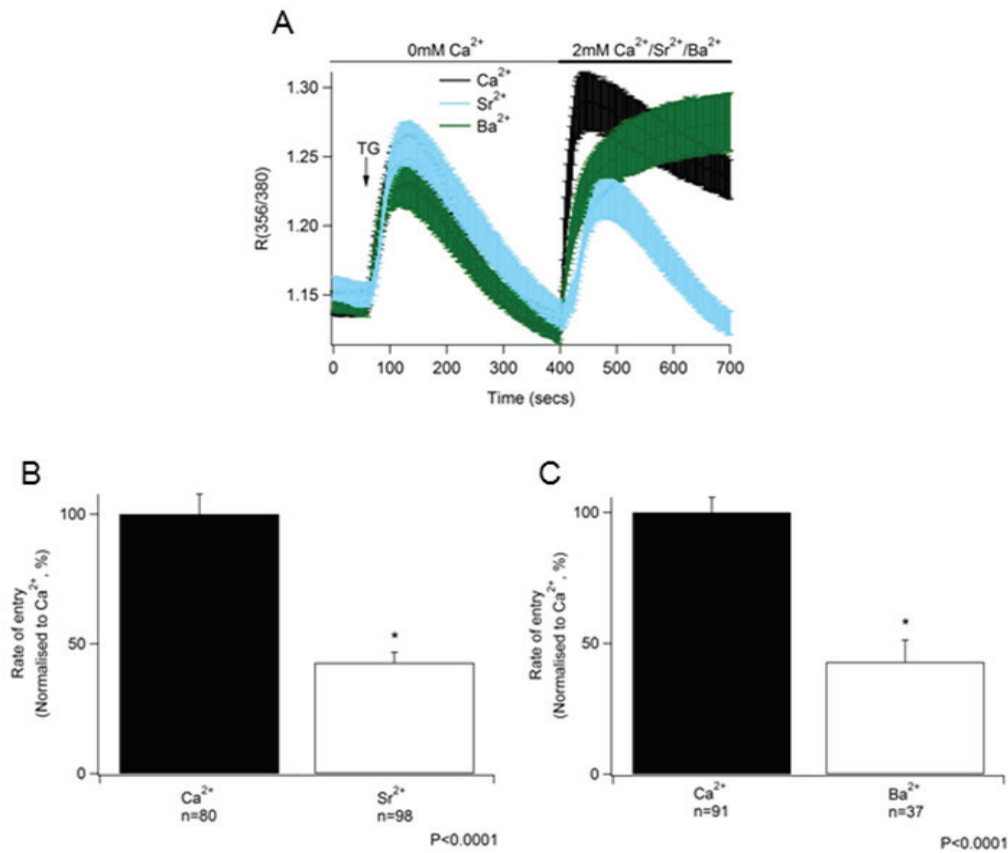
The selectivity of the CRAC channel is better defined by electrophysiology than fluorescence imaging; however due to the small conductance of endothelial I<sub>CRAC</sub>, electrophysiological measurements are only possible in divalent-free solution [1]. Selectivity of CRAC channels in endothelia can therefore not be assessed by this method.

The entry of divalent ions after induction of SOCE in sEnd.1 was examined. TG depletion of stores was followed by addition of 2mM external Ca<sup>2+</sup>, Sr<sup>2+</sup>, or Ba<sup>2+</sup> (Fig.3.4A). In sEnd.1 cells, the rate of both Sr<sup>2+</sup> and Ba<sup>2+</sup> entry are significantly reduced to 43%±4% and 43%±8%, respectively, compared to Ca<sup>2+</sup> entry (Fig.3.4B-C). This indicates that sEnd.1 SOCE discriminates against divalent ions other than Ca<sup>2+</sup>, similarly to CRAC channels. Fura-2 is sensitive to Sr<sup>2+</sup> and Ba<sup>2+</sup> eliciting similar excitation spectra to Ca<sup>2+</sup> [181]. However, Fura-2 displays a lower affinity for both Ba<sup>2+</sup> and Sr<sup>2+</sup> than Ca<sup>2+</sup>, with a 33 times greater affinity for Ca<sup>2+</sup> over Sr<sup>2+</sup> [362]. This must be considered when examining specificity of CRAC channels for divalent ions measured in Ca<sup>2+</sup> imaging experiments. Although this may indicate that detected changes in rate may be due to a reduced ability to detect rises in Ba<sup>2+</sup> and Sr<sup>2+</sup>, it should be considered that Ba<sup>2+</sup> does not affect the same

mechanisms as  $\text{Ca}^{2+}$ .  $\text{Ba}^{2+}$  is neither exported from the cytoplasm by PMCA and NCX, nor does it lead to  $\text{Ca}^{2+}$ -dependent inactivation of the CRAC channel; both of these differences would offset the lower affinity for Fura-2 [12]. Nonetheless, the results show a reduced signal to both  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  relative to  $\text{Ca}^{2+}$ , which is consistent with a difference in CRAC channel permeability.

I noticed that cytoplasmic  $\text{Sr}^{2+}$  removal was considerably faster than that of  $\text{Ca}^{2+}$ , returning to baseline within 300secs of stimulation. This far exceeds the loss of  $\text{Sr}^{2+}$  signal seen in RBL-1 under the same conditions (data not shown) ( $n=32$ ).  $\text{Sr}^{2+}$  is susceptible to the same extrusion mechanisms as  $\text{Ca}^{2+}$ . With the lower bulk rise of  $\text{Sr}^{2+}$  compared to  $\text{Ca}^{2+}$ , this extrusion may provide insight into the  $\text{Ca}^{2+}$  handling mechanisms operating in sEnd.1, by indicating a prominent role for the high affinity low capacity  $\text{Ca}^{2+}$  pump PMCA. The  $\text{Ca}^{2+}$  handling mechanisms present in sEnd.1 cells shall be investigated.  $\text{Ba}^{2+}$  signals remain sustained as  $\text{Ba}^{2+}$  is not susceptible to  $\text{Ca}^{2+}$  removal mechanisms [207].

With the combination of the profile of sEnd.1 pharmacological inhibition and the discrimination of the sEnd.1 store-operated  $\text{Ca}^{2+}$  channel against other divalent ions, the evidence I present here suggests that sEnd.1 SOCE is dependent on the CRAC channel. Further evidence involving targeted knock-down of STIM-1 will be presented in chapter 4. This work adds to the accumulating evidence of the requirement of CRAC channels for endothelial SOCE.



**Figure 3.4** sEnd.1 SOC channel exhibits selectivity for  $\text{Ca}^{2+}$

(A) A representative experiment. Store depletion by TG ( $2\mu\text{M}$ ) followed by add-back of 2mM  $\text{Ba}^{2+}$ ,  $\text{Sr}^{2+}$ , and  $\text{Ca}^{2+}$  after  $\text{Ca}^{2+}$  recovery, shows conduction of  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$  consistent with CRAC channel selectivity.  $\text{Sr}^{2+}$  extrusion rate is increased compared to  $\text{Ca}^{2+}$ . (B-C) Rates of  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  entry are both reduced by 57% compared to  $\text{Ca}^{2+}$ .

### 3.2.4 ATP and histamine can both induce store-operated $\text{Ca}^{2+}$ entry in sEnd.1

TG-induced store depletion is non-physiological. To ensure the biological relevance of endothelial SOCE being investigated in sEnd.1, two known agonists ATP and

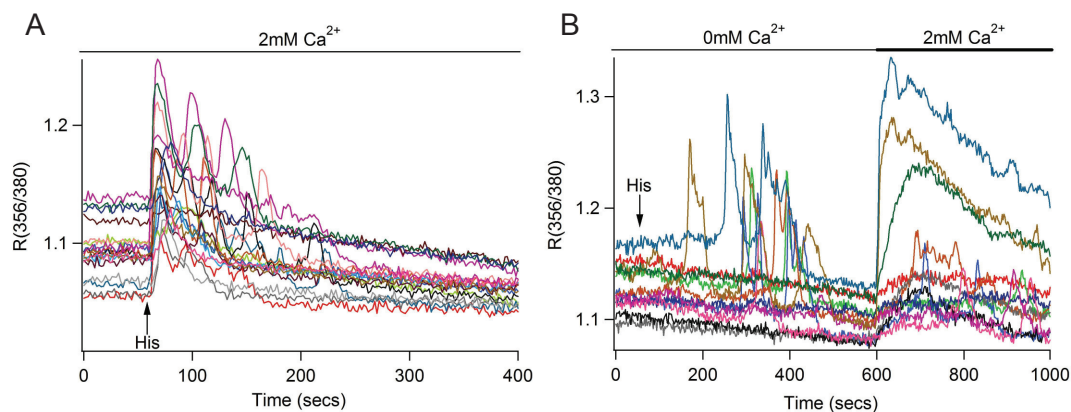
histamine were investigated [243, 249, 342]. Both ATP and histamine activate CRAC channels, via GPCR-dependent production of  $\text{IP}_3$  which results in  $\text{IP}_3\text{R}$ -dependent depletion of ER  $\text{Ca}^{2+}$  stores. Histamine-induced  $\text{Ca}^{2+}$  responses have recently been shown to require CRAC channels in HUVEC, with inhibition of influx with CRAC channel inhibitors including 2-APB, BTP-2, and  $\text{La}^{3+}$  [375]. Both histamine and ATP are known eNOS agonists in endothelial cells, and are therefore relevant to later work within this thesis [123, 186].

The concentration of histamine applied to endothelial cells dictates the type of intracellular  $\text{Ca}^{2+}$  rises observed. It has been reported that concentrations  $<10\mu\text{M}$  induces  $\text{Ca}^{2+}$  oscillations, whereas higher histamine concentrations results in a sustained plateau of  $\text{Ca}^{2+}$  in the presence of extracellular  $\text{Ca}^{2+}$  [62, 157, 243].

Stimulation of sEnd.1 cells with  $50\mu\text{M}$  histamine resulted in repetitive spikes of intracellular  $\text{Ca}^{2+}$  in  $\sim 50\%$  of cells in the presence or absence of external  $\text{Ca}^{2+}$  (Fig.3.5). Increases in  $\text{Ca}^{2+}$  were irregular and not consistent with true  $\text{Ca}^{2+}$  oscillations. With  $2\text{mM}$  extracellular  $\text{Ca}^{2+}$ , histamine elicited multiple spikes on a background of elevated  $\text{Ca}^{2+}$ . Amplitude of spikes were reduced over time and the total duration of response was  $<200\text{secs}$ . sEnd.1 cells failed to reach a sustained plateau phase, as previously described in the literature (Fig.3.5A) [62, 157, 243].

In the absence of extracellular  $\text{Ca}^{2+}$ , histamine evoked irregular  $\text{Ca}^{2+}$  spikes, which returned to baseline prior to the generation of another spike. Timing of  $\text{Ca}^{2+}$  spikes was variable, with some cells generating only one  $\text{Ca}^{2+}$  spike within the duration of the experiment. The application of external  $\text{Ca}^{2+}$  resulted in an intracellular  $\text{Ca}^{2+}$  rise similar to that seen upon  $\text{Ca}^{2+}$  add-back after TG store release (Fig.3.5B). A  $\text{Ca}^{2+}$  rise occurred in 75% of cells, which had previously responded to histamine treatment in the absence of external  $\text{Ca}^{2+}$ . Observed rises subsequently decayed slowly. The extent of  $\text{Ca}^{2+}$  entry varied in both amplitude and rate of the rise (Fig.3.5B).

Low  $\mu\text{M}$  ATP in the presence of extracellular  $\text{Ca}^{2+}$  stimulates a spike of intracellular  $\text{Ca}^{2+}$  associated with  $\text{IP}_3$ -dependent release of  $\text{Ca}^{2+}$  from ER stores, followed by a sustained plateau due to the opening of SOC channels in the plasma membrane [244,342]. Treatment of sEnd.1 cells with  $5\mu\text{M}$  ATP in presence of external  $\text{Ca}^{2+}$  generated this expected response (Fig.3.6A). Dissection of ATP signals with readmittance of external  $\text{Ca}^{2+}$  after store release, as previously described in this chapter, demonstrated a rapid spiked increase in cytosolic  $\text{Ca}^{2+}$  upon extracellular ATP administration in  $\text{Ca}^{2+}$ -free solution, which returned quickly to baseline. Application of external  $\text{Ca}^{2+}$  demonstrated  $\text{Ca}^{2+}$  entry, albeit at a reduced rate compared with TG-induced SOCE. A reduced rate is likely due to the incomplete depletion of stores by agonist, and therefore reduced numbers of CRAC channels activated within a cell (Fig.3.6B). All cells imaged responded to ATP stimulation.

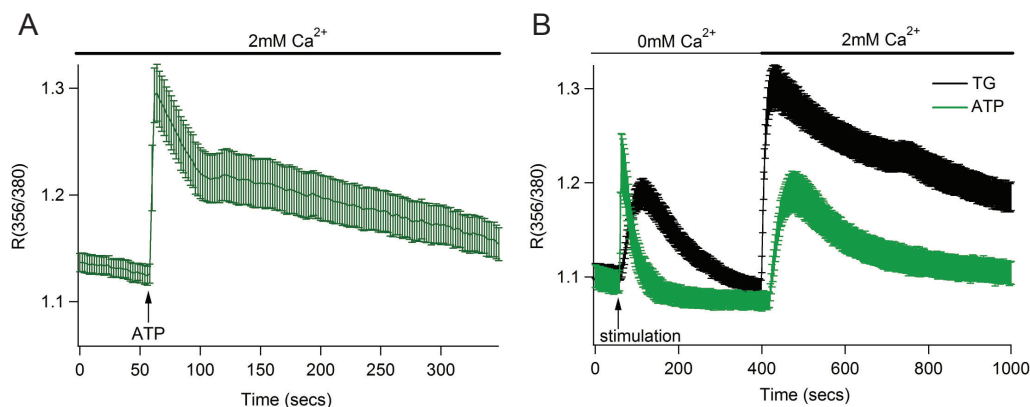


**Figure 3.5** Histamine induces fluctuating rises in cytosolic  $\text{Ca}^{2+}$  and is capable of eliciting a store-dependent  $\text{Ca}^{2+}$  entry response

Data displayed as individual waves of a representative experiment. (A) sEnd.1 treated with 50 $\mu\text{M}$  histamine in presence of 2mM external  $\text{Ca}^{2+}$ . 50% response rate over all experiments ( $n=113$ ). (B) Cells stimulated with 50 $\mu\text{M}$  histamine in absence of external  $\text{Ca}^{2+}$ . Cell response rate was 47% ( $n=136$ ), of which 75% subsequently permitted  $\text{Ca}^{2+}$  entry with the addition of 2mM extracellular  $\text{Ca}^{2+}$  ( $n=64$ ).

Here, I have shown that sEnd.1 cells can support both histamine- and ATP-induced SOCE responses. However, response to histamine is varied and therefore cannot be used reliably to ascertain downstream consequences of physiological SOCE. ATP stimulation, however, resulted in a strong biphasic increase in intracellular  $\text{Ca}^{2+}$ , which is consistent with SOCE. ATP shall, therefore, be used to investigate whether physiological agonists can induce CRAC channel-dependent responses.





**Figure 3.6 ATP induces SOCE**

(A) Treatment of sEnd.1 in 2mM external  $\text{Ca}^{2+}$ , with physiological agonist ATP (5 $\mu\text{M}$ ) elicited a rapid rise in cytosolic  $\text{Ca}^{2+}$  with a prolonged plateau phase consistent with extracellular  $\text{Ca}^{2+}$  entry. (B) Dissection of  $\text{Ca}^{2+}$  signal in response to ATP (5 $\mu\text{M}$ ), shown in comparison to 2 $\mu\text{M}$  TG add-back protocol. ATP induces a store-operated  $\text{Ca}^{2+}$  entry response. ATP induced  $\text{Ca}^{2+}$  entry has a smaller amplitude and slower rate to TG-induced entry, indicative of partial store depletion, and thus activation of fewer CRAC channels.

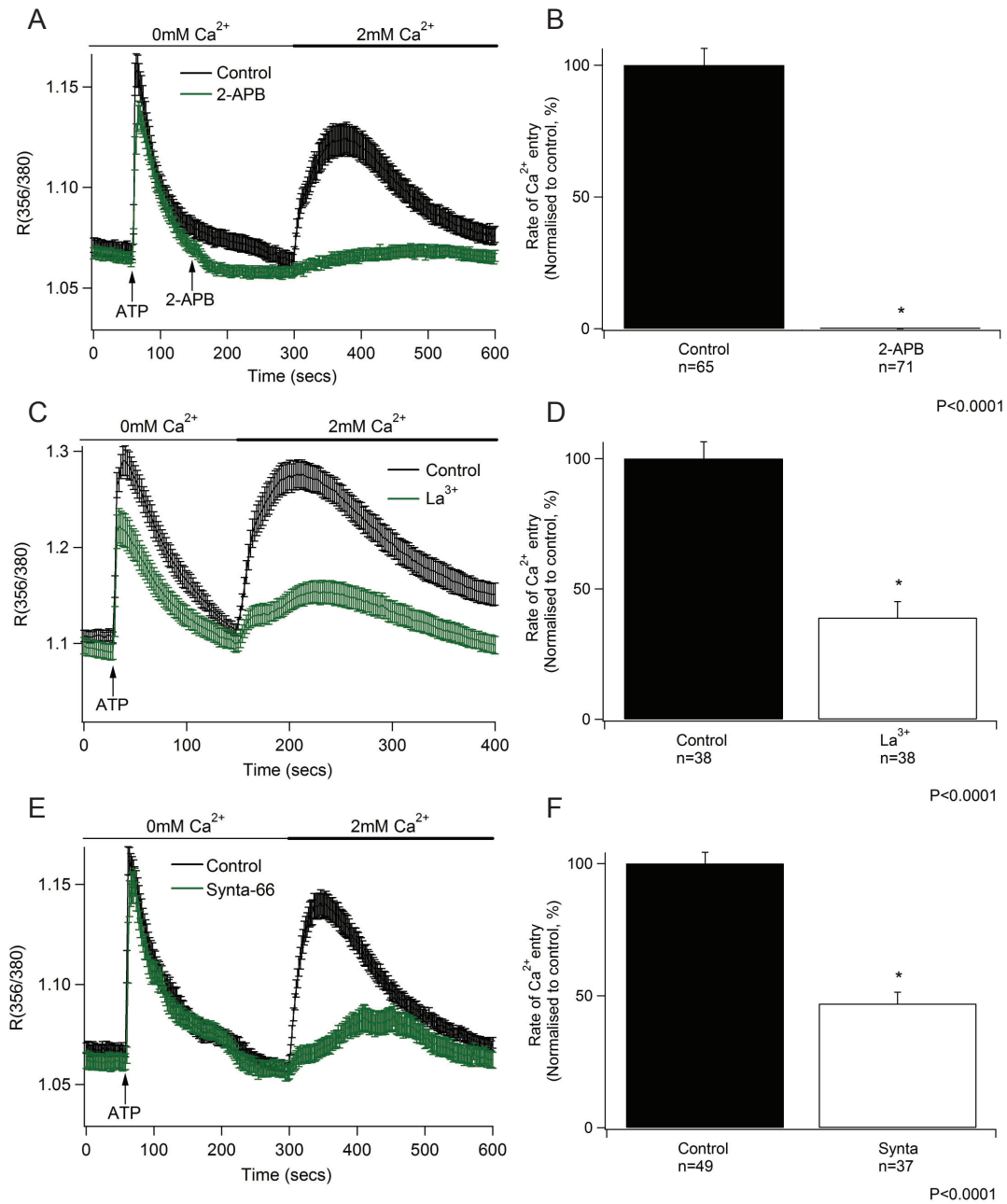
### 3.2.5 ATP-induced store-operated $\text{Ca}^{2+}$ entry is dependent on CRAC channel activity

ATP evoked increase in intracellular  $\text{Ca}^{2+}$  has previously been linked to both CRAC and TRPC channels [208]. To examine if ATP signals are dependent on CRAC channel activity in sEnd.1 cells, pharmacological inhibitors of CRAC channels and selectivity of the  $\text{Ca}^{2+}$  conducting channel were used as previously described for TG.

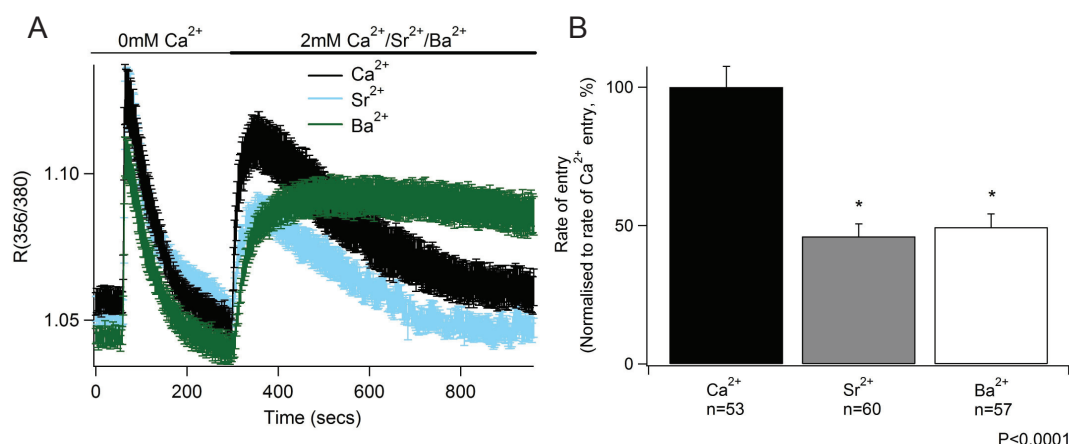
Synta-66 (10 $\mu\text{M}$ ), 2-APB (20 $\mu\text{M}$ ), and  $\text{La}^{3+}$  (50 $\mu\text{M}$ ) all reduced  $\text{Ca}^{2+}$  influx induced by ATP, with 2-APB being more effective at reducing ATP- than TG-evoked  $\text{Ca}^{2+}$  entry (see Fig.3.3 for TG inhibition). 2-APB showed almost complete abrogation

of entry, and so  $\text{Ca}^{2+}$  entry rates were too low to accurately determine (Fig.3.7A-B). 2-APB is a known inhibitor of  $\text{IP}_3\text{R}$  [214]. The increased effectiveness of 2-APB in inhibiting SOCE in sEnd.1 with physiological agonist therefore may be due to the requirement of  $\text{IP}_3\text{R}$  to empty the store and elicit SOCE. However, with this protocol 2-APB was added after store-depletion with ATP, allowing us to bypass the effect of 2-APB on  $\text{IP}_3\text{R}$ . Pre-treatment (5mins) with  $\text{La}^{3+}$  ( $50\mu\text{M}$ ) reduced the rate of entry by  $61\%\pm 6\%$  (Fig.3.7C-D). Pre-incubation with Synta-66 ( $10\mu\text{M}$ ) decreased the rate of  $\text{Ca}^{2+}$  entry to ATP by  $53\%\pm 4\%$  (Fig.3.7E-F).

Pharmacological inhibitors strongly infer that ATP induces  $\text{Ca}^{2+}$  entry through the CRAC channel, to further confirm this  $\text{Ca}^{2+}$  selectivity of ATP induced SOCE was investigated. Depletion of ER  $\text{Ca}^{2+}$  stores with ATP followed by addition of  $2\text{mM}$   $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$ , and  $\text{Ba}^{2+}$  resulted in similar reductions in rates of entry as seen with TG-treated cells (see Fig.3.4 for TG selectivity).  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$  displayed rates of entry which were  $46\%\pm 5\%$  and  $50\%\pm 5\%$  the rate of  $\text{Ca}^{2+}$  entry respectively (Fig.3.7). The rates of  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$  did not show any significant differences.



**Figure 3.7 Known CRAC channel inhibitors attenuate ATP-induced SOCE.** (A-B) 2-APB (20 $\mu\text{M}$ ) applied 150secs prior to  $\text{Ca}^{2+}$  readmission almost abolished all  $\text{Ca}^{2+}$  entry. (C-D) Pre-incubation (5mins) with a high dose of  $\text{La}^{3+}$  (50 $\mu\text{M}$ ) reduces rate of  $\text{Ca}^{2+}$  entry by 61%. (E-F) Pre-incubation (8mins) with the CRAC channel inhibitor Synta-66 (10 $\mu\text{M}$ ) reduced rate of entry by 53%.



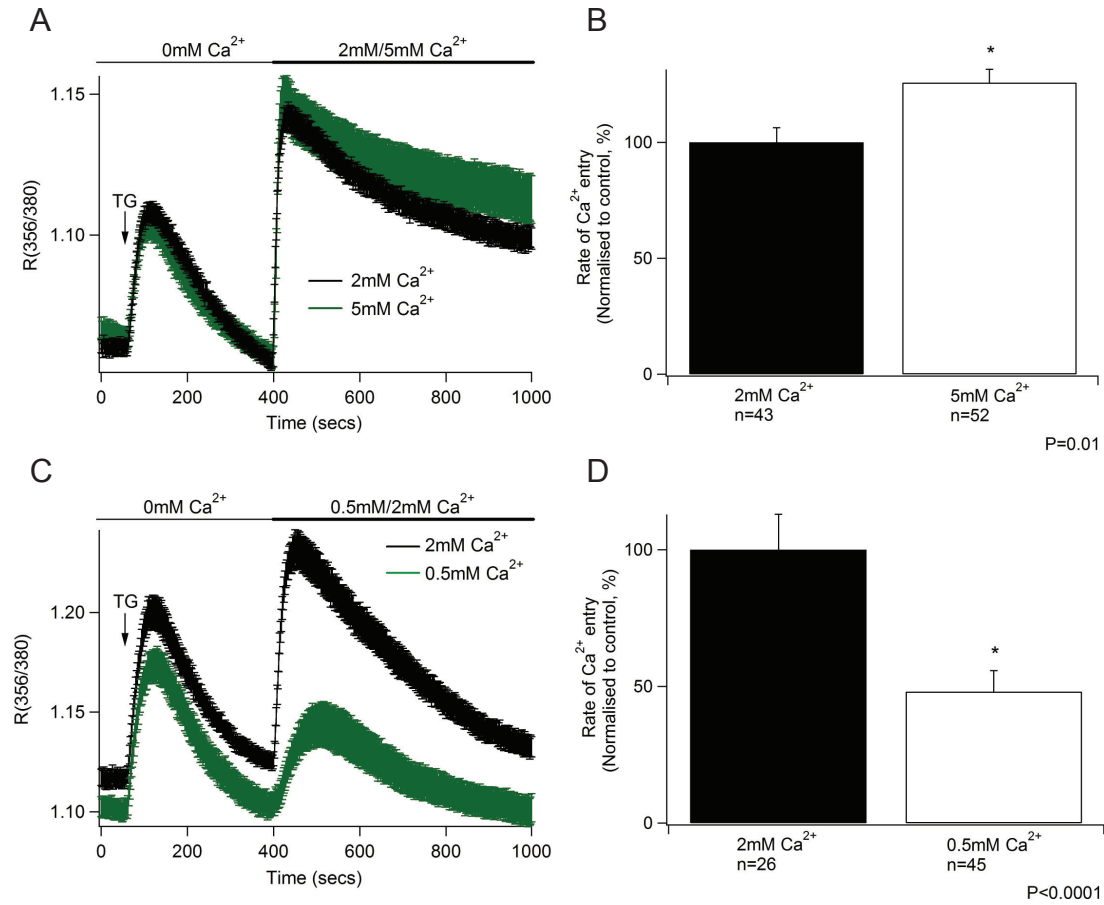
**Figure 3.8 ATP activates a channel with  $\text{Ca}^{2+}$  selectivity consistent with the CRAC channel**

(A) A representative experiment. Store depletion by ATP ( $5\mu\text{M}$ ) followed by add-back of 2mM  $\text{Ba}^{2+}$ ,  $\text{Sr}^{2+}$ , and  $\text{Ca}^{2+}$ . (B) Rates of  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$  entry are reduced by 54% and 51% respectively compared to  $\text{Ca}^{2+}$  control. No statistically significant differences are seen between  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  entry.

### 3.2.6 CRAC channel $\text{Ca}^{2+}$ conductance can be modulated by extracellular $\text{Ca}^{2+}$ concentration in sEnd.1

The low capacity of endothelial SOCE has previously been overcome somewhat by increasing external  $\text{Ca}^{2+}$  [342]. With in an increase in external  $\text{Ca}^{2+}$  from 2mM to 5mM, the rate of store-operated entry increased by  $26\% \pm 6\%$ . The bulk rise in cytosolic  $\text{Ca}^{2+}$  was both increased in amplitude and slightly more sustained (Fig.3.9A-B). Conversely, reduction of extracellular  $\text{Ca}^{2+}$  from 2mM to 0.5mM reduced the rate of  $\text{Ca}^{2+}$  entry by  $40\% \pm 8\%$ . The overall bulk rise in cytosolic  $\text{Ca}^{2+}$  was reduced and intracellular  $\text{Ca}^{2+}$  concentration returned to baseline within 400-600secs (Fig.3.9C-D). The reduction in external  $\text{Ca}^{2+}$  concentration resulted in a  $\text{Ca}^{2+}$  entry profile which was similar to that observed with the addition of

2mM extracellular  $\text{Sr}^{2+}$  (Fig.3.4A-B). Flux through CRAC channels is therefore susceptible to changes in extracellular  $\text{Ca}^{2+}$ , providing a useful tool for manipulating the local and bulk  $\text{Ca}^{2+}$  rise in sEnd.1 cells.



**Figure 3.9 Rate of  $\text{Ca}^{2+}$  entry is dependent on external  $\text{Ca}^{2+}$  concentrations** (A-B) Increased external  $\text{Ca}^{2+}$  to supra-normal concentration (5mM) increased the rate of entry by  $26\% \pm 6\%$ . (C-D) Reduced external  $\text{Ca}^{2+}$  (0.5mM) reduced the rate of entry by  $40\% \pm 8\%$ . Time taken to return to basal intracellular  $\text{Ca}^{2+}$  levels was reduced with lowered external  $\text{Ca}^{2+}$ .

### 3.2.7 Role of PMCA and mitochondrial $\text{Ca}^{2+}$ buffering in sEnd.1 $\text{Ca}^{2+}$ handling

$\text{Ca}^{2+}$  removal mechanisms are critical in determining the size and duration of an intracellular  $\text{Ca}^{2+}$  signal, ensuring cytosolic  $\text{Ca}^{2+}$  returns to basal levels. Common  $\text{Ca}^{2+}$  handling mechanisms have been previously discussed in this thesis (see chapter 1), and include  $\text{Ca}^{2+}$  extrusion by PMCA and NCX, SERCA-dependent  $\text{Ca}^{2+}$  re-uptake into ER  $\text{Ca}^{2+}$  stores, and mitochondrial  $\text{Ca}^{2+}$  buffering.

The signals generated by the addition of extracellular  $\text{Sr}^{2+}$  (2mM) and low  $\text{Ca}^{2+}$  (0.5mM) were noted earlier in this chapter for their shorter duration, and rapid return to baseline levels (see Fig.3.4 and 3.9). The fast removal of  $\text{Sr}^{2+}$  in Figure 3.4 in the presence of TG may be indicative of a prominent role for PMCA in sEnd.1 cells, which is effective following modest increases in cytosolic  $\text{Ca}^{2+}$ . PMCA, therefore, may be critical in handling CRAC channel induced  $\text{Ca}^{2+}$  changes, which are characteristically small in endothelial cells [1]. The role of PMCA in sEnd.1 was addressed using  $\text{La}^{3+}$ , a rapid inhibitor of the pump at mM concentrations [133]. Addition of  $\text{Sr}^{2+}$  post-TG store depletion resulted in a slow rise in intracellular fluorescence which peaked  $\sim 50$ secs after  $\text{Sr}^{2+}$  application (Fig.3.10A).  $\text{La}^{3+}$  (1mM) applied at the peak in  $\text{Sr}^{2+}$  signal dramatically reduced extrusion, although a small gradual decline in  $\text{Sr}^{2+}$  levels was still present (Fig.3.10A). Conversely, the addition of  $\text{La}^{3+}$  (1mM) after  $\text{Ca}^{2+}$  add-back protocol, following TG-induced store depletion, had little effect on the rate of  $\text{Ca}^{2+}$  extrusion (Fig.3.10B). Consistent with a role for PMCA in handling small increases in cytosolic  $\text{Ca}^{2+}$ , pre-treatment of sEnd.1 with  $\text{La}^{3+}$  (1mM) before TG exposure in  $\text{Ca}^{2+}$ -free solution led to a

large and sustained  $\text{Ca}^{2+}$  rise (Fig.3.10D).

It is well established that there is redundancy in the plasma membrane  $\text{Ca}^{2+}$  extrusion mechanisms, with both PMCA and NCX contributing to removing  $\text{Ca}^{2+}$  from the cell [30]. Reduction of external  $\text{Na}^+$  limits the driving force for  $\text{Ca}^{2+}$  export via NCX exchange across the plasma membrane. Low  $\text{Na}^+$  alone did not alter  $\text{Ca}^{2+}$  extrusion (Fig.3.10C). However, when combined with  $\text{La}^{3+}$  block of PMCA, extrusion was substantially slowed (Fig.3.10C). The requirement of the presence of both  $\text{La}^{3+}$  and low  $\text{Na}^+$  in order to see a reduction in  $\text{Ca}^{2+}$  extrusion is indicative of compensation between  $\text{Ca}^{2+}$  extrusion mechanisms. Low external  $\text{Na}^+$  reduces the internal  $\text{Na}^+$  concentration, and therefore may also affect mitochondrial  $\text{Ca}^{2+}$  buffering. This was further investigated.

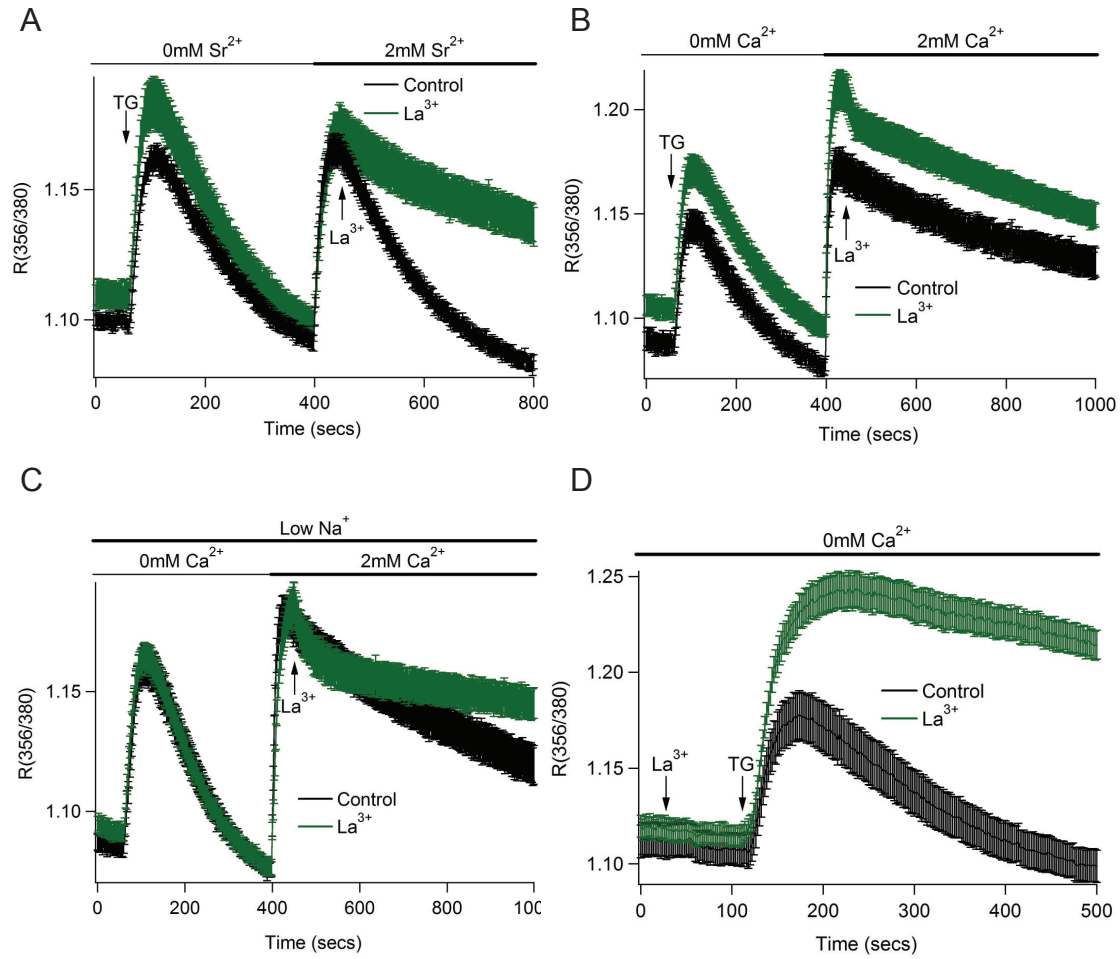
Mitochondrial buffering of cytosolic  $\text{Ca}^{2+}$  is also sensitive to changes in intracellular  $\text{Na}^+$ . Mitochondria have previously been demonstrated to be critical to  $\text{Ca}^{2+}$  homeostasis subsequent to CRAC channel activation in other cell types, including pancreatic acinar cells, modulating both local and global  $\text{Ca}^{2+}$  transients [160]. Mitochondrial  $\text{Ca}^{2+}$  uptake is mediated by an electrochemical gradient which facilitates  $\text{Ca}^{2+}$  entry into mitochondria via a low-affinity  $\text{Ca}^{2+}$  uniporter, the mitochondrial  $\text{Ca}^{2+}$  uniporter (MCU) [68]. The MCU has recently been shown to be important in maintaining physiologically-stimulated SOCE, and downstream consequences including gene expression [293]. A rise above basal  $\text{Ca}^{2+}$  levels is required for mitochondrial  $\text{Ca}^{2+}$  uptake, with the location of mitochondria defining their contribution to intracellular  $\text{Ca}^{2+}$  buffering. A close interaction with the ER results in buffering not only of  $\text{Ca}^{2+}$  influx but also  $\text{Ca}^{2+}$  released during store depletion [285]. Mitochondrial  $\text{Ca}^{2+}$  is, subsequently, re-released slowly into the

cytosol by NCLX, the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger [253], and extruded from the cell by alternative mechanisms. Low extracellular  $\text{Na}^+$ , for a sustained period, additionally depletes intracellular  $\text{Na}^+$  concentration. The combined block of extrusion shown by  $\text{La}^{3+}$  and low  $\text{Na}^+$  could therefore be due to a reduction in mitochondrial  $\text{Ca}^{2+}$  buffering.



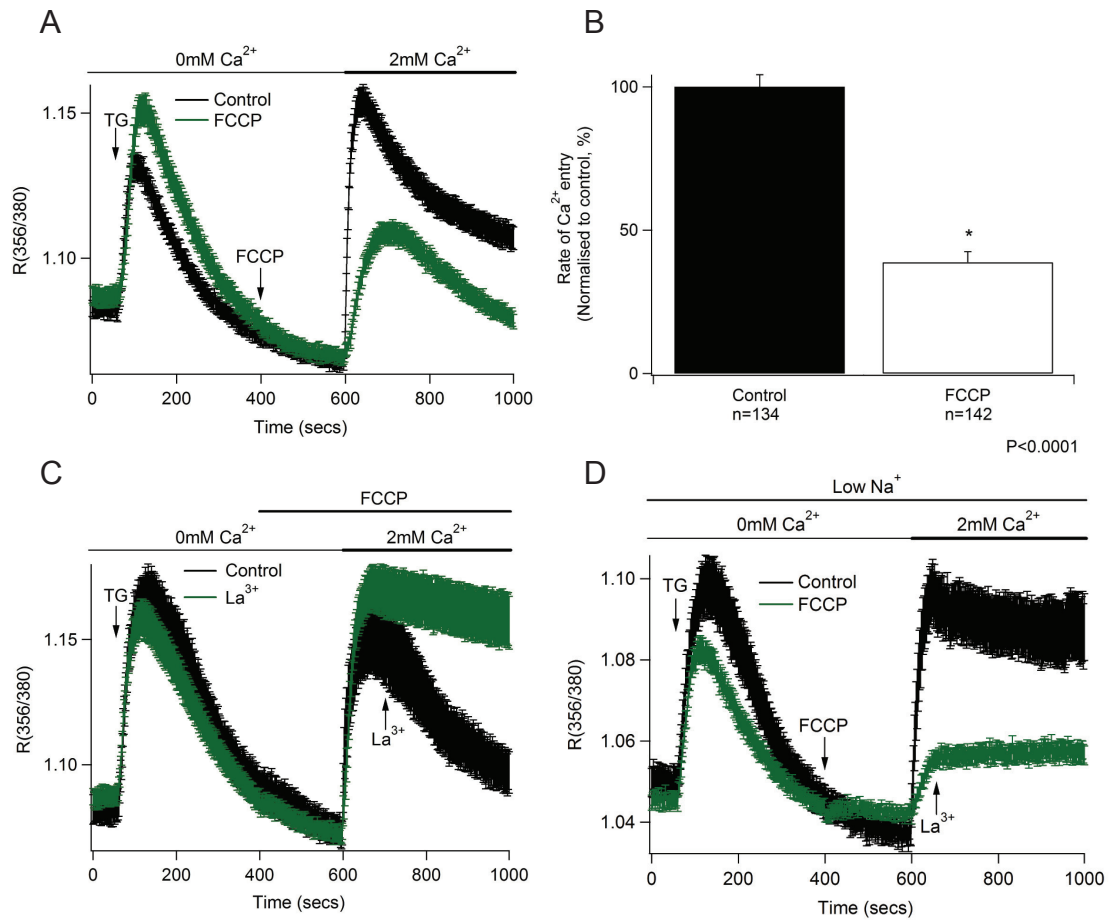
Mitochondrial  $\text{Ca}^{2+}$  buffering not only contributes to  $\text{Ca}^{2+}$  handling, but additionally facilitates prolonged  $\text{Ca}^{2+}$  flux through CRAC channels by reducing slow inactivation elicited by negative feedback of  $\text{Ca}^{2+}$  [121, 125]. Disturbance of mitochondrial function abolishes  $\text{Ca}^{2+}$  buffering, increasing CRAC channel slow inactivation, thus reducing overall observable response; another hallmark of CRAC channels [255].  $\text{Ca}^{2+}$  uptake into mitochondria is driven by the large electrochemical gradient, which is created by the pumping of protons out of the mitochondrial matrix into the intermembrane space by complexes I, III, and IV. Trifluorocarbonylcyanide phenylhydrazone (FCCP) is a protonophore which collapses the mitochondrial membrane potential and abrogates  $\text{Ca}^{2+}$  buffering. Addition of FCCP (5  $\mu\text{M}$ ) after TG store depletion reduced rate of  $\text{Ca}^{2+}$  entry to  $38\% \pm 4\%$  of control (Fig. 3.11 A-B). The bulk rise observed after FCCP treatment was also diminished, and the duration of  $\text{Ca}^{2+}$  response was reduced. This is consistent with decreased CRAC channel activation.

The combined abrogation of ER store refilling (TG), mitochondrial buffering (FCCP) and PMCA ( $\text{La}^{3+}$ ) almost completely abolished  $\text{Ca}^{2+}$  extrusion (Fig. 3.11C), although a slow residual decline in  $\text{Ca}^{2+}$  signal was still observable. Presence of reduced external  $\text{Na}^+$ , in addition to TG, FCCP and  $\text{La}^{3+}$ , resulted in no extrusion over a 350sec time period (Fig. 3.11D). Cumulatively, this data suggests a role for mitochondrial buffering, PMCA, and NCX in sEnd.1 cells, with compensation between the mechanisms meaning that all must be inhibited for extrusion to be abolished. Due to compensation, the relative contributions of each mechanism in sEnd.1 cannot be easily established experimentally.



**Figure 3.10** PMCA is responsible for extrusion of small rises in cytosolic  $Ca^{2+}$ . Extrusion of large cytosolic  $Ca^{2+}$  rises is independent of NCX.

(A-D) Stores were depleted with  $2\mu M$  TG in absence of external  $Ca^{2+}$  and  $Sr^{2+}$ . (A-C)  $La^{3+}$  was applied after divalent ion entry reached a maximum peak at 450secs. (A) Add-back of  $2mM Sr^{2+}$  evoked influx through CRAC channels. Blockade of PMCA with  $1mM La^{3+}$  partially inhibited  $Sr^{2+}$  extrusion. (B) Addition of  $1mM La^{3+}$  post- $Ca^{2+}$  readmission failed to reduce the  $Ca^{2+}$  extrusion rate. (C) Low external  $Na^+$  blocks NCX. NCX block alone failed to inhibit extrusion. Combined  $La^{3+}$  and low  $Na^+$  treatment reduced removal of  $Ca^{2+}$  indicating an alternative  $Na^+$ -sensitive  $Ca^{2+}$  removal pathway may be operating. (D) Application of  $1mM La^{3+}$  prior to store depletion blocks  $Ca^{2+}$  extrusion. Small cytosolic rises in  $Ca^{2+}$  are managed by PMCA. Larger cytosolic  $Ca^{2+}$  rises are extruded via an NCX independent manner.



**Figure 3.11** Depolarisation of the mitochondrial membrane potential reduces  $\text{Ca}^{2+}$  entry and indicates a role of mitochondrial  $\text{Ca}^{2+}$  buffering in sEnd.1  $\text{Ca}^{2+}$  handling.

(A-B) Application of the protonophore FCCP ( $5\mu\text{M}$ ), prior to addition of external  $\text{Ca}^{2+}$ , uncouples mitochondria and reduces the rate of  $\text{Ca}^{2+}$  entry by increasing  $\text{Ca}^{2+}$  inactivation of the CRAC channel. Rate of entry was reduced to 39% of control. (C) Combined inhibition of  $\text{Ca}^{2+}$  extrusion mechanisms with TG,  $\text{La}^{3+}$ , and FCCP reveals the requirement of mitochondrial  $\text{Ca}^{2+}$  uptake in sEnd.1  $\text{Ca}^{2+}$  extrusion. FCCP treatment abolishes mitochondrial  $\text{Ca}^{2+}$  buffering prior to the addition of external  $\text{Ca}^{2+}$ .  $\text{La}^{3+}$  block of PMCA reduces the rate of  $\text{Ca}^{2+}$  extrusion. Despite the simultaneous block of three  $\text{Ca}^{2+}$  buffering mechanisms, a slow decline in  $\text{Ca}^{2+}$  signal still occurs. (D) Low external  $\text{Na}^{+}$  limits the driving force for  $\text{Ca}^{2+}$  extrusion via NCX. Low  $\text{Na}^{+}$  in combination with block of SERCA uptake, mitochondrial  $\text{Ca}^{2+}$  buffering and PMCA complete abrogates  $\text{Ca}^{2+}$  extrusion.

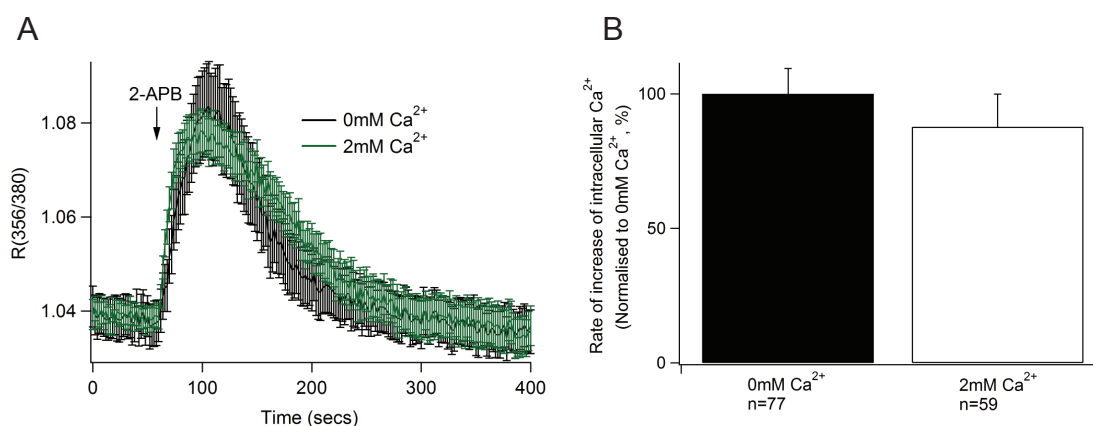
### 3.2.8 No role of Orai-3 in sEnd.1 $\text{Ca}^{2+}$ signalling

Orai-3 also interacts with STIM-1 to form a SOC channel, albeit less effectively than Orai-1 channels [308]. Orai-3 was previously shown in this chapter to be expressed in sEnd.1 cells (Fig.3.2). I therefore investigated whether Orai-3 contributes to SOCE in sEnd.1 cells.

2-APB directly activates  $\text{Ca}^{2+}$  entry through Orai-3 with an  $\text{EC}_{50}$  of  $15\mu\text{M}$ , and unlike Orai-1 and -2, does not show signs of inhibition of entry at any concentration [201,262]. With the presence of  $2\text{mM}$  external  $\text{Ca}^{2+}$ , a transient  $\text{Ca}^{2+}$  signal was observed after stimulation with  $50\mu\text{M}$  2-APB (Fig.3.12A). This increase could be attributable to the activity of Orai-3, however, stimulation of sEnd.1 cells with 2-APB ( $50\mu\text{M}$ ) in the absence of external  $\text{Ca}^{2+}$  did not reduce the  $\text{Ca}^{2+}$  signal (Fig.3.12A). Furthermore the rate of  $\text{Ca}^{2+}$  increase did not differ with the presence or absence of external  $\text{Ca}^{2+}$  (Fig.3.12B). But the increase in intracellular  $\text{Ca}^{2+}$  is in itself interesting and has been observed in other cell types, including RBL-2H3, within the Parekh laboratory, and has additionally been reported in literature, albeit at higher doses of 2-APB [214]. It is clear from the similar  $\text{Ca}^{2+}$  rises with and without the presence of external  $\text{Ca}^{2+}$ , that  $\text{Ca}^{2+}$  release from intracellular stores is occurring. This unintentional response highlights the complexities of using 2-APB as a SOCE inhibitor. 2-APB action on cells is complex with multiple targets.

2-APB was originally identified as an  $\text{IP}_3\text{R}$  antagonist. Although concentration required for block varies across cell type, endothelial  $\text{IP}_3\text{R}$  signalling has previously been reported to be almost completely abrogated at  $50\mu\text{M}$  [29]. However,

2-APB additionally prevents loading of ER stores, resulting in passive leak in a similar action to that of TG by block of SERCA [227]. It is possible here that the increase in intracellular  $\text{Ca}^{2+}$  rise is through passive leak of ER stores. The amplitude of release is smaller than that seen with TG inhibition, and the duration of response is less sustained. Nevertheless, this may be indicative of a less effective block of SERCA by 2-APB than TG, limiting the capacity for passive depletion. Although alternative  $\text{Ca}^{2+}$  stores such as the mitochondria, and acidic stores exist within cells,  $\text{Ca}^{2+}$  release from these stores has not been shown to be inhibited by 2-APB [32, 117].



**Figure 3.12 Orai-3 does not contribute to SOCE in sEnd.1 cells**

(A) A representative experiment. High dose 2-APB applied in the presence of external  $\text{Ca}^{2+}$  failed to induce a sustained  $\text{Ca}^{2+}$  entry, as compared to absence of external  $\text{Ca}^{2+}$ . Lack of prolonged  $\text{Ca}^{2+}$  signal is indicative of no role for Orai-3. (B) Presence of external  $\text{Ca}^{2+}$  did not significantly alter the rate of increase in intracellular  $\text{Ca}^{2+}$ .

### 3.3 Key findings of this chapter

- sEnd.1 can support CRAC channel-dependent SOCE
- ATP and histamine can induce SOCE in sEnd.1
- ATP SOCE is dependent on CRAC channels
- $\text{Ca}^{2+}$  handling in sEnd.1 is governed by the combined action of ER store refilling, PMCA, NCX, and mitochondrial  $\text{Ca}^{2+}$  buffering

### 3.4 Discussion

$\text{Ca}^{2+}$  imaging experiments in sEnd.1 cells demonstrated modest store-operated  $\text{Ca}^{2+}$  entry compared to immune cells, in spite of robust expression of both the main CRAC channel components Orai-1 and STIM-1. This is consistent with previously reported CRAC channel  $\text{Ca}^{2+}$  conductance in endothelia [1, 4, 90]. Expression of Orai-1 in sEnd.1 is comparable to that in RBL-1, however STIM-1 is significantly reduced, although remains clearly detectable. It has previously been reported that STIM-1 limits endothelial SOCE [1]. Although not directly tested in this thesis, insufficient STIM-1 appears likely to be the limiting factor in sEnd.1 SOCE. Roles for NCX, PMCA, and mitochondrial  $\text{Ca}^{2+}$  buffering have all been identified in removing cytosolic  $\text{Ca}^{2+}$  rises induced by TG-depleted ER stores. It is feasible that the small amplitude of  $\text{Ca}^{2+}$  rise could be exacerbated by enhanced extrusion in endothelia. Endothelial  $\text{Ca}^{2+}$  levels must be strictly controlled in order to maintain vascular homeostasis, due to the plethora of  $\text{Ca}^{2+}$ -regulated vasoactive molecules produced by endothelia. Indeed I noted that the level of basal

$\text{Ca}^{2+}$  in endothelial cells is lower than other commonly used cell types. This is reflected within the literature [1, 324]. Intracellular  $\text{Ca}^{2+}$  concentration is generally reported at  $\sim 100\text{nM}$ , however endothelia have been shown to have resting concentrations at  $30\text{nM}$  [6].

Here, I have identified CRAC channels as mediators of sEnd.1 SOCE by pharmacological methods of inhibition; supporting the notion in the literature that CRAC channels provide the major  $\text{Ca}^{2+}$  entry pathway in endothelia [1, 192]. However, the efficacy of the pharmacological approach to CRAC channel identification needs to be considered carefully.

The development of specific blockers of the CRAC channel has been significantly hindered by a lack of knowledge of the identity of CRAC channel proteins, as a result only two inhibitors have been reported to be specific for CRAC channels: BTP-2 and Synta-66. BTP-2 is a pyrazole derivative which was discovered prior to Orai and STIM, and has been suggested to be a CRAC channel specific inhibitor, blocking TG-induced  $\text{Ca}^{2+}$  influx, with an  $\text{IC}_{50}$  in the low nanomolar range [155, 378]. Further to this, BTP-2 does not affect  $\text{Ca}^{2+}$  release from the ER stores, nor  $\text{Ca}^{2+}$  extrusion mechanisms [378]. However, it has also been shown to potentiate TRPM-4 channels, indicating off-target effects, which could contribute to phenotypes observed in the presence of BTP-2 [327]. A study has also shown BTP-2 can block DAG-induced signalling by TRPC-3 [130]. Despite these indications, BTP-2 is considered more specific than most other CRAC channel inhibitors.

Crucially, Synta-66 has never been linked to TRPC signalling nor indeed any off target effects. Furthermore, unpublished data from the Parekh laboratory

indicates that at concentrations  $>10\mu\text{M}$  no block of TRPC channels is observable. However the potency of Synta-66 is lower than BTP-2, with  $\mu\text{M}$  concentrations usually required [7, 240]. It has previously been reported that Synta-66 has a higher potency for vascular CRAC channels with an  $\text{IC}_{50}$  of just 26nM, however work was carried out in VSMC, and reported higher potency may not be applicable to endothelia [192]. The 50% block of sEnd.1  $\text{Ca}^{2+}$  influx at  $10\mu\text{M}$  Synta-66 is more consistent with other cell types, where CRAC channels have been demonstrated [240]. Synta-66 was therefore used over BTP-2 as a selective inhibitor of CRAC channels, although clearly unknown off-target effects may still be an issue.

A lack of specific inhibitors for CRAC channel has historically resulted in the combined use of many non-specific inhibitors to demonstrate a CRAC channel specific profile. As this is a well-characterised approach, it was used here to confirm the findings of Synta-66 inhibition. Cumulatively, inhibition by 2-APB,  $\text{La}^{3+}$ , and FCCP all provided evidence of CRAC channel activity.

The action of 2-APB is multifarious, as previously discussed throughout this chapter. The compound is capable of inhibiting SOC channels independently of its action on  $\text{IP}_3\text{R}$  and SERCA pumps, acting on extracellular regions of SOC channels [9]. 2-APB potentiates and inhibits both Orai-1 and -2, but additionally similar concentrations of 2-APB, used on CRAC channels, can also inhibit TRPC-3,-6, and -7 [196, 262]. The different activation of these TRPCs from CRAC channels, allows their activity to be dissected. Activation of store depletion by TG circumvents the activation of PLC. Activation of SOCE by this protocol immediately rules out contributions by both TRPCs-3 and -6, which are dependent on PLC product DAG [137, 344]. TRPC-7 is regulated by SERCA independently of store



depletion, however still requires PLC for activation [70]. TRPCs can therefore be discounted in this protocol.

$\text{La}^{3+}$  is a promiscuous inhibitor of  $\text{Ca}^{2+}$  channels, having been reported to block L-type, and T-type channels, TRPCs, TRPVs, and CRAC channels, as well as  $\text{Ca}^{2+}$  extrusion by PMCA, but at much higher concentrations. The specificity of  $\text{La}^{3+}$  raises concerns regarding its block of CRAC channels. Induction of CRAC channels with TG largely discounts  $\text{La}^{3+}$  effects on many  $\text{Ca}^{2+}$  entry channels. Moreover voltage-gated channels aren't highly expressed within the endothelium, and  $\text{La}^{3+}$  has previously been reported to inhibit these channels at very low  $\mu\text{M}$  concentrations [275]. At even the lowest concentrations used to inhibit CRAC channels voltage-gated channels would be inhibited. However induction of TRPC channels should be considered, especially with regards to physiological activation, which occurs through stimulation. Interestingly,  $\text{La}^{3+}$  potentiates two non-selective TRPC channels: TRPC-4 and -5 at  $\mu\text{M}$  concentrations. The concentration of  $\text{La}^{3+}$  required is crucial in differentiation of these channels. Although the  $\text{IC}_{50}$  for  $\text{La}^{3+}$  block in sEnd.1 ( $8\mu\text{M}$ ) is higher than that previously reported in immune cells ( $\sim 1\mu\text{M}$ ), here I have shown that  $50\mu\text{M}$   $\text{La}^{3+}$  almost completely abrogates  $\text{Ca}^{2+}$  entry, below the reported levels of inhibition for most TRPCs at  $>100\mu\text{M}$   $\text{La}^{3+}$  [77, 275]. As previously discussed TRPC-1 and -4 are the only two TRPCs which still have some credence as being capable of supporting store-operated currents under certain conditions, although a study by the Trebak group strongly refutes this [1]. TRPC-1 has been reported to be blocked by  $\text{La}^{3+}$  with an  $\text{IC}_{50}$  of  $1\text{-}10\mu\text{M}$ , a similar range to observations in sEnd.1. TRPC-1, however, is not permeable to  $\text{Sr}^{2+}$ , whereas I have clearly detected  $\text{Sr}^{2+}$  entry in sEnd.1 after

store depletion [22]. This gives confidence that the channel being inhibited is the CRAC channel.

Mitochondrial buffering has previously been demonstrated as a positive regulator of  $I_{\text{CRAC}}$  independently of its promotion of store depletion [121]. Removal of intracellular  $\text{Ca}^{2+}$  in the vicinity of active CRAC channels reduces negative feedback and slow  $\text{Ca}^{2+}$ -dependent inactivation of CRAC channels. Use of the protonophore FCCP de-energises mitochondria and prevents  $\text{Ca}^{2+}$  buffering. Feedback of  $\text{Ca}^{2+}$  on the channel itself therefore inactivates CRAC channels. FCCP clearly reduces  $\text{Ca}^{2+}$  influx in sEnd.1 (Fig.3.11).  $\text{Ca}^{2+}$ -dependent inactivation is considered a hallmark of CRAC channels, being mediated by the CRAC modulatory domain on STIM-1 [74].

The combined power of FCCP, Synta-66,  $\text{La}^{3+}$ , and 2-APB in reducing  $\text{Ca}^{2+}$  entry in sEnd.1 in response to TG store depletion gives confidence that the sEnd.1 SOCE signal is dependent on the CRAC channel. Furthermore, the pharmacological profile of ATP-induced SOCE is similar to that of TG. However, knock-down of CRAC channel components would provide further evidence. The knock-down of  $\text{Ca}^{2+}$ -store sensor STIM-1 will be addressed in chapter 4.

CRAC channel agonists, ATP and histamine, induced store-dependent  $\text{Ca}^{2+}$  signals in sEnd.1 cells. However, high dose of histamine failed to induce sustained  $\text{Ca}^{2+}$  entry as described in the literature [62, 157, 243]. This may be indicative of low H1 receptor expression in sEnd.1, although this has not been examined. ATP  $\text{Ca}^{2+}$  influx, however, has been shown to have the same pharmacological profile as TG-induced  $\text{Ca}^{2+}$  entry showing CRAC channel activation in sEnd.1 can be induced through physiological  $\text{IP}_3$  signalling.

## Chapter 4

# Knockdown of the CRAC channel component STIM-1 in sEnd.1

### 4.1 Introduction

The presence of CRAC channels in sEnd.1 cells, and their contribution to sEnd.1 SOCE was described in chapter 3. However, due to the lack of a selective CRAC channel blocker, a multitude of structurally distinct small molecule inhibitors were utilised. Each one, at a maximally effective concentration, reduced  $\text{Ca}^{2+}$  entry to similar extents, consistent with the view that the same pathway is being targeted. Although Synta-66 has not been linked to block of any other channel, both  $\text{La}^{3+}$  and 2-APB have been shown to inhibit some members of the TRPC  $\text{Ca}^{2+}$  channel family [196]. Rather than a direct effect on the channel itself, FCCP acts through increasing  $\text{Ca}^{2+}$ -dependent slow inactivation of the CRAC channel by abolishing mitochondrial  $\text{Ca}^{2+}$  uptake. Although slow inactivation is viewed as a hallmark of

CRAC channels, it remains possible that other  $\text{Ca}^{2+}$ -permeable channels are also effected by the reduction in mitochondrial buffering. Taken together, the pharmacological tools used indicate that the CRAC channel is present and functional in sEnd.1 cells. However, direct evidence can be provided by knockdown of the channels, for example by use of an siRNA directed strategy.

RNA interference ( $\text{RNA}_i$ ) is a powerful tool which reduces the expression of a particular protein of interest, allowing the role of that protein in a specific process or its contribution to a cell phenotype to be established. Indeed, its importance was recognised in the 2006 Nobel prize for medicine, which was awarded to Fire and Mello for the discovery of  $\text{RNA}_i$  [99].  $\text{RNA}_i$  is in fact a physiological process which limits expression of mRNA into protein, acting as a post-transcriptional control of protein expression. Transfection of double stranded RNA (dsRNA) was proposed by the Mello group in 1998 as being the most effective method of inducing  $\text{RNA}_i$  in cells [99]. dsRNAs are processed *in vivo* to form siRNAs. siRNAs increase degradation of mRNA, by acting as guides to recruit degradation machinery to target mRNAs.

The use of exogenous siRNA results in a transient reduction in protein expression, which can be transmitted to some extent to successive generations of cells. The effect of siRNA knockdown is gradually lost over time, with the transfection of siRNAs typically resulting in an optimum knock-down of protein between 36-72hrs post-transfection.

A more stable knock-down of protein can be achieved through transfection of a plasmid, which integrates into the host genome, and expresses short hairpin RNAs (shRNA). shRNAs are processed to form siRNAs capable of reducing mRNA levels

of the target protein. shRNAs are typically used to establish knock-down cell lines, knock-down in animal models, or to induce a longer lasting knock down in *ex vivo* cells. siRNAs conversely are used for a transient reduction in the target protein.

In this chapter I have used an RNA<sub>i</sub> approach to knock-down STIM-1, and then investigated the effect of this on Ca<sup>2+</sup> influx.

## 4.2 Results

### 4.2.1 Transfection of sEnd.1 - difficulties encountered

Only one study has previously reported successful transient transfection in sEnd.1 cells, showing both overexpression of dihydrofolate reductase (DHFR) and knock-down of GTP cyclohydrolase I and DHFR [60]. Overexpression of protein was robust, with a 5-fold increase reported. Furthermore, a strong reduction in protein level was shown by Western blot, although the level of knockdown was not quantified. The paper additionally did not state the transfection method used or transfection efficiency, although this must have been reasonable because knock-down was seen in a Western blot, which measures protein levels over a population of cells. Alternative studies have shown successful establishment of stable sEnd.1 cell lines with initial transfection mediated by poly-L-ornithine with DMSO shock, and liposomal transfection reagent Lipofectamine [47, 108]. Although these methods can successfully introduce nucleic acids into sEnd.1 cells, transfection efficiency is not a consideration, because cells are selected by transfection success before development of the stable cell line. Transfection methods for sEnd.1 were therefore

not available in the literature, which complicated establishing an approach for knockdown of STIM-1.

Transfection methods typically are either biological, through viral transduction, chemical, with liposomal and non-liposomal reagents, and physical through electroporation, microinjection, and laser-based transfection [171]. Here I addressed transfection of sEnd.1 with a combination of chemical and physical approaches commonly used to transfect cell lines, and other endothelial cell types. Viral transduction, although known to have significantly higher efficiency than other transfection methods, was not considered for this thesis. Both time-constraints in generating the viral vectors, cost of the technique, and data in the laboratory that showed viral transfection (lentivirus) increased basal  $\text{Ca}^{2+}$  levels in RBL-cells, were limitations. Six transfection methods were tested as briefly outlined in Table.4.1.

The key tenets of cell transfection are cell survival, transfection efficiency, and for this thesis, low basal  $\text{Ca}^{2+}$ . Attempts at transfection in sEnd.1 cells were plagued with issues, with low transfection efficiency, assessed by eGFP expression detected by fluorescence microscopy, and low cell viability, determined by cell counts compared to untransfected controls. Establishing a transfection protocol for sEnd.1 cells was therefore a difficult and laborious process.

Cell health and active proliferation are crucial for the success of transfection. Infection of cell cultures can significantly affect cell physiology, causing inhibition of proliferation, and apoptosis, through consumption of nutrients, including L-arginine by the action of arginine deiminase [242]. The effect of arginine deiminase present in mycoplasma would also affect investigations into NO production undertaken later in this thesis; depleting levels of available substrate for NOS, and

increasing L-citrulline, a detectable by-product of NO production.

The absence of a cell wall, together with its small size, makes mycoplasma infections hard to detect, and additionally allows for a more fluid cell structure, meaning that mycoplasma may remain within culture medium even after sterilisation using filters as small as 0.2 $\mu$ M. Further to this, mycoplasma are resistant to commonly used laboratory antibiotics, which often target murein, which forms cell walls. These characteristics lead to mycoplasma infection being common, and therefore a major issue, in laboratory cell cultures [242].

Because of the difficulty in transfecting sEnd.1 cells, and with mycoplasma infection being a common problem which inhibits nucleic acid uptake, I checked for its presence in this sEnd.1 cell culture. One of the recommended techniques for mycoplasma detection is staining with the fluorescent DNA dye 4',6-diamidino-2-phenylindole (DAPI), which binds to A-T rich regions of DNA [242]. DAPI staining will detect any DNA present within the culture, identifying eukaryotic nuclei and free DNA present in prokaryotes, such as mycoplasma. The mycoplasma is identifiable as an extracellular blue fluorescence, at the edges of a cell, or occurs freely in aggregates [340]. The staining of sEnd.1 cell cultures with DAPI did not show either of these hallmarks of mycoplasma infection. This demonstrates that difficulty in sEnd.1 transfection is not due to an underlying infection, and instead shows sEnd.1 cells as being a hard-to-transfect cell line.

Poor transfection efficiency is not uncommon in endothelial cells, and has been repeatedly reported [150,178]. One potential reason for this may be the propensity for endothelial cells to form a monolayer, increasing cell-cell contacts even at a low confluency. Cell-cell interaction is known to be inhibitory to nucleic acid

uptake. Electroporation methods were therefore investigated next, as they require suspensions of cells and thus reduces cell-cell interactions.

Electroporation methods with sEnd.1 caused substantial cell death, immediately after transfection. The remaining surviving cells often showed altered morphology, and eGFP expression within such a cell was very high, which likely contributed to poor cell health. Electroporation was first approached using the Amaxa (Lonza) technique, which is well-established within the lab. Poor cell survival immediately after transfection, resulted in fewer sEnd.1 cells remaining post transfection. sEnd.1 cells, as with endothelial cells in general, show decreased proliferation when present at low cell density. This therefore exacerbated the low cell count post-transfection.

The Neon transfection technique (Invitrogen) is a newer method of electroporation which allows better optimisation of electroporation parameters, and creates a more even electrical field than a standard cuvette-based approach. The system consists of a specialised pipette tip containing a central wire electrode. A cell suspension is taken up into the tip for electroporation. A single wire electrode improves transfection efficiency by minimising the surface area of the electrode in contact with cell suspension [170].

Neon transfection showed a high efficiency of transfection with  $>85\%$  cells expressing eGFP after 24hrs, when between  $1 \times 10^5$  cells are electroporated with 3 pulses of 1500V at 10ms intervals (data not shown). Although transfection was very successful, cell viability was severely diminished with a surviving cell count between 20% and 50% of control. Furthermore, the morphology of surviving cells was different from untransfected control cells. This indicates that electroporation



is not appropriate for sEnd.1 transfection. A high level of cell death and changes in cell morphology may imply a fragile cell membrane in sEnd.1 cells, which is easily perturbed by electroporation. Unusual cell morphology led to concerns that transfection itself may alter the physiology of sEnd.1 cells; I therefore investigated chemical techniques of transfection.

Four widely employed methods were considered: Lipofectamine 2000, FuGene 6, FuGene HD and NanoJuice. Lipofectamine was selected as it was previously used to establish a stable transfection in sEnd.1 [108]. Chemical methods of transfection have been reported to have relatively low transfection efficiencies in endothelial cells, with reports between 20%-40% [150]. This was confirmed in sEnd.1 where transfection efficiencies <50% for all four reagents were routinely observed. Cell counts were additionally reduced with all four transfections. It was not determined whether a diminished cell population was due to cell death or growth inhibition by transfection.

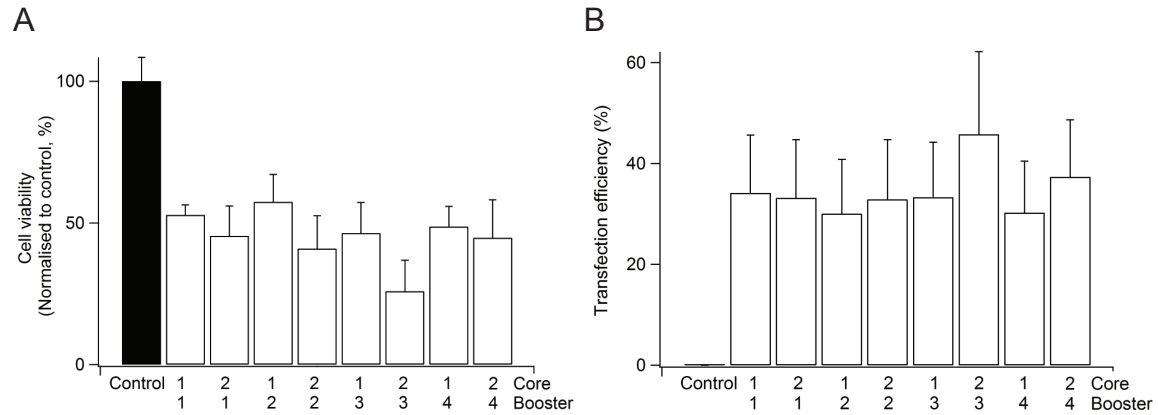
**Table 4.1 Comparison of transfection methods in sEnd.1**

| Transfection method  | Transfection type | Transfection outcome             |
|----------------------|-------------------|----------------------------------|
| <b>Amaxa</b>         | Electroporation   | Low cell viability               |
| <b>Neon</b>          | Electroporation   | Low cell viability               |
| <b>FuGene 6</b>      | Non-liposomal     | Poor transfection efficiency     |
| <b>FuGene HD</b>     | Non-liposomal     | Poor transfection efficiency     |
| <b>Lipofectamine</b> | Liposomal         | Variable transfection efficiency |
| <b>NanoJuice</b>     | Liposomal         | Successful transfection          |

NanoJuice gave the best and most consistent transfection out of the three chemical reagents trialled (Table.4.1). Transfection efficiency of sEnd.1 with Lipofectamine and FuGene 6 and FuGene HD was assessed by GFP transfection, and was calculated as  $23\% \pm 7\%$ ,  $25\% \pm 12\%$ , and  $24\% \pm 2\%$  respectively. Furthermore, transfection with Lipofectamine efficiency was tested on multiple occasions, showing a variable transfection efficiency, ranging between 7% and 42%.

Optimisation of NanoJuice was therefore undertaken. NanoJuice consists of a core transfection reagent, and a booster reagent. The ratio of these reagents is reported by the manufacturer as a key factor in determining the success of the transfection. The cell count 48hrs post-transfection ranged between 41% and 58% cell survival with the exception of a 2:3 ratio, which showed markedly reduced cell survival or proliferation with a cell count  $26\% \pm 11\%$  of untreated control (Fig.4.1 A). Transfection efficiency ranged between 30% and 46% (Fig.4.1 B). The 2:3 ratio

of core reagent to booster reagent also resulted in the highest transfection efficiency at  $46\% \pm 16\%$ . Due to the low viability and large variation in transfection efficiency with this ratio, this protocol was not selected. A 2:4 ratio of core:booster gave the best overall outcome in cell count, and transfection efficiency after 48hrs with  $45\% \pm 3\%$  and  $37\% \pm 11\%$  respectively.



**Figure 4.1 Nanojuice transfection method optimisation**

(A) Neon reduces cell viability to an average of  $45\% \pm 3\%$ . All transfection protocols reduce cell viability to similar extents. (B) Transfection efficiency ranged from 30% to 45%.

### 4.2.2 Knock-down of STIM-1

The establishment of a transfection method for sEnd.1 allowed both the knock-down of Orai-1 and STIM-1 to be addressed. It should be considered that CRAC channels are known to be important in endothelial cell proliferation, which may further exacerbate difficulties in obtaining a high transfection efficiency and adequate cell count in sEnd.1 [1].

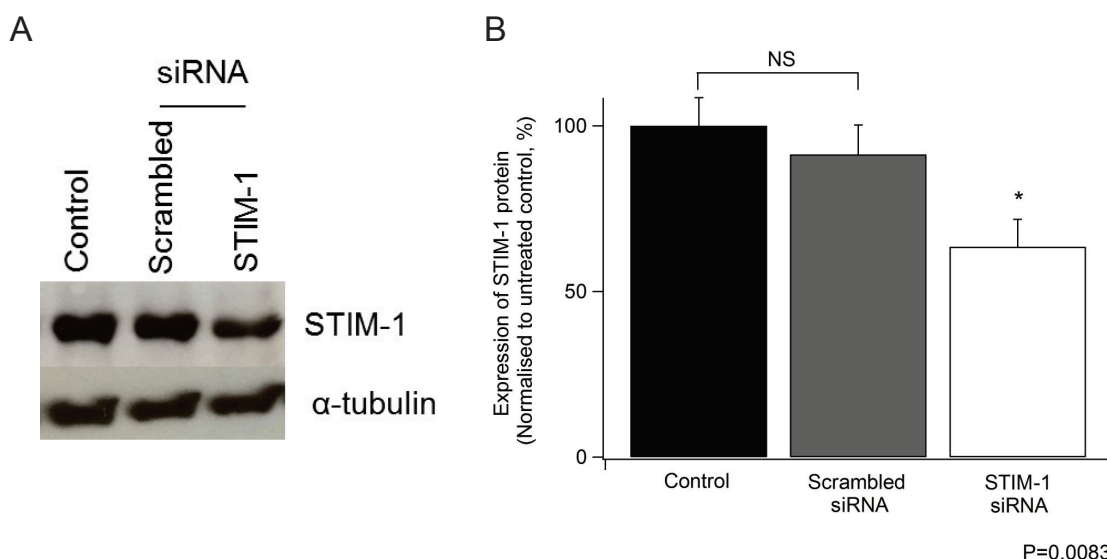
Orai-1 expression in sEnd.1 cells is comparable to that in RBL-1, however STIM-1 cells is markedly reduced in comparison (see Fig.3.2). It is therefore likely that

STIM-1 is a limiting factor to the formation of CRAC channels, as is seen in other endothelial cell types [1]. A larger reduction in protein levels is therefore likely to be required in order to view a functional difference in  $\text{Ca}^{2+}$  entry. Such a large knock-down would be difficult in sEnd.1 cells where transfection efficiency was  $<50\%$ . STIM-1, as the limiting factor in endothelial SOCE, is, on the other hand, an ideal target for a reduction in CRAC channels, and the associated  $\text{Ca}^{2+}$  entry. STIM-1 knock-down was achieved with transfection of 30nM STIM-1 siRNA, and use of cells around 48-72hrs, post-transfection dependent on cell confluency after transfection.

Although RNA<sub>i</sub> targets mRNA levels, overall protein content is a more appropriate read-out of STIM-1 knockdown, for functional studies. Detection of mRNA considers only the potential for protein production, but does not consider the existing pool of protein. The size of the existing pool is determined by the protein's half-life. The half-life of STIM-1 is 19hrs, considerably shorter than the average protein at 46hrs [291, 299]. Successful knockdown of STIM-1 using the NanoJuice transfection reagent in sEnd.1 was therefore assessed by Western blot (Fig.4.2). STIM-1 expression was reduced to  $64\% \pm 8\%$  of untransfected control. Expression of STIM-1 in cells transfected with scrambled siRNA did not differ from untreated controls, demonstrating that STIM-1 expression is unaffected by the transfection process itself.

This knockdown considers the reduction of protein over the whole cell population. With a transfection efficiency of  $37\% \pm 11\%$ , it can be assumed that knockdown of STIM-1 expression in successfully transfected cells will be significantly higher than that predicted by Western blot. Western blot is a useful indicator for a semi-

quantitative assessment of protein levels in a population of cells. However work within this thesis primarily utilises single-cell measurements. Single cell Western blot has recently been reported [149]. This could provide an exciting new approach to quantify individual cell protein levels, although the technology will need to be further developed to make it available for diagnostics and use in basic science, and will likely highlight heterogeneity within cells.

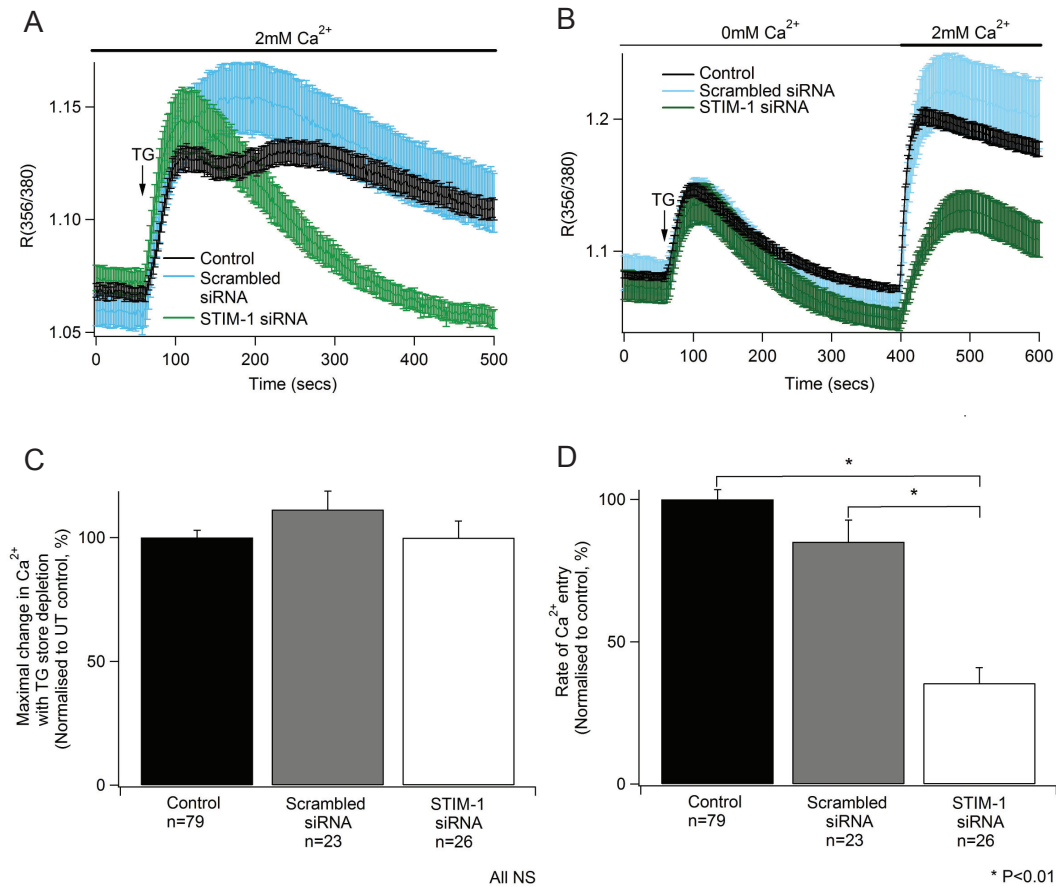


**Figure 4.2** Expression of STIM-1 can be reduced following RNA<sub>i</sub> knockdown

(A) A representative Western blot. (B) 30nM STIM-1 siRNA reduces protein expression to 64%±8% of control. Scrambled siRNA has no effect on protein expression (91%±9%).

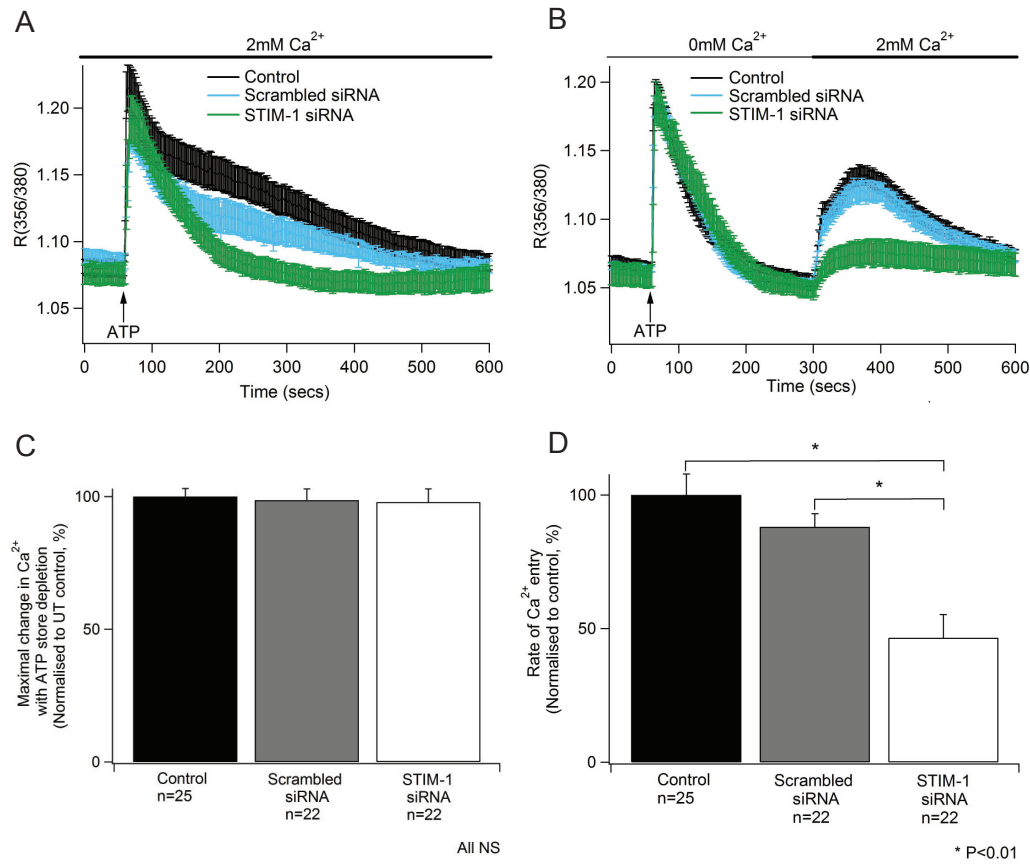
After establishing that STIM-1 knock-down reduced protein levels, the functional effect on  $\text{Ca}^{2+}$  signals in sEnd.1 was investigated (Fig.4.3 and 4.4). Cells were exposed to TG (2μM) or ATP (5μM) in the presence of external  $\text{Ca}^{2+}$ . Following

an initial rise in  $\text{Ca}^{2+}$ , intracellular  $\text{Ca}^{2+}$  returned to basal levels quicker than that seen in untreated control cells, and cell transfected with a scrambled siRNA (Fig.4.3A and 4.4A). In order to confirm that the reduced time of  $\text{Ca}^{2+}$  entry was due to a reduction in STIM-1,  $\text{Ca}^{2+}$  store-release and entry were dissected, as previously described in this thesis (Fig.4.3B and 4.4B), by stimulating in  $\text{Ca}^{2+}$ -free solution and then readmitting 2mM external  $\text{Ca}^{2+}$ . STIM-1 knockdown reduced  $\text{Ca}^{2+}$  entry to  $35\%\pm 5\%$  and  $47\%\pm 9\%$  of untreated control with TG and ATP respectively (Fig.4.3D and 4.4D). This level of functional knock-down corresponds with previous knock-downs of STIM-1 in endothelia [4]. Treatment with scrambled siRNA did not significantly alter the rate of  $\text{Ca}^{2+}$  entry in either ATP or TG stimulated cells (Fig.4.3D and 4.4D). Neither transfection of scrambled siRNA nor STIM-1 siRNA altered the amplitude of store depletion with TG or ATP (Fig.4.3C and 4.4C). The amplitude was calculated by change in fluorescence from point of stimulation to maximum peak height.



**Figure 4.3 STIM-1 RNA<sub>i</sub> knockdown reduces SOCE in sEnd.1**

(A) Stimulation with TG in the presence of external  $\text{Ca}^{2+}$  shows loss of classic biphasic  $\text{Ca}^{2+}$  signal. (B) Dissection of  $\text{Ca}^{2+}$  signals with  $\text{Ca}^{2+}$  add-back protocol demonstrates reduced rate of  $\text{Ca}^{2+}$  entry but no change in store-release. (C) Store depletion by TG is unaffected by transfection, with no significant differences being observed. (D) Rate of TG-induced  $\text{Ca}^{2+}$  entry reduced to  $35\% \pm 5\%$  of control by STIM-1 KD. Rate of  $\text{Ca}^{2+}$  entry is not significantly altered by transfection of a scrambled siRNA ( $P < 0.05$ ).



**Figure 4.4 STIM-1 RNA<sub>i</sub> knockdown reduces SOCE in sEnd.1**

(A) Stimulation with ATP in the presence of external  $\text{Ca}^{2+}$  shows loss of classic biphasic  $\text{Ca}^{2+}$  signal. (B) Dissection of  $\text{Ca}^{2+}$  signals, with readmission of  $\text{Ca}^{2+}$  after ATP stimulation, demonstrates reduced rate of  $\text{Ca}^{2+}$  entry but no change in store-release. (C) Amplitude of store depletion by ATP is unaffected by transfection, with no significant differences being observed. (D) Rate of TG-induced  $\text{Ca}^{2+}$  entry reduced to  $35\% \pm 5\%$  of control by STIM-1 KD. The rate of  $\text{Ca}^{2+}$  entry is not significantly altered by transfection of a scrambled siRNA ( $P < 0.05$ ).



### 4.3 Key findings of this chapter

- Protein knock-down in sEnd.1 cells can be achieved with NanoJuice
- STIM-1 knock-down reduces  $\text{Ca}^{2+}$  entry in response to both TG and ATP, but does not affect  $\text{Ca}^{2+}$  store release

### 4.4 Discussion

The knockdown of STIM-1 and concurrent reduction in  $\text{Ca}^{2+}$  entry demonstrates a major role in mediating sEnd.1 SOCE. This adds to the previous pharmacological evidence that CRAC channels are required for SOCE in sEnd.1. Orai-1 knock-down would further support this, but requires much improved knock-down efficiency as Orai-1 is present in excess.

Co-knock-down of STIM-1 and Orai-1 in endothelial cells has previously been reported in the literature [4]. The Frieden group demonstrated an 80% reduction in Orai-1 protein levels which, when knocked down alone, yielded a 40% decrease in  $\text{Ca}^{2+}$  entry. A 67% reduction in STIM-1 protein in contrast resulted in a 60% decrease in  $\text{Ca}^{2+}$  entry. When STIM-1 and Orai-1 were knocked down together, no further decrease in  $\text{Ca}^{2+}$  entry was seen [4]. This strongly corroborates the finding by Trebak's group, that STIM-1 is the limiting component in the endothelial CRAC channel [1]. Expression data of Orai and STIM proteins in sEnd.1 compared to RBL-1 (see Fig.3.2) showed similar expression of Orai-1 in sEnd.1 and RBL-1, but a reduction in STIM-1 expression level in sEnd.1. This indicates that STIM-1 is limiting in sEnd.1, as in other endothelial cells. It is likely therefore,

that co-knock-down of STIM-1 and Orai-1 in sEnd.1 would not further reduce the  $\text{Ca}^{2+}$  entry, with the reduction dependent on levels of STIM-1 knock-down.

There have been suggestions that Orai-1 and in particular STIM-1 interacts with TRPC channels, thus mediating  $\text{Ca}^{2+}$  entry by non-store-operated mechanisms. Additionally TG has been reported to activate TRPC channels in a manner dependent on STIM-1 [4, 368]. Reports began to emerge shortly after the discovery of STIM and Orai, that TRPC interacted with these proteins, and are still being published, with a recent report determining that TRPC-1, -4, and -5 interact with STIM-1 via the STIM-1 Orai activating domain (SOAR) [187]. However, others find no role for STIM-1 or store depletion in TRPC gating [70].

TRPC-1, -4 and -5 have been suggested to associate with STIM-1 through electrostatic interaction, and furthermore TRPC-1 interaction with other TRPCs, including TRPC-3, has been proposed to infer store-dependency on otherwise store-independent TRPCs [187, 368, 369]. The TRPC-1 channel has been largely at the centre of discussions involving TRPC interaction with the CRAC channel. A recent review has suggested that TRPC-1 may act as a channel regulator, rather than permeating  $\text{Ca}^{2+}$  itself [79]. Indeed, TRPC-1 has also been reported to interact with a wide variety of other TRP channels, and additionally introduction of TRPC-1 homotetramers shows no increase in  $\text{Ca}^{2+}$  entry above control. TRPC-1 as a regulator of  $\text{Ca}^{2+}$  entry is possible, nonetheless two key studies published by prominent groups within the field have collectively discounted the contribution of all TRPCs in CRAC channel conductance [1, 70].

Work by the Putney and Trebak groups in HUVECs, HPAECs, and HEK293 cells, demonstrate that TRPC function is independent of the presence of Orai-1

and STIM-1 [1, 70]. The Trebak group, who worked with endothelia, approached the conundrum of STIM-Orai-TRPC interactions by considering TRPC-1 and -4 requirement for maintaining  $I_{CRAC}$  [1]. Knock-down by siRNA of both TRPCs showed no effect on electrophysiological measurement of  $I_{CRAC}$  nor on  $Ca^{2+}$  entry assessed by  $Ca^{2+}$  imaging. Furthermore, knock-down of both STIM-1 and Orai-1 nearly abolished  $I_{CRAC}$ . Putney took this further by investigating TRPC-3, -5, -6, and -7 with STIM-1, whereby co-expression did not alter  $Gd^{3+}$ -insensitive carbochol-induced  $Ca^{2+}$  entry [70]. TRPC-1 was additionally investigated in this work, however overexpression failed to generate a  $Gd^{3+}$ -insensitive  $Ca^{2+}$  entry [70]. These reports convincingly contend suggestions of TRPC involvement. However, it is possible that TRPCs contribute to  $Ca^{2+}$  entry perhaps as regulators, and their involvement could be more prominent following receptor stimulation.

Nevertheless, the cumulative evidence reported in this thesis with the use of CRAC channel inhibitors and STIM-1 knock-down shows the role of CRAC channels in supporting endothelial SOCE. Further to this, it provides a wide range of tools for probing one of the critical SOCE-driven endothelial functions: NO production. Similar pharmacology of  $Ca^{2+}$  influx to TG and ATP acting on  $P_2Y$  receptors, suggests both use the STIM-Orai pathway. Unpublished data in the lab and elsewhere shows Synta-66 does not block TRPCs at concentrations over 10 $\mu$ M, further arguing against a contribution from TRPC channels under our conditions.

Due to difficulties in transient transfection of sEnd.1 cells, the establishment of stable cell lines for future work would be preferable. Stable knock-downs can be achieved through the use of shRNA as previously discussed in the introduction of this chapter. A further alternative to the knock-down of Orai-1, considering the

difficulties faced in use of RNA<sub>i</sub> in this model, would be transfection of a dominant negative Orai-1 protein [146]. Establishment of stable sEnd.1 cell lines, expressing either mutant channels or stable knock-down in the future, could help to elucidate further downstream functions of CRAC channels in endothelia.

## Chapter 5

# Calcium entry through the CRAC channel increases production of nitric oxide in sEnd.1

### 5.1 Introduction

The field of nitric oxide is dense with publications. A PubMed search for nitric oxide raises over 130,000 results and for endothelial nitric oxide synthase specifically over 20,000 papers are available. It has been known since 1987 that  $\text{Ca}^{2+}$  is required for the activation of endothelial NO production, and further to this there is a requirement of SOCE [31, 109, 197, 234, 252]. Although there has been no direct evidence of Orai-STIM requirement in eNOS activation, in recent years some circumstantial evidence has arisen.

VEGF-induced  $\text{Ca}^{2+}$  fluxes in endothelia have been shown to be CRAC channel-dependent and important in endothelial cell proliferation and angiogenesis [83, 192, 202]. In turn, VEGF signalling promotes endothelial migration, which is inhibitable by blocking NOS [89]. VEGF is a known NOS activator and has been extensively used to investigate eNOS [110, 111, 254].

Extracellular ATP was one of the first recognised agonists of endothelial production of NO and vasodilation [123]. Stimulation of isolated vessels with ATP demonstrated a relaxation in vessel tension, which was abrogated by vessel denudation [67]. Within this thesis I have demonstrated that ATP-induced SOCE in endothelia is dependent on CRAC channels (Fig.3.7,4.3 and 4.4), implying ATP-induced NO production may be reliant on CRAC channel activity. This is corroborated in the literature [172].

Evidence of altered CRAC channel presence and activity in endothelial disease models is increasing. Diabetic mouse vasculature exhibit reduced ACh-induced vasorelaxation and are reported to have diminished expression of STIM-1 and SERCA-3 [88, 210]. The overexpression of STIM-1 in the vascular endothelium rescued vasodilation in diabetic vessels. This work demonstrates that STIM-1 is key in maintaining normal resting ER  $\text{Ca}^{2+}$  levels, however, confusingly, that reduced ER  $\text{Ca}^{2+}$  store release in diabetic endothelia is responsible for diminished NO production and thus vasodilation [88]. This contradicts the widely accepted work by the Rodman group, which shows  $\text{Ca}^{2+}$  entry rather than release is crucial for eNOS activity [197]. Although DAF imaging of NO production is shown to be stimulated by store depletion in the absence of extracellular  $\text{Ca}^{2+}$ , it is unclear whether vessel tension experiments were performed in the presence or absence

of extracellular  $\text{Ca}^{2+}$ . This therefore makes the overall conclusions of the paper unclear [88].

Robust identification of NO production is required in order to address the mechanism behind eNOS activation. It is clear that the mechanism of  $\text{Ca}^{2+}$  activation of eNOS must be better defined. However, NO is arguably one of the most difficult second messengers to reliably detect. This chapter will consider the difficulties in NO detection and utilise two distinct methods to investigate production of NO in sEnd.1 cells, using SERCA blocker TG and physiological agonist ATP.

## 5.2 Results

### 5.2.1 Detecting NO

Detecting NO is largely complicated by its low physiological concentrations and short half life in biological systems, with NO quickly oxidised to form nitrites and nitrates [40]. Detection methods (as outlined in Fig.5.1) often utilise indirect detection to determine intracellular NO levels. Early detection of NO synthesis relied on measurements of downstream consequences such as cGMP production, however detection methods have improved since this time [104]. Mechanisms of detection commonly used at present vary from measurement of only nitrites (Griess reaction), and use of the conversion of L-arginine to L-citrulline as a read-out of NO production (radioactive L-arginine to citrulline assay), to direct detection of NO (electron paramagnetic resonance and amperometry).

No NO detection method is perfect or even preferred, with different methods advantageous in different situations. Specificity of detection for NO production, sensitivity of detection, possibility of real-time measurements, whether single cells or populations are measured, and equipment availability within a laboratory are all considerations when selecting a detection method. The requirements of the experiment should be closely considered too. Detection of NO production in non-cultured tissue samples is more suited to a population-focused approach, in order to detect overall changes in the material and improve sensitivity of detection. Diseased tissues typically display reduced NO production, especially in the presence of high levels of oxidative stress. In these cases detection sensitivity is the key consideration, as well as specificity for NO. Detection methods such as the fluo-



rescence of the NO dye DAF-2 would overestimate the production of NO in such models. This is because reaction of  $O_2^-$  and NO forms  $ONOO^-$ , which reacts more efficiently with the DAF family of dyes, increasing their fluorescence, and thus overestimating available cellular NO [87, 161, 289]. It should also be considered whether the amount of NO produced, the quantity of biologically available NO, or NOS activity is the desired variable to be measured. Indirect methods, such as cGMP activity, are able to demonstrate the amount of available NO for downstream signalling. Detection of conversion of L-arginine to L-citrulline on the other hand can determine the level of NOS activity. When considering disease models, small considerations such as these can elicit huge changes in the conclusions drawn from experimental work. The investigation of the signalling mechanisms and regulation of NOS activity is most accurately dissected using single-cell real-time detection methods. Fortunately, despite the constraints of NO detection, two methods are readily available: NO dyes, such as the DAF family developed in 1998 by the Nagano lab; and amperometry, which utilises NO reactivity to transfer an electron from NO to an electrode, producing a detectable current [176, 211, 329].

Here, I aim to test the hypothesis that induction of NO production via eNOS is dependent physiologically on CRAC channels. Furthermore, I will address the mechanism behind this activation. Use of amperometry and NO dyes were therefore primarily considered. The use of NO dye, such as DAF-2, has been shown to be marginally more sensitive than amperometry and additionally has the advantages of higher throughput, and the possibility of co-imaging with  $Ca^{2+}$ -sensitive dyes [211, 325, 366]. DAF-2 imaging, although a recognised and valid NO detection method, has limitations. In order to confirm that detected rises in DAF fluores-

cence are truly due to increase in intracellular NO, it is prudent to adopt a second, distinct, method of detection. The L-arginine to L-citrulline assay provides a completely distinct method of measuring NO production by virtue of the equimolar production of L-citrulline and NO in the NOS reaction [65]. The advantages and disadvantages of both of these NO detection methods will be discussed later in this chapter.

**Table 5.1 Common NO detection methods**  
(continued on following page)

| Detection method                                             | Method description                                                                                                                                                                                                                                                      | Detection type               | Single cell/<br>population?      | Real-time<br>detection?                                                          | NO<br>sensitivity | Direct NO<br>detection?                                                                                                               |
|--------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------|----------------------------------|----------------------------------------------------------------------------------|-------------------|---------------------------------------------------------------------------------------------------------------------------------------|
| Griess<br>reaction                                           | Detection of NO breakdown product nitrites through a diazotisation reaction with sulphanilamide, HCl, and <i>N</i> -(10-naphthyl)ethylenediamine, resulting in a detectable chromophore product. Nitrites can be reduced to nitrites in order to detect total NO [119]. | Spectrophotometer            | Population                       | No                                                                               | 3 $\mu$ M         | No                                                                                                                                    |
| NO dyes<br>E.g.<br>DAF-2-DA                                  | NO dye DAF-2 reacts with NO and NO derivatives, undergoing nitrosation of the diamino group producing a fluorescent triazole compound detectable at 490nm [176].                                                                                                        | Imaging<br>Spectrophotometer | Single cell<br>and<br>Population | Yes                                                                              | 5nM               | Detects NO, nitrites, and nitrates.<br>However, is additionally susceptible to reaction with O <sub>2</sub> <sup>-</sup> derivatives. |
| Electropara-<br>magnetic<br>resonance<br>& Spin-<br>trapping | NO is a diatomic free radical which is detectable by EPR. In biological solutions NO is under the detectable limit. Trapping NO species with other radicals or more commonly iron ions is therefore required.                                                           | Spectroscopy                 | Population                       | Has been used<br><i>in vivo</i> .<br>Generally not used for real-time detection. |                   | Can differentiate between NO and NO derivatives.                                                                                      |

| Detection method                          | Method description                                                                                                                                                                                                                                                                                                                                                                                                     | Detection type                         | Single cell/<br>population? | Real-time<br>detection? | NO<br>sensitivity                                                                      | Direct NO<br>detection?                                                                           |
|-------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------|-----------------------------|-------------------------|----------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|
| L-arginine<br>to<br>L-citrulline<br>assay | NO and citrulline are produced from L-arginine by NOS with a 1:1 stoichiometry. Detection of the more stable citrulline can be used as a read-out of NO production. Citrulline and arginine can be resolved from one another using ion exchange columns. Use of $^3\text{H}$ - or $^{14}\text{C}$ -L-arginine can distinguish between endogenous citrulline and that produced during the experimental time frame [65]. | High-performance liquid chromatography | Population                  | No                      | Not comparable. NO production is defined by % radioactivity converted into citrulline. | No, but provides a NOS specific read-out of activity which is directly relevant to NO production. |
| Amperometry                               | Electrochemical detection using the ability of NO to react with metallic or carbon electrodes. Upon reaction one electron is transferred from NO forming a nitrosonium ion, which degenerates to nitrite. Electron transfer to the anode generates a detectable current which is directly proportional to NO present in the cell [211, 329].                                                                           | Electrochemical                        | Single cell                 | Yes                     | 10nM [211]                                                                             | Yes                                                                                               |

### 5.2.2 DAF-2 detection of NO

4,5-dimaminofluorescein (DAF-2) is a non-ratiometric fluorescent indicator which binds NO and NO derivatives irreversibly. DAF-2 was developed in order to measure the production of NO with the spatial and temporal resolution, and sensitivity that is lacking in many other NO detection methods. Its design allows direct real-time bio-imaging of the production of NO [176,177].

The specificity of DAF-2 has previously been called into question by the Chatton group, with indications of photoactivation and dependency of the fluorescent response on divalent cation concentration. Work utilising the NO donors *s*-nitrosocysteine, DETA NONOate, and SNP showed large increases in DAF-2 fluorescence only in the presence of NO donor and mM concentrations of divalent ion. Chatton deduced that DAF-2 sensitivity to NO was increased in the presence of  $Mg^{+}$  and  $Ca^{2+}$  [37]. However, increase in DAF-2 fluorescence in presence of divalent ions did not correlate with the quantity of nitrites produced by the NO donors as determined by the Griess reaction. Further to this, the Nagano group refuted this conjecture by demonstrating that use of solutions bubbled with NO gas resulted in  $Ca^{2+}$  and  $Mg^{+}$  independent detection of NO. This work indicated that the observed effect of  $Ca^{2+}$  and  $Mg^{+}$  on fluorescence intensity was due to an increase in NO donor instability, resulting in greater release of NO [325].

Light sensitivity of DAF-2 was also proposed by Chatton, with enduring high-intensity light pulses of up to 25secs resulting in increased DAF fluorescence in the presence of  $Ca^{2+}$  and NO donor [37]. However, the work failed to address the potential for photodecomposition of NO donors and increased fluorescence cannot

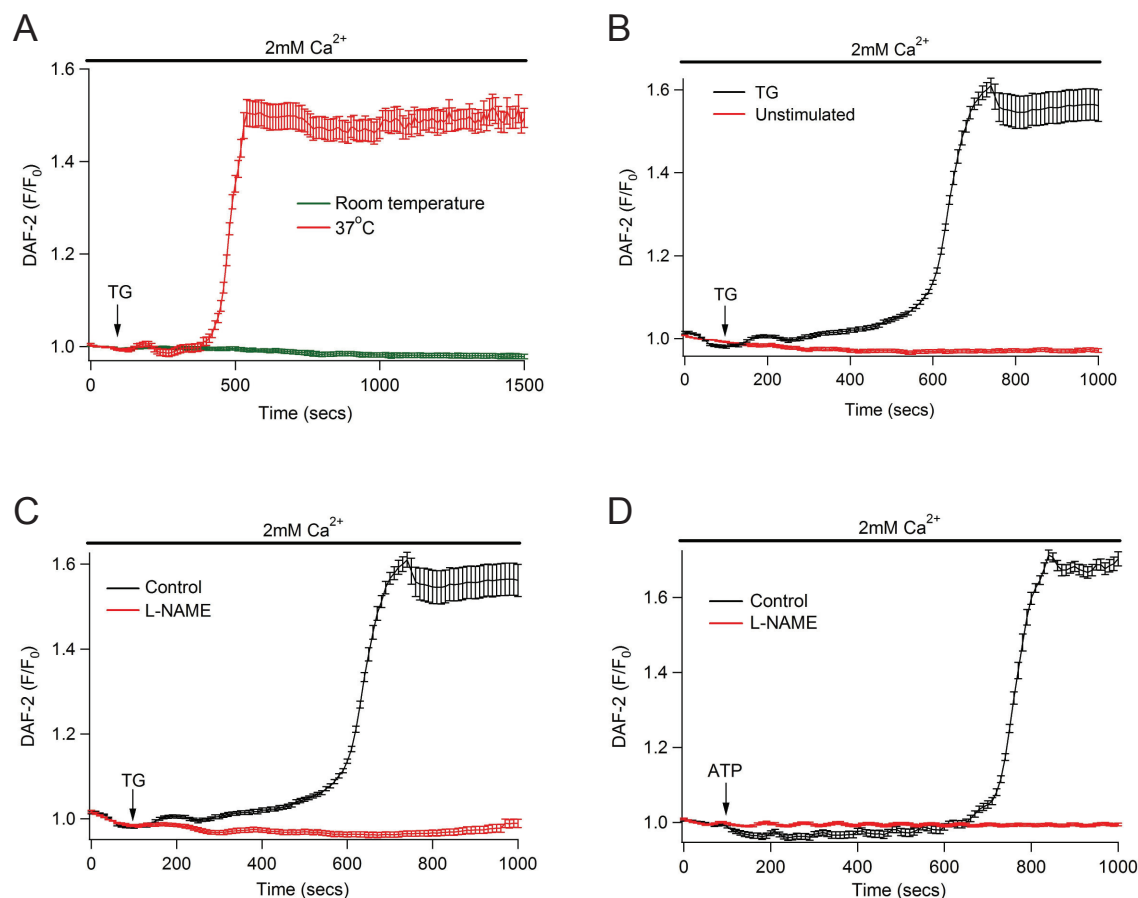
be affirmatively attributed to a sensitivity in DAF-2. Although the potential for photoactivation of DAF-2 should still be a concern to some protocols utilising higher light intensity such as confocal imaging, the duration and light intensity of pulses used within these experiments would have no effect on experimental data obtained during a normal bio-imaging experiment. Light activation of DAF-2 can therefore be disregarded in respect to the work within this thesis. DAF-2-DA is still regularly used to detect NO within the field and is considered to be reliable in measuring increased NO and nitrosating products [189, 268, 283].

With the advantages of DAF-2 in addressing the production of NO in a single cell on a real-time basis, I aimed to use this NO-sensitive dye to further establish the involvement of SOCE in eNOS activation. Previously in this thesis it has been established that both SERCA inhibitor TG and physiological SOCE agonist ATP require CRAC channels to mediate  $\text{Ca}^{2+}$  entry in sEnd.1 cells; this model was therefore used to address the hypothesis that SOCE can activate eNOS.

DAF-2 was loaded into sEnd.1 at 37°C for 30mins in standard external solution containing 2mM  $\text{Ca}^{2+}$  and additionally 100 $\mu\text{M}$  L-arginine. This solution was used for all further NO experiments. Neither the presence of L-arginine in imaging solution or NOS inhibitor N-nitro L-arginine methyl ester (L-NAME), had an effect on  $\text{Ca}^{2+}$  signals, either by amplitude or rate of  $\text{Ca}^{2+}$  entry (data not shown) (*L-arginine*  $n=53$ , *L-NAME*  $n=48$ ). Cells were imaged at room temperature as per the protocol used for previous  $\text{Ca}^{2+}$  imaging experiments, with stimulations performed at 100secs to ensure a sufficient  $F/F_0$  baseline was established. Stimulations with both TG (2 $\mu\text{M}$ ) and known NO agonist ATP (5 $\mu\text{M}$ ) failed to induce any increase in DAF-2 fluorescence (Fig.5.1A. ATP data not shown ( $n=39$ )). This lack

of response was surprising considering the previous reports of robust NO production upon ATP stimulation and, additionally, the evidence presented by Lin et al regarding the role of SOCE in eNOS activation [67, 197]. It would have been easy to assume that SOCE was not playing a role in NO production and that ATP was contributing to eNOS activation through an alternative pathway independent of  $\text{Ca}^{2+}$  entry. However, these suppositions do not stand up to the existing evidence and fail to solve why I saw no response. NO detection experiments, in contrast to  $\text{Ca}^{2+}$  experiments, are typically carried out at physiological temperature, which led us to question whether this was due to ambient temperature not being sufficient to support NO activation. To address whether ambient temperature was affecting NO production, experiments were repeated at 37°C. Interestingly, the increase in temperature to physiological levels resulted in an increase in DAF-2 fluorescence with both TG and ATP stimulation (Fig.5.1A). However, a response was seen in both cases only after significant time delay- typically up to 10mins (Fig.5.1A). The increase in DAF-2 fluorescence only at 37°C was intriguing. To determine that the signal was dependent on NO production by NOS and not due to an effect on NO itself, cells loaded with DAF-2 were imaged in the absence of stimulation (Fig.5.1B). No signal was observed, indicating that the mobilisation of  $\text{Ca}^{2+}$  stores and subsequent  $\text{Ca}^{2+}$  influx were responsible for the increase in fluorescence. To further establish that these signals were dependent on NO production by eNOS, I pre-incubated DAF-2 loaded sEnd.1 cells with the NOS inhibitor L-NAME (100 $\mu\text{M}$ ) for 5mins. Presence of L-NAME inhibited both ATP and TG-induced DAF-2 signals. It can therefore be concluded that TG and ATP are able to induce NO production in sEnd.1 cells.

## CHAPTER 5. CALCIUM ENTRY THROUGH THE CRAC CHANNEL INCREASES PRODUCTION OF NITRIC OXIDE IN sEnd.1



**Figure 5.1** An L-NAME inhibitable increase in NO production can be induced with TG and ATP at physiological temperature

(A) Addition of TG (2μM) to DAF-2-DA loaded sEnd.1 cells, in the presence of 2mM external Ca<sup>2+</sup> and 100μM L-arginine, induced increased NO production at 37°C (*room temperature (RT)*  $n=17$ ;  $37^{\circ}C$   $n=16$ ) (B) Absence of TG stimulation at physiological temperature failed to increase DAF-2 fluorescence, indicating response is due to increase in cytosolic NO and NO derivative levels rather than light activation of DAF-2 (*unstimulated*  $n=58$ ; *TG stimulated*  $n=55$ ) (C) Competitive antagonist of eNOS L-NAME (100μM) abrogated TG-induced NO response (*control*  $n=26$ ; *L-NAME*  $n=65$ ) (D) Physiological activator of CRAC channels ATP (5μM) induced increased eNOS activity, which was inhibitable by L-NAME (*control*  $n=27$ ; *L-NAME*  $n=39$ ).

The signals observed are themselves interesting, with a sigmoidal shape, consisting of a slow increase from time of activation, followed by rapid production of NO,



and eNOS inactivation when fluorescence reaches a maximum amplitude. The maximum peak response was determined to be dependent on the amount of NO produced and not saturation of the dye. The NO donor SNP was applied to DAF-2 loaded cells, and reached levels of fluorescence 4 times greater than that seen upon agonist stimulation (data not shown). Activation of NO synthesis has been shown here to take minutes, and it should be noted that the time taken to stimulate the fast rise in NO is variable. eNOS activation is a complex process as previously discussed in chapter 1. Variability in time taken to induce rapid NO production is not necessarily surprising as a multitude of intracellular activities are required in order to achieve efficient NO production by eNOS, including release of caveolin inhibition,  $\text{Ca}^{2+}$ -CaM binding, and phosphorylation and de-phosphorylation of specific eNOS residues among other processes [50, 103, 114, 216].

The distinctive lag time taken to induce rapid NO production is of interest. Treatment of endothelia with  $\text{Ca}^{2+}$  ionophores (commonly A23187) shows NO production, through detection with amperometry, within seconds [38, 39, 41]. Ionophores, however, allow excess  $\text{Ca}^{2+}$  entry into cells; eNOS activation under these conditions would be significantly quicker than activation of physiological  $\text{Ca}^{2+}$  entry processes, creating a surplus of  $\text{Ca}^{2+}$ -CaM. Manipulation of eNOS  $\text{Ca}^{2+}$ -dependency through slow processes such as phosphorylation would not be required under these high  $\text{Ca}^{2+}$  conditions [50, 103, 216]. A time delay prior to full eNOS activation, when induced by physiological  $\text{Ca}^{2+}$  entry pathways, has not to our knowledge been commented on in the literature. Real-time detection of NO in the literature frequently uses time courses with poor resolution, showing measurements every  $\sim 5$ mins, or alternatively reporting total NO production within a denoted time pe-

riod [197,239]. Representation of data in this manner may overlook both variation in time to activation and a time lag itself. Kimura et al, however, treated bovine aortic endothelial cells with ATP (1 $\mu$ M) in the presence of 1.5 mM external  $\text{Ca}^{2+}$ , demonstrating an increase in NO production which occurred with a similar profile to that observed in sEnd.1 [172]. Observed rate of NO production in sEnd.1 however, appears to be quicker; this could be explained by the high eNOS production in sEnd.1 cells, and/or higher ATP concentration used within this work. Crucially, Kimura et al observed a time delay before NO production was detectable. This time delay was shorter at around 200-300secs, than often observed in sEnd.1 cells, however, I notice a variation in activation time, with 200-300secs well within our observed range. A variation in response times was not reported by Kimura.

The Bird group has demonstrated simultaneous imaging of NO and  $\text{Ca}^{2+}$  using the dyes Fura-2 and DAF-FM in uterine endothelial cells, and showed representative traces [366]. They demonstrate NO production as being detectable from the point of activation, with a rapid spike in the rate of NO production which declines quickly at first, and then slowly returns to an inactive state, with NO response ceasing in some cell types within 200secs. This is in surprising contrast to our data, and time courses of alterations in protein recruitment and post-translational modifications on eNOS, which will be discussed later in this thesis. Interestingly, the Fura signals shown were also starkly different from those obtained in this thesis, with TG inducing sustained  $\text{Ca}^{2+}$  increases in the presence of 2mM external  $\text{Ca}^{2+}$  for 1000secs with no indication of CRAC channel inactivation.  $\text{Ca}^{2+}$  increases were additionally around 5 times greater than those observed in this thesis, and in other reports of endothelial cell CRAC channel activity [1]. Furthermore, simultaneous

imaging of  $\text{Ca}^{2+}$  and NO may alter NO production, as Fura-2 acts as a  $\text{Ca}^{2+}$  chelator, and therefore may alter the internal  $\text{Ca}^{2+}$  profile. This study therefore may not be a useful comparison to the work within this thesis.

When considering mechanisms of NOS activation, and additional evidence in terms of physiological effects of NO, an activation period of low NO production is not unexpected. NO-dependent vasorelaxation in response to ACh, which generates NOS-activating  $\text{Ca}^{2+}$  fluxes, occurs slowly over 5mins [112]. Further to this, modifications of eNOS, which increase eNOS activity are slow, with phosphorylation, de-phosphorylation, and binding of Hsp90 all occurring over time periods of  $\sim 5$ mins [50, 103, 114, 216]. These time frames are consistent with the time lag observed within this work, with activation usually occurring, upon ATP or TG stimulation in the presence of 2mM external  $\text{Ca}^{2+}$ , within 500secs. The time frame to activation will be more extensively investigated in chapter 6, as a potential hallmark of eNOS regulation.

### **5.2.3 L-arginine to L-citrulline assay detects two-fold increase in NO production**

With the previously discussed limitations of DAF-2 NO detection, it is prudent to demonstrate the activation of eNOS by a second method. For this reason, I again addressed the question of SOCE activation of eNOS using an alternative technique of  $^{14}\text{C}$ -L-arginine to L-citrulline conversion.

All NOS enzymes catalyse a reaction between L-arginine and 2 molecules of  $\text{O}_2$  to form NO and the amino acid L-citrulline in an equimolar ratio. The produc-

tion of L-citrulline can therefore be used as an indirect but accurate measure of total NO production by NOS. The replacement of external L-arginine with a radioactive form ( $^{14}\text{C}$ -L-arginine) allows detection of the production of a radioactive L-citrulline product, thus differentiating between basal cellular L-citrulline and that produced subsequent to NOS activation.  $\text{Ca}^{2+}$  mobilising agents, commonly  $\text{Ca}^{2+}$  ionophores, are used to activate the enzyme eNOS. HPLC separation is then used to distinguish reaction products based on elution times from the column, which is dependent on product interaction with the sorbent. A scintillation detector reveals quantity of radioactivity detected over time. Here I normalise the level of radioactivity at each peak to the total radioactivity, thus calculating % conversion of  $^{14}\text{C}$ -L-arginine [65].

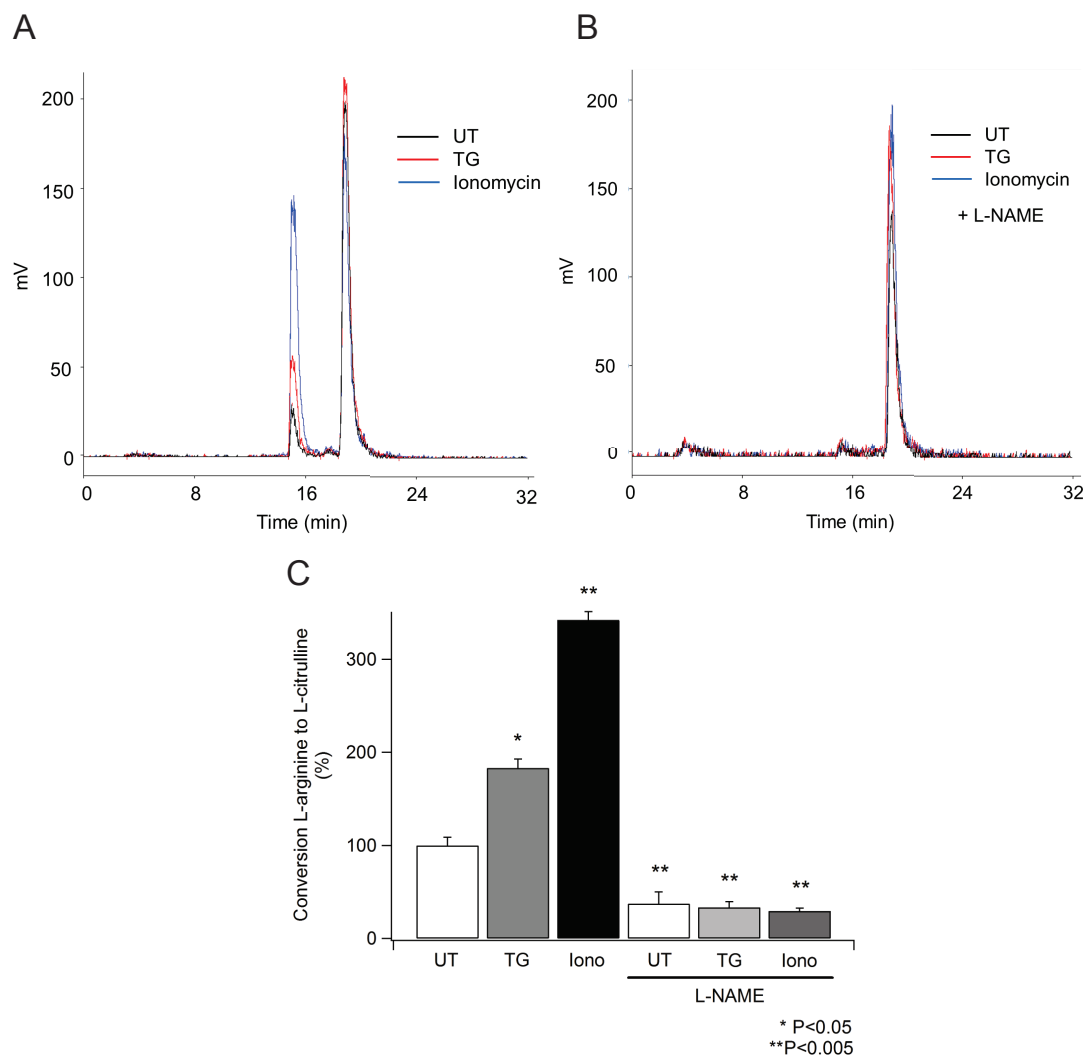
Detection of L-citrulline over NO is advantageous as it is able to circumvent the difficulties presented with the high reactivity of NO, therefore measuring NOS activity over available NO or NO derivatives. However, the detection sensitivity of this method is not reported in literature as it relies on the % conversion of radioactive product. Defining exact quantities of radioactivity in the samples is difficult, as protein quantification cannot be easily determined with these samples. Variances in total radioactivity in each sample cannot therefore be defined. % conversion can therefore be considered more accurate than calculation of total L-citrulline. I can determine, however, that detection sensitivity is limited as the technique relies on cell populations and the use of  $\text{Ca}^{2+}$  ionophores, commonly A23817, to detect conversion. However, this method is commonly used to detect changes in diseased vascular tissue, which classically display low production of NO [5, 82]. This implies that this technique will be sensitive enough to detect

NO production induced by physiologically relevant  $\text{Ca}^{2+}$  entry i.e. SOCE, in a endothelial cell line model.

Along with the requirement of a population of cells and unknown detection limits, the  $^{14}\text{C}$ -L-arginine conversion assay cannot be used for real-time detection of NO production. Real-time measurements are not possible, as samples require de-proteinisation prior to HPLC separation. It is therefore less well suited to investigations of signalling mechanisms where real-time single cell detection is clearly advantageous. In combination, however,  $^{14}\text{C}$ -L-arginine conversion and DAF-2 would provide a strong evidence base for increased eNOS activity post-CRAC channel activation.

sEnd.1 cells were pre-treated with arginase inhibitor Nor-NOHA (100 $\mu\text{M}$ ), and  $^{14}\text{C}$ -L-arginine for 5mins at room temperature, prior to stimulation with TG, or a positive control of  $\text{Ca}^{2+}$  ionophore ionomycin (2 $\mu\text{M}$ ). Cells were incubated for 2hrs at 37°C. Cell lysates were de-proteinised prior to HPLC separation. Radioactive products were resolved on HPLC, detected via a scintillation counter, and differentiated based on elution times compared to radioactively-labelled controls for urea, ornithine, arginine, and citrulline [65]. The increased conversion of  $^{14}\text{C}$ -L-arginine to citrulline was readily observable in sEnd.1 cells with a  $242\% \pm 9\%$  increase above basal conversion with ionomycin treatment. Induction of SOCE with TG additionally showed a detectable increase in L-citrulline, reaching  $183\% \pm 10\%$  of untreated (UT) control (Fig.5.2). Treatment of all conditions with L-NAME reduced  $^{14}\text{C}$ -L-arginine conversion by almost two-thirds from basal level (*UT*  $37\% \pm 13\%$ ; *TG*  $33\% \pm 6\%$ ; *Ionomycin*  $29\% \pm 3\%$ ) confirming eNOS activity is responsible for increased conversion. The reduction of detectable L-citrulline in untreated samples

demonstrates the constitutive activity of eNOS in sEnd.1 cells and was expected. The increase of detectable L-citrulline after TG treatment and increase in fluorescence of DAF-2 were both found to be within the same range of 1.5-2 fold, which gives additional confidence to the reliability of both methods of detection. It can therefore be concluded that SOCE increases NOS activity to produce NO, confirming the findings of Lin et al [197].



**Figure 5.2 SOCE increases conversion of  $^{14}\text{C}$ -L-arginine to L-citrulline**  
(A-B) Representative HPLC traces.  $^{14}\text{C}$ -labelled products of intracellular metabolism of  $^{14}\text{C}$ -L-arginine elute at different times. The elution times of detectable products are: urea (5mins), ornithine (19mins), citrulline (15.5mins) and remaining arginine (21mins). (C) Treatment of sEnd.1 cells increase conversion of  $^{14}\text{C}$ -L-arginine to L-citrulline almost two-fold compared to basal. Treatment with NOS inhibitor L-NAME abrogates this increase and reduces basal NO production.

### 5.2.4 Induction of SOCE increases $O_2^-$ production, but doesn't fully 'uncouple' eNOS

DAF-2 reacts with biologically relevant nitrates, nitrites, and reactive nitrogen oxide species, including  $ONOO^-$ . It is well defined that in the presence of oxidative stress, the ratio of essential eNOS cofactor  $BH_4$  to its inactive oxidised counterpart  $BH_2$  is reduced.  $BH_4$  and  $BH_2$  both bind eNOS at the same site with the same affinity; however, only  $BH_4$  is catalytically active and able to couple electron transfer between the reductase and oxygenase domains of NOS. Binding of  $BH_2$  results in 'uncoupling' of the the two NOS domains. The electron is no longer passed between domains, but is instead transferred directly to the  $O_2^-$  bound in the NOS catalytic site forming  $O_2^-$  [343]. The ratio of  $BH_4$  to  $BH_2$  therefore determines the 'coupled' state of eNOS [58, 60].  $BH_2$  is converted back to  $BH_4$  by DHFR, which maintains the ratio in favour of NO production [60]. sEnd.1 cells have previously been reported to express high levels of DHFR and eNOS is considered 'coupled' within these cells [59].

Production of  $O_2^-$  by eNOS is of concern both physiologically and when assessing NO production using DAF-2 DA. The equimolar reaction of NO and  $O_2^-$  produces  $ONOO^-$ . DAF-2 has previously been reported as being more sensitive to  $ONOO^-$ ; its presence results in a 7 times greater increase in the formation of the detectable fluorescent product: DAF-2-triazole (DAF-2-T).  $ONOO^-$  oxidises DAF-2, forming a non-fluorescent intermediate, which allows a more rapid reaction with the NO free radical to form DAF-2T [87, 161, 289]. An increased presence of  $O_2^-$  could alter the amplitude of detected fluorescence and it is therefore prudent to ascertain that



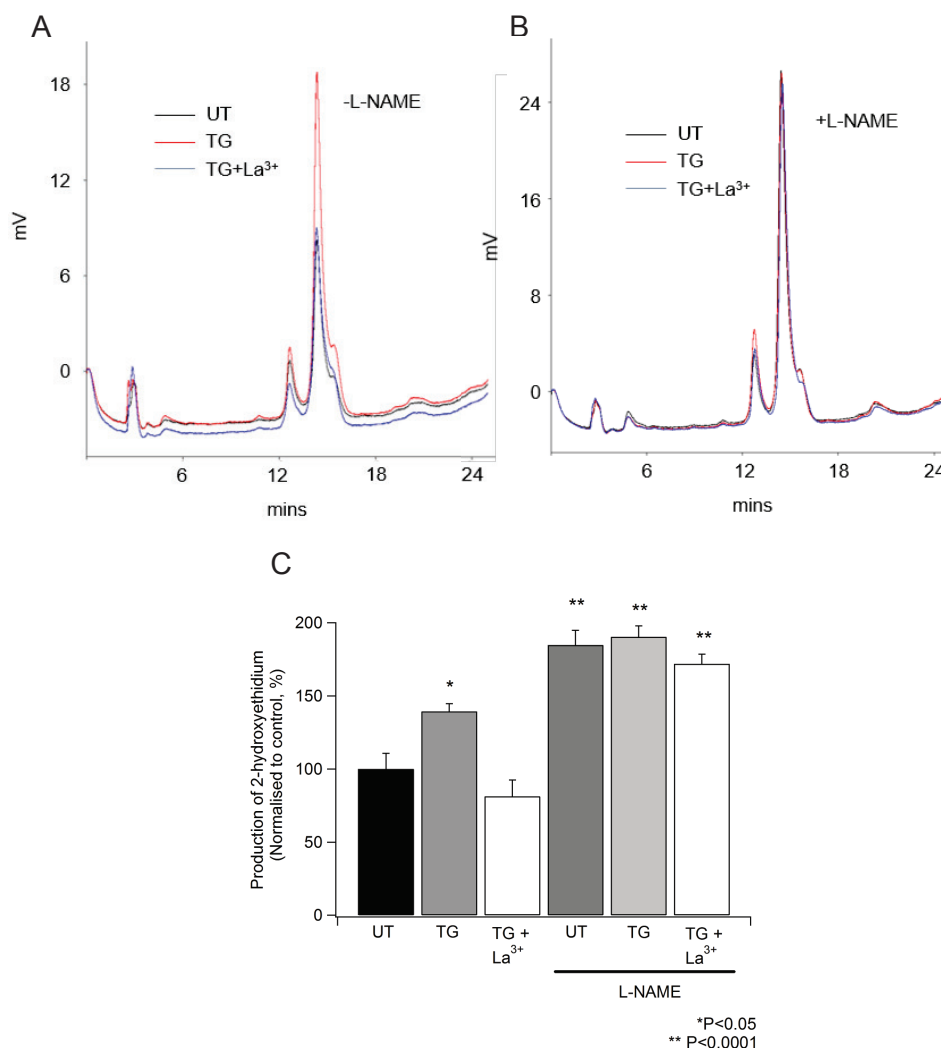
eNOS remains ‘coupled’ after stimulation of SOCE.

The conversion of dihydroethidium (DHE) to ethidium and 2-hydroxyethidium is used as a measure of superoxide production in cells. The production of 2-hydroxyethidium is directly dependent on oxidation of DHE by  $O_2^-$  and presents a specific measure of intracellular  $O_2^-$  levels [98]. Separation of reaction products by HPLC allows quantification of ethidium produced.

sEnd.1 cells were pre-incubated in presence or absence of NOS inhibitor L-NAME (1mM) in standard NO solution for 5 mins at room temperature prior to stimulation with TG (2 $\mu$ M). Cells were incubated at 37°C for 15mins before addition of DHE (50 $\mu$ M) and a further 20mins incubation protected from light at 37°C. Cell lysates were de-proteinated prior to separation of HPLC utilising electrochemical detection. TG increased detectable  $O_2^-$  to 139% $\pm$ 5% compared to UT; an increase which was abrogated with pre-incubation  $La^{3+}$  (50 $\mu$ M). Pre-incubation with 1mM L-NAME increased superoxide production almost two-fold under all conditions (*UT* 184% $\pm$ 10%; *TG* 190% $\pm$ 7%; *TG+La<sup>3+</sup>* 172% $\pm$ 7%) (Fig.5.3).

L-NAME inhibits NO synthesis. The presence of intracellular NO reduces detectable  $O_2^-$  due to NO scavenging. The increase in ethidium detected with L-NAME is due to a lack of eNOS activity and therefore diminished availability of cellular NO. This is indicative of ‘coupled’ eNOS. It appears basal levels of NO production are able to compensate for cellular  $O_2^-$  production. Although TG demonstrated a detectable increase in  $O_2^-$ , use of L-NAME indicated NOS is still largely ‘coupled’ in sEnd.1 cells; however, ONOO $^-$  will be increased post-stimulation. The contribution of ONOO $^-$  presence in increased DAF-2 fluorescence should be considered in interpretation of DAF-2 data.

The similar fold-change measured by both methods of NO detection may help to give more confidence to interpretation of the amplitude of DAF-2 fluorescence increase. Considering that  $\text{ONOO}^-$  was reported to increase DAF fluorescence 7 times more effectively than NO alone, if the increase in  $\text{O}_2^-$  was significantly affecting overall fluorescence in this system, then one would expect a much higher fold increase in DAF-2 when compared to L-citrulline production.



**Figure 5.3 TG-treatment increases oxidative stress in sEnd.1**

(A-B) Representative HPLC traces. Three peaks are visible in the chromatographs separated by elution times: DHE (4mins), 2-hydroxyethidium (13mins) and ethidium (16mins). 2-hydroxyethidium is considered specific for increased  $O_2^-$  over other oxidative species. (C) Treatment with TG increased  $O_2^-$  production by  $39\% \pm 5\%$ , whereas TG with  $La^{3+}$  do not significantly alter  $O_2^-$  production in sEnd.1. Treatment with L-NAME inhibits eNOS.  $O_2^-$  production is significantly increased compared to UT control in absence of L-NAME (*UT L-NAME*  $185\% \pm 10\%$ ; *TG L-NAME*  $190\% \pm 7\%$ ; *TG La<sup>3+</sup> L-NAME*  $172\% \pm 7\%$ )  $n=7$  for all treatments.

### 5.2.5 Effect of physiological temperature on $\text{Ca}^{2+}$ signals

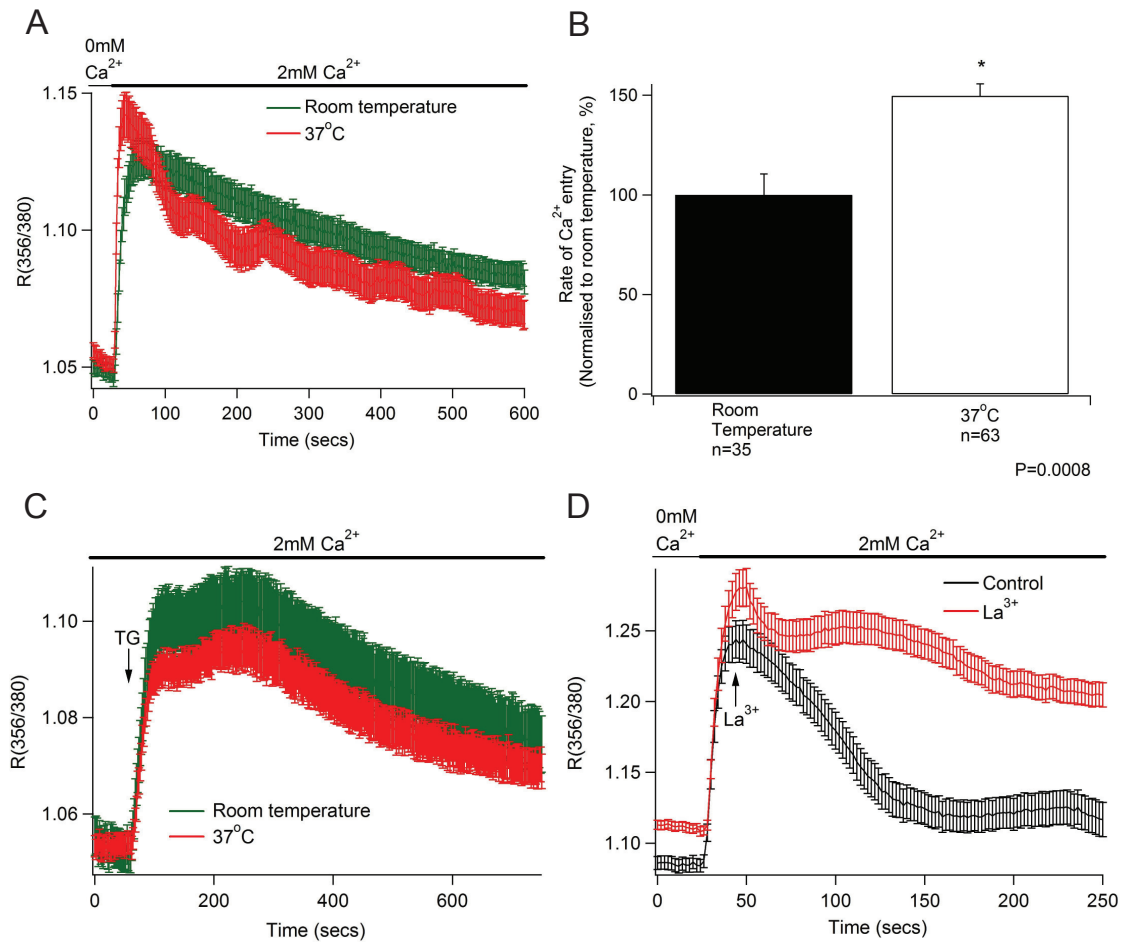
Temperature dependence of NO production in sEnd.1 indicated that CRAC channels may also show temperature dependent changes in  $\text{Ca}^{2+}$  conductance. Historically there have been no major indications of the requirement of physiological temperature for the development of  $I_{\text{CRAC}}$ . The CRAC channel and  $I_{\text{CRAC}}$  were extensively profiled prior to the discovery of Orai/STIM. The distinctive  $I_{\text{CRAC}}$  current was noted for low conductance and high selectivity, but temperature-sensitivity was never reported [142, 143, 272, 274, 379]. Patch-clamp and  $\text{Ca}^{2+}$  imaging experiments are conducted within the field at ambient temperature and  $\text{Ca}^{2+}$  influx is prominent [10, 96, 163, 192, 379]. STIM-1 has been reported to be sensitive to changes in temperature environment, and at hyperthermic temperatures can activate  $I_{\text{CRAC}}$  independently of store-depletion. STIM temperature sensitivity is considered an adaptation to environmental stress [212, 361]. I therefore investigated whether temperature altered CRAC channel activity in sEnd.1 cells to see if this explained the striking temperature-dependence of NO production. Temperature was maintained on a heated chamber between 36°C and 37.9°C.

The characterisation of CRAC channels was re-addressed at physiological temperature. The rate of  $\text{Ca}^{2+}$  entry after store depletion with TG (2 $\mu\text{M}$ ) and ATP (5 $\mu\text{M}$ ) was compared between the temperatures. Physiological temperature increased both the rate of  $\text{Ca}^{2+}$  entry and the rate of  $\text{Ca}^{2+}$  extrusion in response to TG (Fig.5.4A-C). Rate of  $\text{Ca}^{2+}$  entry was increased by  $50\% \pm 6\%$ , a substantial increase. However, bulk rise was less sustained.  $\text{La}^{3+}$  (1mM) block of PMCA largely abrogated this increased  $\text{Ca}^{2+}$  extrusion (Fig.5.4D).  $\text{Ca}^{2+}$  entry in response to ATP was also increased at 37°C. Increased temperature had a strikingly smaller

effect on ATP SOCE, increasing the rate of entry by only  $20\% \pm 5\%$  (Fig.5.5). The temperature coefficient ( $Q_{10}$ ) for rate of  $\text{Ca}^{2+}$  entry between room and physiological temperature in this system is therefore low, with TG and ATP having values of 1.36 and 1.15 respectively. The smaller increase seen with ATP compared to TG may be due to the increased rates of  $\text{Ca}^{2+}$  re-uptake into ER  $\text{Ca}^{2+}$  stores, and  $\text{Ca}^{2+}$  extrusion masking the increase in rate. Rate of  $\text{Ca}^{2+}$  entry was consistently increased at physiological temperature, implying increased conductance. This would result in enhanced  $\text{Ca}^{2+}$  microdomains. Although increased conductance would play a role in ability of eNOS to activate at physiological temperature, the overall increases of only 20 and 50% are relatively small, especially taking into account the enhanced  $\text{Ca}^{2+}$  removal, which affects overall bulk  $\text{Ca}^{2+}$  rise. It is therefore likely that other mechanisms are also having an effect, and eNOS activation at physiological temperature is not solely dependent on increased cytosolic  $\text{Ca}^{2+}$ .

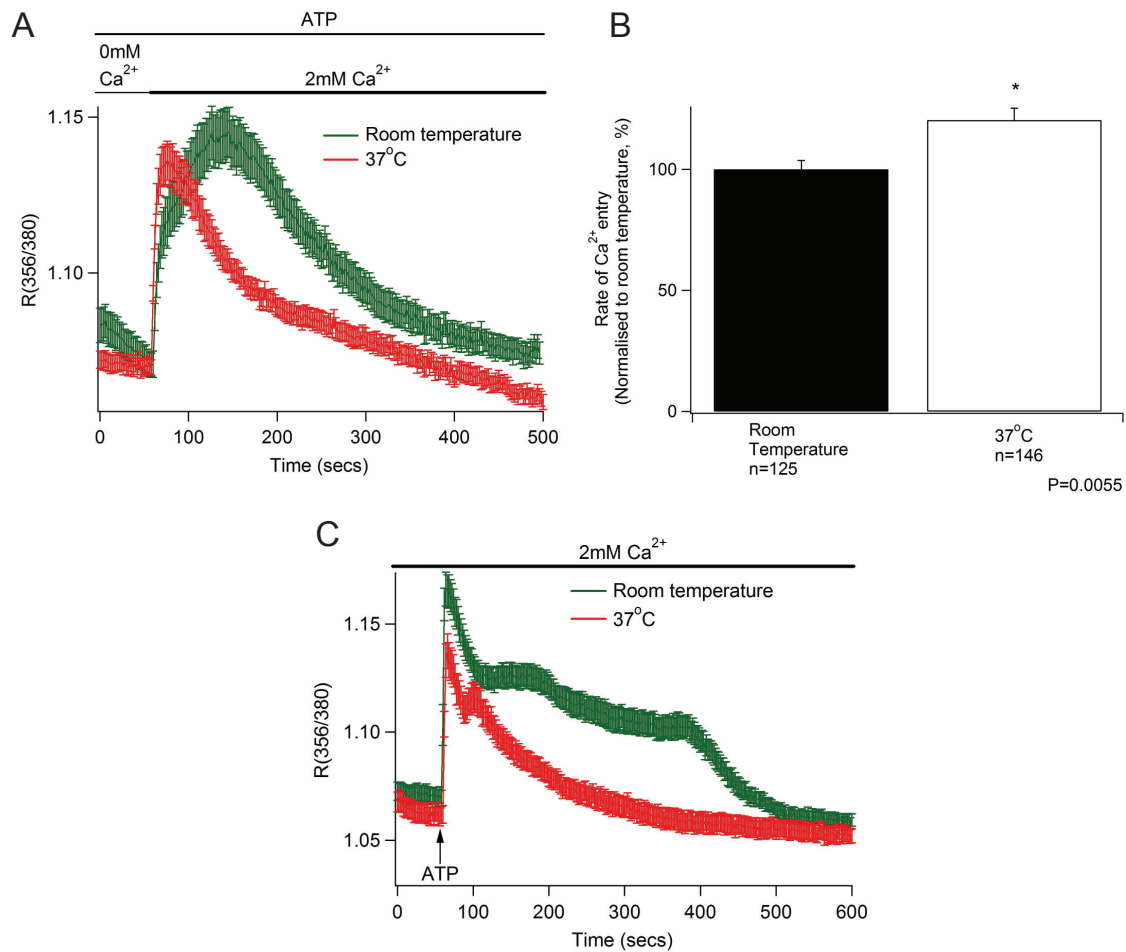
The enhanced removal of  $\text{Ca}^{2+}$  being largely abrogated by PMCA inhibition may be indicative of increased  $\text{Ca}^{2+}$ -CaM activity [35]. Indeed, this could be a contributory factor in eNOS activation at physiological temperature. Potential reasons for presence of activation at  $37^\circ\text{C}$  only will be discussed later within this chapter.

## CHAPTER 5. CALCIUM ENTRY THROUGH THE CRAC CHANNEL INCREASES PRODUCTION OF NITRIC OXIDE IN SEND.1



**Figure 5.4** Physiological temperature increases the rate of  $Ca^{2+}$  entry after store-release triggered by TG stimulation, but reduces the duration of the  $Ca^{2+}$  rise.

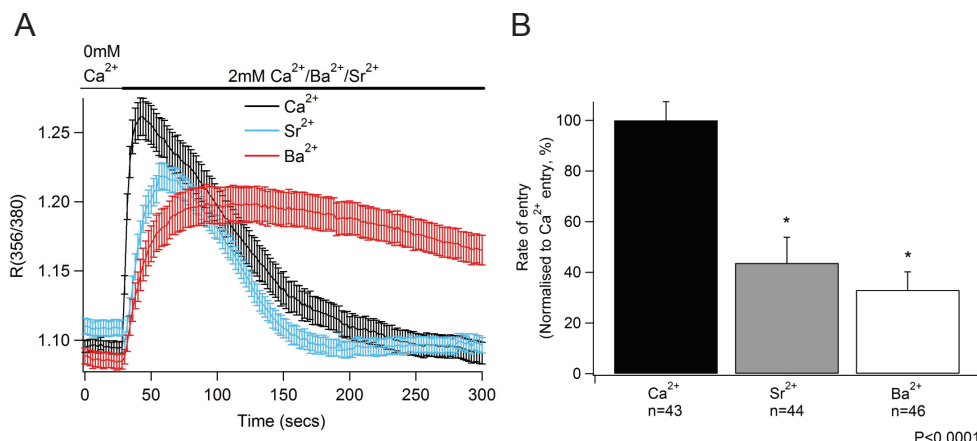
(A) (A) A representative experiment. (A-B) Cells were stimulated with TG in the absence of  $Ca^{2+}$  (not shown). 2mM  $Ca^{2+}$  was added at 60secs at room temperature (RT) (20-25°C) or 37°C. Cells demonstrated a 50% increase in the rate of  $Ca^{2+}$  entry.  $Ca^{2+}$  removal appeared increased at physiological temperature. (C) A representative experiment. TG stimulation in the presence of external  $Ca^{2+}$ . Increased  $Ca^{2+}$  removal appears more apparent in the continual presence of extracellular  $Ca^{2+}$ . (D) Inhibition of PMCA with 1mM  $La^{3+}$  reduced the increased extrusion seen at physiological temperature in TG-treated cells after addition of external  $Ca^{2+}$ .



**Figure 5.5** Increased temperature increases the rate of  $\text{Ca}^{2+}$  entry after store-release by ATP stimulation, but response is less sustained due to increased  $\text{Ca}^{2+}$  removal

(A) A representative experiment. Cells were stimulated with ATP in the absence of  $\text{Ca}^{2+}$  (not shown). 2mM  $\text{Ca}^{2+}$  was added at 60secs at RT or 37°C. Cells demonstrated an increased rate of  $\text{Ca}^{2+}$  entry as well as increased  $\text{Ca}^{2+}$  removal. Bulk rise was less sustained at physiological temperature. (B) Rate of  $\text{Ca}^{2+}$  entry was increased by 20% compared to RT. (C) A representative experiment of ATP stimulation in presence of extracellular  $\text{Ca}^{2+}$ . Increased  $\text{Ca}^{2+}$  removal results in a shorter and more transient rise in bulk  $\text{Ca}^{2+}$ .

Higher temperature increases conductance of CRAC channels in sEnd.1 cells, but does not alter selectivity of the channel;  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$  rate of entry are  $44\% \pm 10\%$  and  $33\% \pm 7\%$  respectively of  $\text{Ca}^{2+}$  control (Fig.5.6). These rates of entry are comparable, when normalised to  $\text{Ca}^{2+}$  entry, to those seen at room temperature ( $\text{Sr}^{2+}$   $43\% \pm 4\%$  and  $\text{Ba}^{2+}$   $43\% \pm 8\%$ ). However, it should be noted that there is a trend towards slower  $\text{Ba}^{2+}$  entry. Increased selectivity may be masked by the increased rate of PMCA activity at higher temperatures. PMCA extrudes both  $\text{Ca}^{2+}$  and  $\text{Sr}^{2+}$ , but is impermeable to  $\text{Ba}^{2+}$ . Increased removal of  $\text{Sr}^{2+}$  and  $\text{Ca}^{2+}$  may be underestimating their rates of entry, and thus hiding enhanced selectivity.

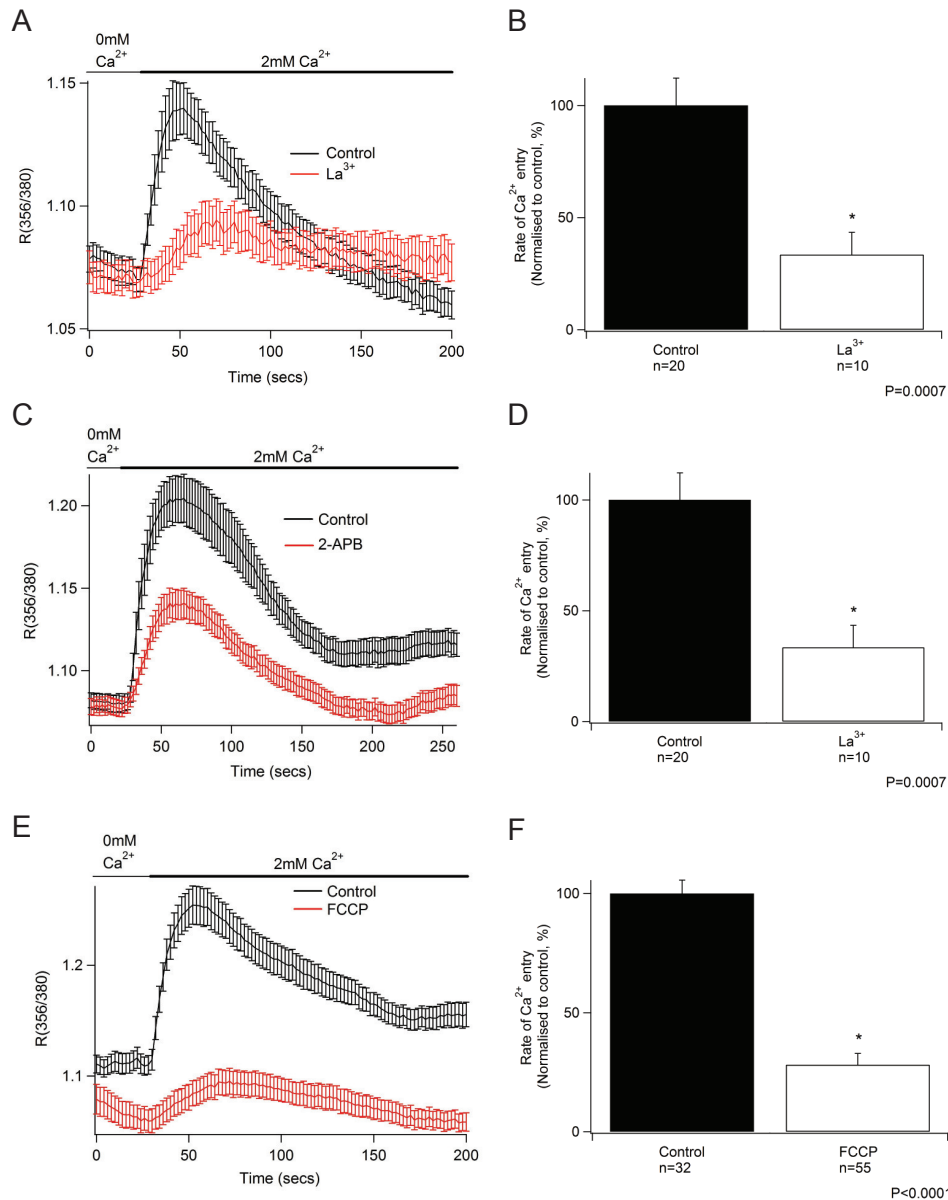


**Figure 5.6** Physiological temperature does not alter the  $\text{Ca}^{2+}$  selectivity of the CRAC channel in sEnd.1

(A) A representative experiment. Cells were stimulated with TG in the absence of  $\text{Ca}^{2+}$  (not shown). Add-back of  $2\text{mM Ca}^{2+}$ ,  $\text{Sr}^{2+}$ , and  $\text{Ba}^{2+}$  is shown. (B) Rate of entry of  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$  are reduced to 44% and 33% of  $\text{Ca}^{2+}$  respectively. The rate of  $\text{Ba}^{2+}$  appears reduced compared to  $\text{Sr}^{2+}$  at increased temperature but doesn't reach significance at  $P < 0.05$ .



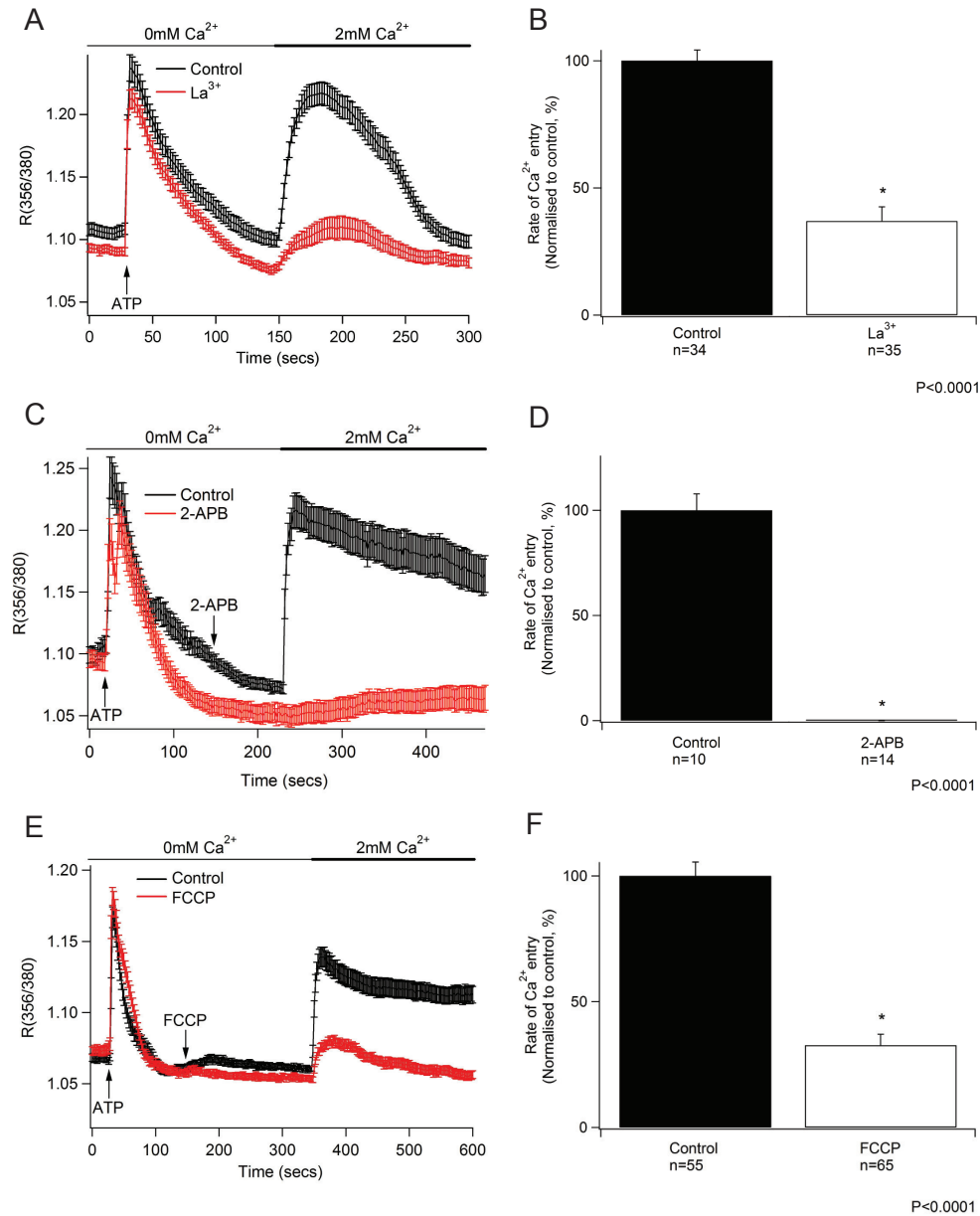
Ensuring CRAC channel inhibitors are still effective at reducing  $\text{Ca}^{2+}$  entry at physiological temperature is important for future experiments to determine CRAC channel role in NO production. All inhibitors remained effective against the elevated rate of  $\text{Ca}^{2+}$  entry at 37°C.  $\text{La}^{3+}$  (50 $\mu\text{M}$ ) blocked TG and ATP SOCE, reducing it by  $67\% \pm 10\%$  and  $63\% \pm 6\%$  of control respectively (Fig.5.7E-F and 5.8E-F). 2-APB (20 $\mu\text{M}$ ) reduced  $\text{Ca}^{2+}$  entry in response to TG to  $50\% \pm 10\%$  of control, and completely abolished ATP-induced SOCE (Fig.5.7C-D and 5.8C-D). FCCP (5 $\mu\text{M}$ ) treatment of ATP and TG treated cells reduced  $\text{Ca}^{2+}$  entry to  $33\% \pm 4\%$  and  $28\% \pm 5\%$  of control entry respectively (Fig.5.8A-B and 5.7A-B). Although not directly comparable, CRAC channel inhibitors, therefore, appear to work with similar efficiency at physiological temperatures, as at room temperature (Fig.3.3 and 3.7). 2-APB again is particularly effective at inhibiting ATP-induced  $\text{Ca}^{2+}$  influx, completely abolishing entry.



**Figure 5.7 CRAC channel inhibitors are effective at physiological temperatures**

(A-E) Cells were treated with TG in the absence of external  $Ca^{2+}$  after pre-incubation with respective CRAC channel inhibitors at  $37^{\circ}C$  (not shown).  $Ca^{2+}$  was administered at 30secs. (A-B) Pre-incubation with  $La^{3+}$  ( $50\mu M$ ) reduced rate of entry to  $33\% \pm 10\%$  compared to untreated control. (C-D) Administration of 2-APB ( $20\mu M$ ) reduced entry by  $50\% \pm 10\%$ . (E-F) Depolarisation of mitochondria with FCCP ( $5\mu M$ ) reduces  $Ca^{2+}$  entry through CRAC channels to  $28\% \pm 5\%$ .

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**Figure 5.8 CRAC channel inhibitors reduce  $\text{Ca}^{2+}$  entry after treatment with physiological SOCE agonist ATP at  $37^\circ\text{C}$**

(A-B) 5min pre-incubation with  $50\mu\text{M}$   $\text{La}^{3+}$  (5min) decreased ATP-induced ( $5\mu\text{M}$ ) SOCE to  $37\% \pm 6\%$ . (C-D)  $20\mu\text{M}$  2-APB administered completely abrogated  $\text{Ca}^{2+}$  entry. (E-F) FCCP ( $5\mu\text{M}$ ) applied at 150secs reduced the rate of external  $\text{Ca}^{2+}$  entry by  $67\% \pm 4\%$ .

### 5.3 Key findings of this chapter

- TG and ATP can stimulate production of NO in sEnd.1 at physiological temperature
- TG increases  $O_2^-$  production in sEnd.1
- Physiological temperature increases both the rate of store-operated  $Ca^{2+}$  entry and increases PMCA-dependent  $Ca^{2+}$  removal
- CRAC channel inhibitors are effective in reducing  $Ca^{2+}$  entry at 37°C

### 5.4 Discussion

Within this chapter, I have demonstrated the activation of NOS-dependent NO synthesis at physiological temperature, as determined by DAF-2 fluorescence and conversion of  $^{14}C$ -L-arginine to L-citrulline. Use of the specific NOS-inhibitor L-NAME completely abrogated responses to both ATP and TG. Establishment of robust detection of NO production will allow clarification of the role of store release and  $Ca^{2+}$  entry in NO production [88, 197]. Real-time detection of NO production through the use of NO-sensitive dye DAF-2 has revealed interesting, and to our knowledge previously unreported, kinetics of NO production with a variable time that precedes a rapid phase NO production. It should be noted though that small rises in NO production were detectable immediately after stimulation of intracellular  $Ca^{2+}$  fluxes. Activation of NO synthesis is a complex process, involving numerous post-translational modifications, and protein interactions [281]. A variable time taken to full activation is not necessarily surprising. However, ability

to manipulate this unexplored variable may help to dissect components of eNOS activation.

### 5.4.1 Superoxide production

The identified increase in  $O_2^-$  production upon TG stimulation should be considered when interpreting the changes in NO production. Altering kinetics of eNOS activation as indicated by a temporal shift in detectable NO production is likely irrespective of  $O_2^-$  production, and the subsequent formation of  $ONOO^-$ . Increased  $O_2^-$  was perturbed with CRAC channel inhibitors, but was further increased above TG-stimulated to equal levels by NOS inhibitors. TG treatment did not further increase  $O_2^-$  in the presence of L-NAME, compared to unstimulated L-NAME treated cells. This indicates that TG is acting to increase  $O_2^-$  via activation of eNOS. Manipulation of the kinetics of eNOS activation would therefore affect both NO and  $O_2^-$  production, making analysis of temporal regulation reliable despite the unexpected increase in  $O_2^-$  production upon TG stimulation. Changes in total NO production, however, should be treated with more caution. Increased  $ONOO^-$  would intensify potentially small differences in total NO production. Perceived differences in total NO production therefore may be dependent on increased  $ONOO^-$ . Further information into  $ONOO^-$  increase with TG stimulation could be obtained through an ELISA sensitive for  $ONOO^-$  [304].

Increased  $O_2^-$  production with TG stimulation is in itself interesting. sEnd.1 cells have previously been reported as having ‘coupled’ eNOS due to sufficient DHFR to maintain  $BH_4 > BH_2$  [330]. Is the increase in  $O_2^-$  TG specific or are sEnd.1 cells more liable to eNOS ‘uncoupling’ than previously thought? Although this thesis

does not address these questions, HPLC detection of  $\text{BH}_4$  and  $\text{BH}_2$  levels after TG treatment in sEnd.1 cells would help clarify their altered levels of  $\text{BH}_4$  and  $\text{BH}_2$  [57]. In addition detection of  $\text{O}_2^-$  and  $\text{BH}_4$  in sEnd.1 and other endothelial cell models with both TG and physiological agonists would assess the role of TG in inducing oxidative stress in endothelial cells. The ability of  $\text{Ca}^{2+}$  influx through CRAC channels to increase endothelial oxidative stress could be interesting in disease. Consideration of animal models of vascular disease states may reveal a role for increased CRAC channel dependent  $\text{O}_2^-$  production in the emergence of endothelial dysfunction.

#### 5.4.2 Temperature dependency of eNOS activation

The presence of NO synthesis at physiological temperature but absence at ambient temperature is intriguing. Increased  $\text{Ca}^{2+}$  conductance was hypothesised to be responsible for this dichotomy. Investigation of CRAC channel  $\text{Ca}^{2+}$  conductance revealed  $\text{Ca}^{2+}$  entry to be increased at 37°C. However, response was less sustained due to enhanced  $\text{Ca}^{2+}$  removal primarily by PMCA. Increased temperature therefore, amplifies local  $\text{Ca}^{2+}$  microdomains, but diminishes bulk  $\text{Ca}^{2+}$  rise. Activation of eNOS through enhancement of  $\text{Ca}^{2+}$  microdomains would support a study by Lin et al which suggested a requirement of local  $\text{Ca}^{2+}$  in eNOS activation [197]. The rate of  $\text{Ca}^{2+}$  entry in response to ATP at 37°C, although increased, still did not reach that of TG-induced  $\text{Ca}^{2+}$  entry at room temperature, which reflects sub-maximal store depletion by ATP. If, increased  $\text{Ca}^{2+}$  entry was the critical determinant of NO production at physiological temperature then ATP would only support NO production by enhanced microdomains. However, CRAC

channel microdomains have been reported to reach low  $\mu\text{M}$  concentration at room temperature [257]. A study has established that  $\text{Ca}^{2+}$  concentration above 300nM provides a close to optimal binding of  $\text{Ca}^{2+}$ -CaM to eNOS, and the  $\text{EC}_{50}$  of eNOS for  $\text{Ca}^{2+}$  is 200nM [34, 218]. Local  $\text{Ca}^{2+}$  rise would have reached these concentrations at ambient temperature, so increased local  $\text{Ca}^{2+}$  alone cannot explain the temperature-dependence of NO production by eNOS.

To test if increased  $\text{Ca}^{2+}$  entry was sufficient to allow NO production at room temperature, Orai-1 and STIM-1 were overexpressed in sEnd.1 cells. Transfected cells were stimulated, in the presence of 5mM external  $\text{Ca}^{2+}$ , with ionomycin (1 $\mu\text{M}$ ). Treatment resulted in a small increase in DAF-2 fluorescence with a  $\Delta F$  of only 0.06 (data not shown) ( $n=7$ ), 10-fold lower than that commonly seen at physiological temperature. Larger rises in  $\text{Ca}^{2+}$  in this protocol, still failed to increase NO production to the levels induced at physiological temperature. This strongly suggests that increased  $\text{Ca}^{2+}$  influxes seen with temperature change are not solely responsible for temperature-induced changes in NO production.

Both PMCA and eNOS are CaM-dependent [35, 104, 269]. Release of eNOS from the inhibitory caveolin by binding of  $\text{Ca}^{2+}$ -CaM activates NO production by facilitating electron flow through the homodimer [103]. The widely-expressed PMCA 1 and 4, have low affinity for CaM, however CaM binding increases PMCA affinity for  $\text{Ca}^{2+}$  by one or two orders of magnitude [35]. I considered whether increased PMCA activity at 37°C (as shown in Fig.5.4D) may be indicative of increased concentrations of  $\text{Ca}^{2+}$ -CaM, or enhanced diffusion within the cell, at physiological temperature, and therefore responsible for NO production at higher temperatures. However, if increased concentrations of  $\text{Ca}^{2+}$ -CaM were responsible for the pres-

ence of NO production at physiological temperature, then increased intracellular  $\text{Ca}^{2+}$  with the overexpression of Orai and STIM in sEnd.1, as previously described, should have been sufficient to activate NO production. Furthermore, the diffusion of  $\text{Ca}^{2+}$ -CaM to sites of action is unlikely to be significantly enhanced by the temperature change from ambient to physiological temperature, with the  $Q_{10}$  of CaM diffusion only  $\sim 1.3$ . Diffusion of  $\text{Ca}^{2+}$ -CaM to sites of eNOS is therefore unlikely to be significantly enhanced by the temperature increase within these experiments. It is likely that PMCA conductance itself is also increased at 37, therefore explaining the dramatically enhanced extrusion, rather than a sole effect of  $\text{Ca}^{2+}$ -CaM

With increased rate of  $\text{Ca}^{2+}$  entry, and enhanced CaM activity and diffusion, all unlikely to solely mediate eNOS activation at physiological temperature, the catalytic activity of eNOS itself was considered. Both the flow of electrons through NOS enzymes, and release of NO after synthesis have been shown to be critical processes in NOS activity. Inability to release NO results in a futile cycle which generates nitrate, and binding of  $\text{BH}_2$  results in  $\text{O}_2^-$  production, due to uncoupling of electron transfer. Could non-physiological temperatures uncouple the flow of electrons or prevent release of NO? Difficulties in eNOS catalysis of NO production seems unlikely. In the literature, NOS biosynthetic mechanism, and calculation of NOS rate constants, are investigated at temperatures ranging between 10°C and 25°C [3, 296]. With complete synthesis and release of NO at these temperatures NOS remains coupled in the presence of sufficient co-factors, and substrate. Further to this, a study demonstrated that both iNOS and nNOS were sensitive to temperature changes *in vitro*, increasing  $V_{\max}$  up to 2-fold with a 12°C rise in tem-



perature. eNOS however was found to be temperature insensitive in the presence of sufficient L-arginine, which would have been met under the experimental conditions used [345]. Although the direct catalytic activity of eNOS has been shown to be temperature-independent, this work was carried out on purified enzymes and may not completely reflect changes in eNOS activity *in vivo* or even *in vitro*. Experimental conditions used to investigate purified enzymes provide co-factors, binding proteins, substrate and  $\text{Ca}^{2+}$  in excess, which do not reconstitute the complexities of the internal cell environment. A study has shown a role for eNOS *in vivo* in the vasodilatory response to local temperature increase in skin [166]. The mechanism for this activation is currently unknown.

It is possible that the increased eNOS activity at higher temperatures may also be dependent on enhanced activity of kinases and phosphatases, and other regulators of eNOS post-translational modification. A combination of increased  $\text{Ca}^{2+}$  entry, CaM activity, and, post-translational modifications could cumulatively result in eNOS temperature-dependent activity. Indeed, small increases in phosphatase activity could have large effects on eNOS activation. Phosphorylation of eNOS on Thr495 limits the enzyme's activity by up to 75%, however saturating levels of  $\text{Ca}^{2+}$  supports full CaM binding even with Thr495 phosphorylation [218, 266]. Slower de-phosphorylation of this residue at room temperature would limit the ability of eNOS to bind  $\text{Ca}^{2+}$ -CaM, during non-maximal  $\text{Ca}^{2+}$  transients. This could, in effect, uncouple eNOS from the rise in  $\text{Ca}^{2+}$  and formation of  $\text{Ca}^{2+}$ -CaM. This model would explain the small increase in NO production observed at room temperature with ionomycin, which although exhibiting raised  $\text{Ca}^{2+}$  levels, would not reach  $\text{Ca}^{2+}$  saturation, as entry is still occurring through a store-operated

mechanism at a concentration of 1 $\mu$ M ionomycin.

Investigation of the phosphorylation profile of key eNOS residues at ambient and physiological temperature, in particular the time frame taken to observe changes, and use of eNOS mutant constructs could dissect out the cause of this temperature dependency. eNOS temperature dependency will not be further addressed within this thesis. However, the presence of eNOS activity only at physiological temperature highlights the importance of using biologically relevant temperatures in experiments. With Ca<sup>2+</sup> fluxes largely being measured at room temperature, consideration should always be taken of the requirement for physiological temperatures in activating downstream processes.

# Chapter 6

## Temporal control of NO synthesis by CRAC channels

### 6.1 Introduction

Store-operated  $\text{Ca}^{2+}$  entry was first proposed to activate eNOS in 2000 by the Rodman group, and that local  $\text{Ca}^{2+}$  microdomains around SOC channels were responsible for activating eNOS activity. External  $\text{Ca}^{2+}$  was varied to evoke similar global  $\text{Ca}^{2+}$  rises in TG- and ionomycin-treated cells. Despite similar global  $\text{Ca}^{2+}$  rises, TG evoked a much larger increase in eNOS activity than ionomycin, which led the authors to propose a role for local  $\text{Ca}^{2+}$  entry near CRAC channels [197]. However, ionomycin depletes stores and activates CRAC channels in addition to its direct ionophore action in transporting  $\text{Ca}^{2+}$  across the plasma membrane, and so it is puzzling that Rodman saw a difference between TG and ionomycin. Nevertheless, the requirement of local  $\text{Ca}^{2+}$  microdomains in activating eNOS fits

with the intracellular distribution of eNOS. Inactive eNOS is tethered to the membrane through a combination of post-translational modifications: palmitoylation and myristoylation, and interaction with the inhibitory protein caveolin, which provides tonic inhibition of eNOS under basal conditions [104, 162, 269]. Upon influx of  $\text{Ca}^{2+}$ , the  $\text{Ca}^{2+}$ -CaM complex is formed which competes with caveolin for binding of eNOS, thus removing inhibition. As a binding partner of both Orai-1 and STIM-1, CaM is located close to the CRAC channel, allowing rapid formation of  $\text{Ca}^{2+}$ -CaM upon the formation of  $\text{Ca}^{2+}$  microdomains around open CRAC channels [16, 238]. The transmission of local  $\text{Ca}^{2+}$  signals in this manner to relieve tonic inhibition provides a mechanistic explanation for the data presented by the Rodman group [197]. Furthermore there is precedence for  $\text{Ca}^{2+}$ -CaM to relay local  $\text{Ca}^{2+}$  signals to activate a downstream process which is spatially segregated. The CRAC channel regulation of NFAT translocation to the nucleus is controlled by CaM-dependent calcineurin dephosphorylation of NFAT. The binding of CaM to Orai has recently been revealed to facilitate this process [165]. Localisation of eNOS at the plasma membrane and cis-Golgi bodies is required for normal eNOS production. Cytosol-localised mutants show reduced NO production, indicating that eNOS needs to be close to the site of  $\text{Ca}^{2+}$  influx to be activated [110].

However, a contribution by bulk  $\text{Ca}^{2+}$  rise in eNOS activation would not necessarily be surprising. The regulation of eNOS localisation is dynamic. eNOS is released from the plasma membrane during activation, allowing inhibitory post-translational modifications, such as nitrosylation, to be reversed [86]. Changes in eNOS localisation exposes the enzyme to different  $\text{Ca}^{2+}$  environments. Maintaining  $\text{Ca}^{2+}$ -CaM in the cytosol would be dependent on the bulk  $\text{Ca}^{2+}$  rise. A role for

both local and global  $\text{Ca}^{2+}$  may therefore be necessary to sustain eNOS activation.

This chapter aims to confirm the CRAC channel dependency of eNOS activation, and further to this the role of both local and global  $\text{Ca}^{2+}$  in eNOS activation. I shall also begin to define post-translational modifications of eNOS induced  $\text{Ca}^{2+}$  signalling through CRAC channels.

## 6.2 Results

### 6.2.1 Store-operated $\text{Ca}^{2+}$ entry and not $\text{Ca}^{2+}$ store release activates eNOS

It has previously been shown that  $\text{Ca}^{2+}$  store release is not sufficient to induce NOS-dependent NO production in endothelial cells [197]. To confirm this in sEnd.1 cells, I compared NO production in DAF-2-DA-loaded cells after challenge with TG ( $2\mu\text{M}$ ) in either the presence or absence of external  $\text{Ca}^{2+}$  (Fig.6.1A). Store-release in the absence of external  $\text{Ca}^{2+}$  entry failed to induce detectable NO production (blue trace Fig.6.1A).

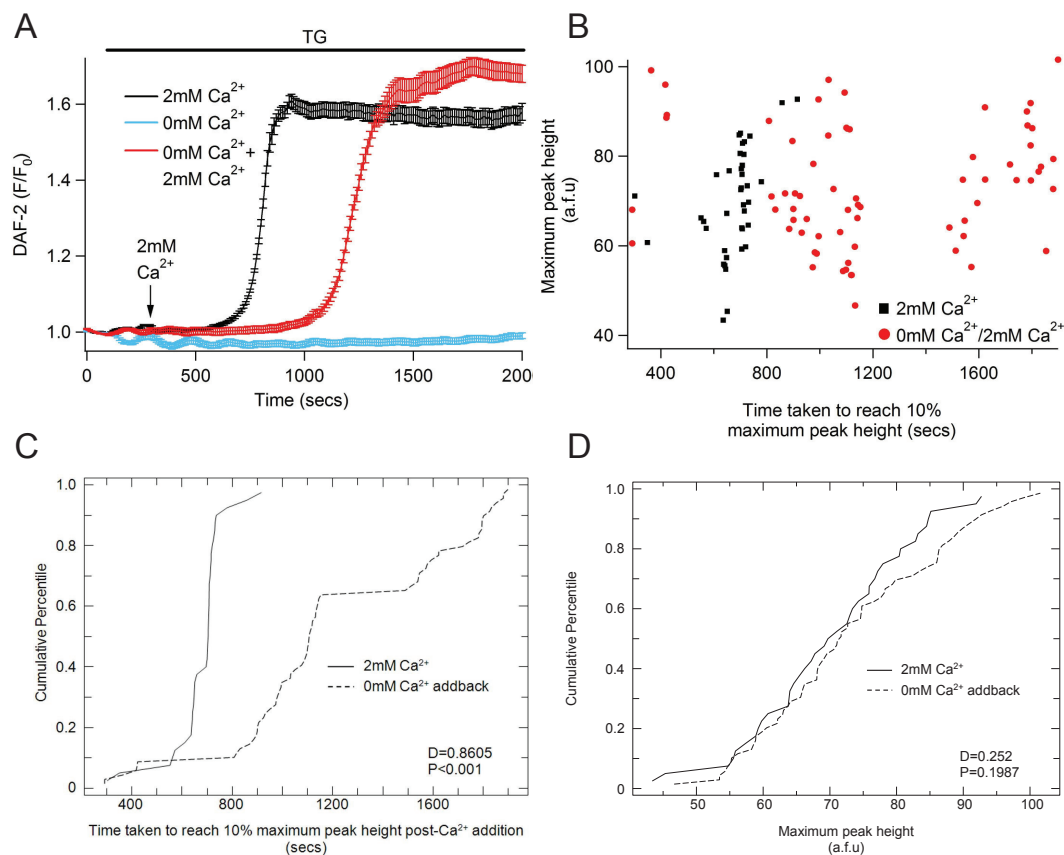
The dissection of  $\text{Ca}^{2+}$  release from  $\text{Ca}^{2+}$  entry, as previously described in chapter 3, was performed to determine whether  $\text{Ca}^{2+}$  entry could stimulate NO production after release had terminated.  $\text{Ca}^{2+}$  was readmitted 200secs after store depletion by TG. Addition of external  $\text{Ca}^{2+}$  now resulted in NO production (red trace Fig.6.1A). In the continuous presence of external  $\text{Ca}^{2+}$  TG stimulation resulted in an increase in NO production as previously shown (black trace Fig.6.1A).

DAF-2 reacts irreversibly with NO to form the fluorescent DAF-2-triazole. The total change in fluorescence from baseline to maximum peak can therefore be taken as a measure of the total quantity of NO produced within the experiment, and can be used as a measure of eNOS activity. The total NO production was unchanged by dissecting  $\text{Ca}^{2+}$  store release from  $\text{Ca}^{2+}$  entry (Fig. 6.1D). Although, considering the increase in  $\text{O}_2^-$  upon TG stimulation this should be treated with caution. In order to determine if dissection of  $\text{Ca}^{2+}$  store release and  $\text{Ca}^{2+}$  entry affected

NO production, total NO produced and changes in the time taken to reach fast production of NO were investigated.

The DAF-2 detected profile of NO production was distinctive, as previously discussed in chapter 5, showing a sigmoidal shape, consisting of a slow activation phase, fast production phase, and deactivation of enzyme activity (Fig.6.1A). The time taken to reach the fast production phase was variable even within experiments under the same conditions. To quantify this, I measured the time taken to reach 10% of the total NO production with TG application taken as  $t=0$ . This can be considered the onset of the fast production phase, correlating with a rapid increase in DAF fluorescence. This time will also be referred to as the ‘NO response time’ within this thesis.

In cells stimulated with TG in presence of  $\text{Ca}^{2+}$ , the NO response time is taken from the point where both TG and external  $\text{Ca}^{2+}$  were present. When  $\text{Ca}^{2+}$  entry was dissected from store release, this was taken as the point of external  $\text{Ca}^{2+}$  addition. The NO response time for different cells is compared in Fig.6.1B. There was a clear tendency for the time to be longer and more variable in experiments where  $\text{Ca}^{2+}$  was readmitted after store depletion, compared with TG stimulation in the continuous presence of 2mM external  $\text{Ca}^{2+}$ . This increased time to activation suggests a requirement for simultaneous store release and entry, which may be indicative of a requirement for bulk  $\text{Ca}^{2+}$  rise in the sustained activation of eNOS.



**Figure 6.1**  $\text{Ca}^{2+}$  entry is required to activate eNOS. Simultaneous store release and  $\text{Ca}^{2+}$  entry is necessary to reduce time lag to eNOS activation.

(A) A representative experiment; 2  $\mu\text{M}$  TG administered at 100secs in presence (black) or absence of external  $\text{Ca}^{2+}$  (blue and red). External  $\text{Ca}^{2+}$  was added 200secs after TG to separate the requirement of external  $\text{Ca}^{2+}$  entry from store release (red). NO production is induced by the addition of external  $\text{Ca}^{2+}$ , with store depletion alone being insufficient to activate eNOS (*control*  $n=39$ ; *absence of  $\text{Ca}^{2+}$*   $n=66$ ). (B) Time taken to reach 10% maximum peak height demonstrated no correlation within either group. (C) Add-back of  $\text{Ca}^{2+}$  post-TG administration increased time taken to reach 10% maximum peak height (taken from point of  $\text{Ca}^{2+}$  add-back). Separation of store release and  $\text{Ca}^{2+}$  addback increased the range of times taken to reach eNOS activation. (D) Distribution of maximum peak height was not different between control cells treated in 2mM external  $\text{Ca}^{2+}$  and cells where external  $\text{Ca}^{2+}$  was readmitted (*control*  $n=39$ ;  *$\text{Ca}^{2+}$  add-back*  $n=68$ ).

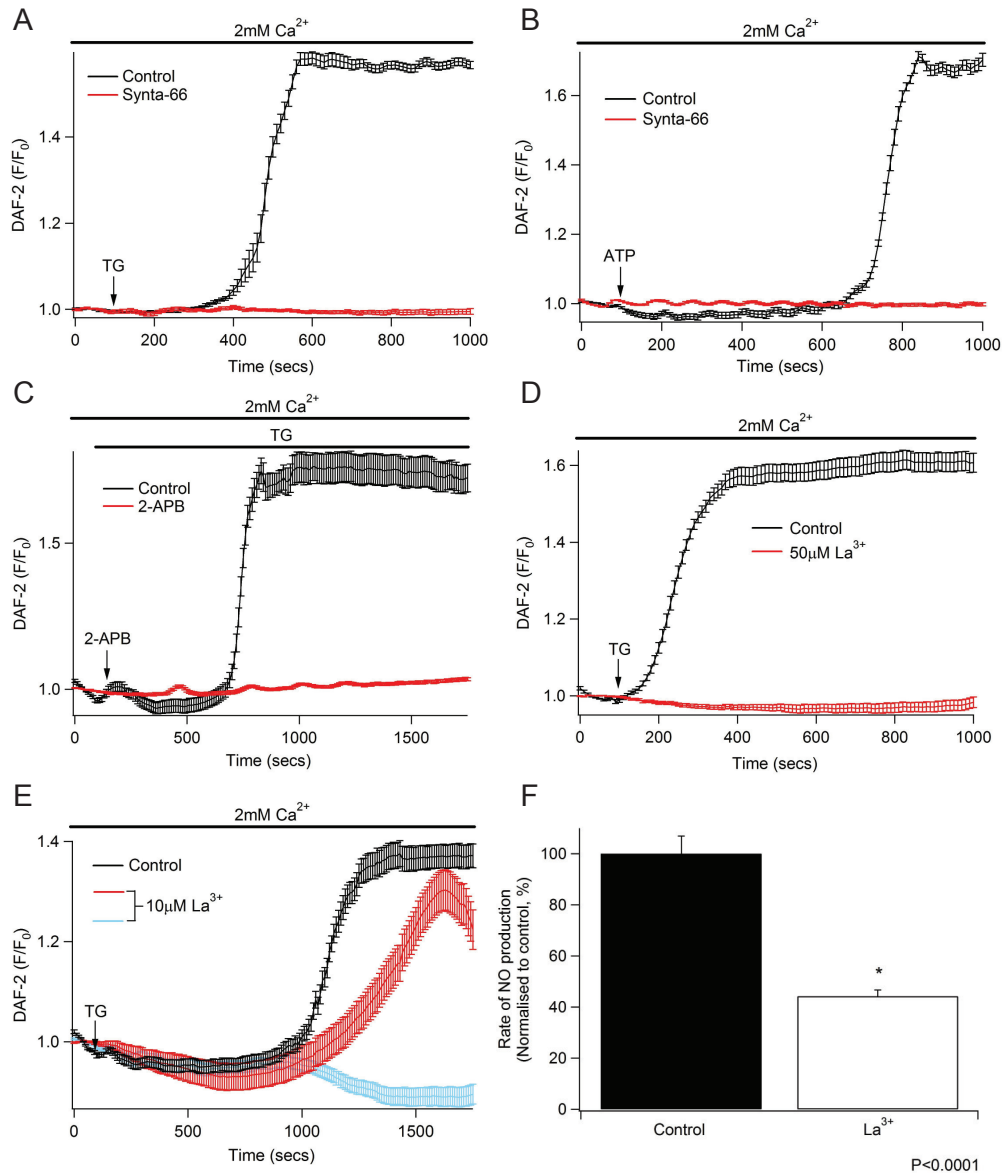


### 6.2.2 Inhibition of the CRAC channel blocks eNOS-dependent NO production

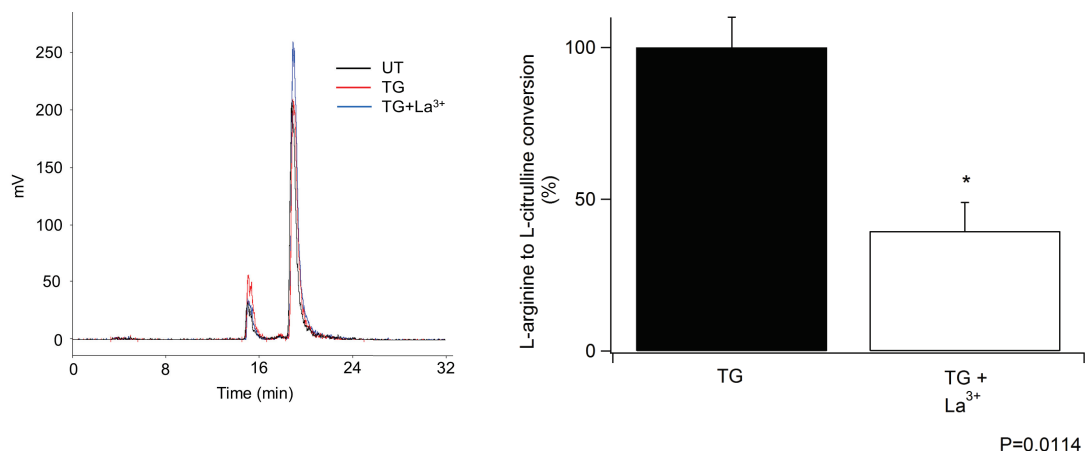
Store-operated  $\text{Ca}^{2+}$  entry clearly activates the production of NO in sEnd.1 cells, as shown by both NO imaging and a  $^{14}\text{C}$ -L-arginine conversion assay (see Fig.5.1 and 5.2). Furthermore, I have previously described in this thesis the effect of known pharmacological inhibitors of CRAC channels, and used these to help define sEnd.1 SOC channels as CRAC channels. Here, I utilise the inhibitors Synta-66, 2-APB, and  $\text{La}^{3+}$ , to demonstrate the CRAC channel-dependency of eNOS production of NO.

sEnd.1 cells were pre-treated with Synta-66 (10 $\mu\text{M}$ ), 2-APB (20 $\mu\text{M}$ ), and  $\text{La}^{3+}$  (50 $\mu\text{M}$  and 10 $\mu\text{M}$ ), which reduced  $\text{Ca}^{2+}$  entry after TG treatment by 50%, 65%, 72% and 34% respectively. DAF-2 loaded sEnd.1 cells were treated with Synta-66, 2-APB or 50 $\mu\text{M}$   $\text{La}^{3+}$  prior to application of TG. All three inhibitors completely abolished NO production within the imaging time (1400 secs from stimulation) (Fig.6.2A,C-E). Furthermore, Synta-66 treatment also abolished agonist-induced NO production (Fig.6.2B). Interestingly, 10 $\mu\text{M}$   $\text{La}^{3+}$  had a variable effect on NO production, either completely blocking (22/29 cells) or partially reducing the rate of NO production (7/29 cells) by  $56\% \pm 3\%$  (Fig.6.2E-F).

To further confirm that pharmacological inhibition reduces eNOS activity, I addressed the effect of CRAC channel inhibition with  $\text{La}^{3+}$  on the conversion of  $^{14}\text{C}$ -L-arginine to L-citrulline. Pre-incubation with  $\text{La}^{3+}$  ( $50\mu\text{M}$ ) for 5mins prior to TG stimulation reduced conversion of  $^{14}\text{C}$ -L-arginine to L-citrulline by  $60\%\pm 9\%$  (Fig.6.3). The conversion of  $^{14}\text{C}$ -L-arginine to L-citrulline in untreated cells did not differ from TG-stimulated cells pre-treated with  $\text{La}^{3+}$  (data not shown), indicating that  $\text{Ca}^{2+}$  increase-dependent portion of NO production was fully inhibited. This further demonstrates the involvement of CRAC channels in activation of NO production.



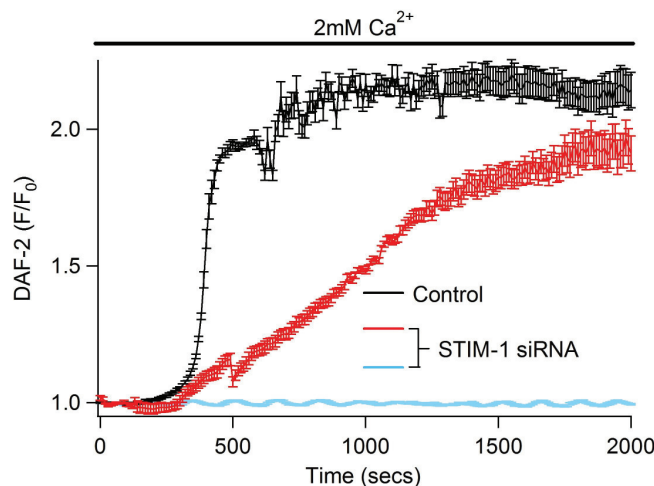
**Figure 6.2 CRAC channel inhibitors abrogate increased NO production.** (A-B) Pre-incubation (8mins) with CRAC channel inhibitor Synta-66 (10μ) inhibits eNOS activation elicited by TG (*control*  $n=53$ ; *Synta*  $n=51$ ) and ATP (*control*  $n=53$ ; *Synta*  $n=39$ ). (C) 20μM 2-APB added 50secs post-TG stimulation blocks increase in eNOS activity (*control*  $n=24$ ; *2-APB*  $n=29$ ). (D) Pre-incubation (5mins) with 50μM La<sup>3+</sup> completely blocks NO response in all cells (*control*  $n=45$ ; *50μM*  $n=21$ ). (E-F) Administration of 10μM La<sup>3+</sup> (5min pre-incubation) completely blocked (blue) ( $n=22$ ) or reduced the rate of NO production (red) ( $n=7$ ) compared to TG-stimulated control ( $n=35$ ). The rate of NO production was calculated only from La<sup>3+</sup>-treated cells, which responded to TG treatment.



**Figure 6.3 Treatment with La<sup>3+</sup> reduces TG-induced <sup>14</sup>C-L-arginine conversion to L-citrulline**

(A) Representative HPLC traces. <sup>14</sup>C-labelled products of intracellular metabolism of <sup>14</sup>C-L-arginine elute at different times. The elution times of detectable products are: urea (5mins), ornithine (19mins), citrulline (15.5mins) and remaining arginine (21mins). (B) 50μM La<sup>3+</sup> treatment reduced <sup>14</sup>C-L-arginine conversion to 40%±9% of TG-treated control cells (n=3).

The effect of STIM-1 knock-down in sEnd.1 cells on NO production was also investigated using DAF-2. Due to the fluorescence overlap of DAF-2-DA with commonly used fluorescent proteins, cells were not co-transfected with GFP and siRNA against STIM-1. It has previously been established in this thesis that the transfection efficiency of 37%±11%, and that knock-down of STIM-1 (30nM STIM-1 siRNA) yielded a 67% reduction in Ca<sup>2+</sup> entry. The detection of NO production by DAF-2 in STIM-1 knock-down sEnd.1 cells showed either reduced the rate of NO production (28/37 cells) or completely abolished the response (9/37) (Fig.6.4). The time taken to reach response could not be quantified, as some cells had not reached a plateau by the end of the experimental time frame, however, STIM-1 knock-down had a clear effect on the rate of production.

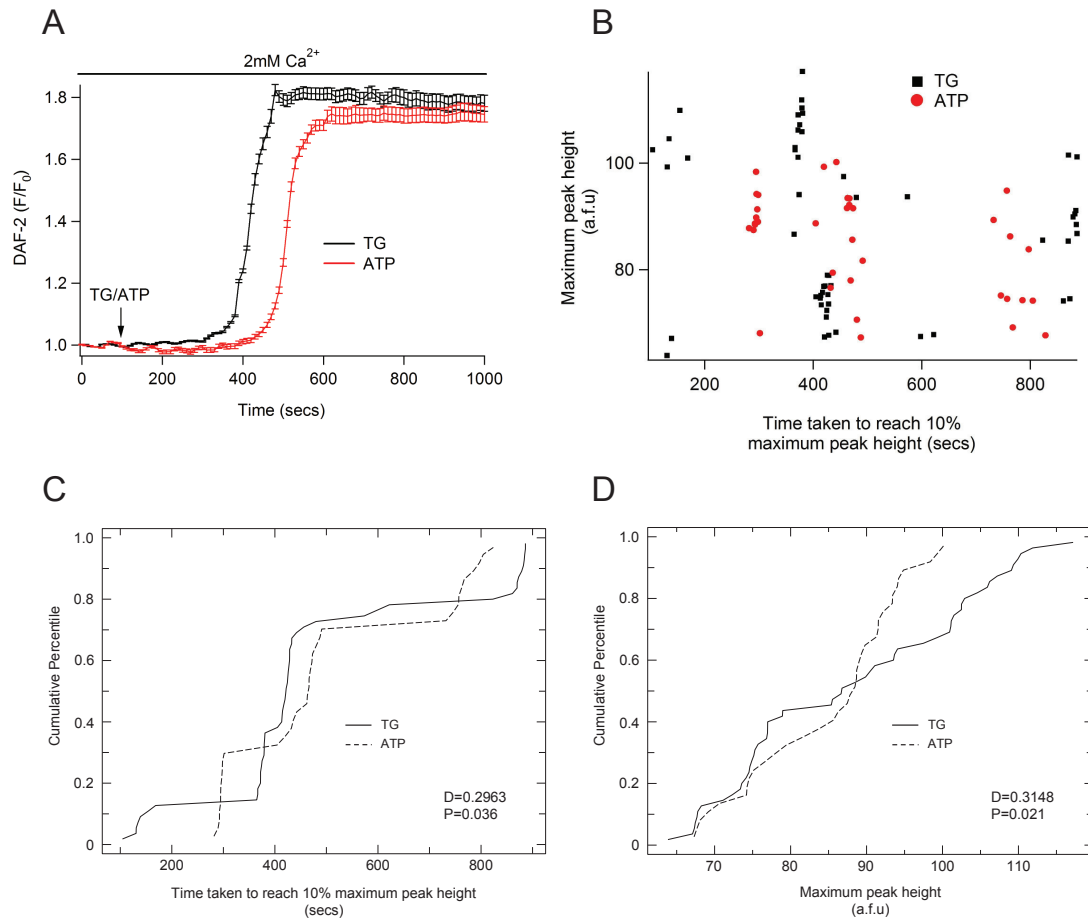


**Figure 6.4 Knockdown of CRAC channel component STIM-1 limits the ability of SOCE agonists to increase NO production** A representative experiment. 30nM STIM-1 siRNA completely abolished or reduced the rate of NO production in sEnd.1 (*control*  $n=30$ ; *STIM-1 KD responded*  $n=28$ ; *STIM-1 KD no response*  $n=9$ ).

NO production was more sensitive to CRAC channel inhibitors than  $\text{Ca}^{2+}$  entry itself. This is consistent with a high  $\text{Ca}^{2+}$  threshold for eNOS activation [335]. Two interesting results, however, were the effects of knockdown of STIM-1 and the use of a sub-maximal dose of inhibitor  $\text{La}^{3+}$  ( $10\mu\text{M}$ ). Instead of complete abrogation of NO production or a shift in the time taken to reach activation of eNOS, both conditions resulted in a lower rate of NO production. A sub-maximal level of eNOS activation may be useful in helping to determine a model for the  $\text{Ca}^{2+}$  requirements of eNOS. This will be further discussed later in this chapter.

### 6.2.3 ATP is less effective at activating NO production than TG

TG and ATP stimulation yield distinctly different intracellular  $\text{Ca}^{2+}$  signals. Incomplete store depletion by ATP results in a reduced rate of  $\text{Ca}^{2+}$  entry, due to the activation of fewer CRAC channels.  $\text{Ca}^{2+}$  transients are therefore smaller, and additionally have a shorter duration due to re-uptake of incoming  $\text{Ca}^{2+}$  into ER  $\text{Ca}^{2+}$  stores (Fig.3.6 and 5.5). Differences in  $\text{Ca}^{2+}$  signals have a modest but significant effect on the extent of NO production both in terms of temporal activation and amplitude. The smaller rise in internal  $\text{Ca}^{2+}$  induced by ATP results in delayed response to activation by  $\sim 43$ secs (calculated from differences in medians) (Fig.6.5C). Furthermore stimulation of NO production with ATP reduced the variability in total NO production and results in a smaller total NO production (Fig.6.5D). There was no correlation between maximum peak height and NO response time after either ATP or TG stimulation (Fig.6.5B).



**Figure 6.5 Activation of eNOS is affected by method of store depletion**  
 (A) A representative experiment; TG (2 $\mu$ M) (black) or ATP (5 $\mu$ M) (red) was administered at 100secs in presence of external Ca<sup>2+</sup>. (B) A weak negative correlation between maximum peak height and time taken to reach 10% maximum peak was seen with ATP stimulation ( $R=-0.47$ ;  $P=0.004$ ) (B-D) Distribution of maximum peak heights and time taken to reach 10% maximum peak height was significantly altered between stimulation types. (TG  $n=54$ ; ATP  $n=36$ )

#### 6.2.4 Does local or global Ca<sup>2+</sup> control eNOS activation?

Despite both direct and circumstantial evidence which implicates local Ca<sup>2+</sup> signals in eNOS regulation I have demonstrated that the time taken to activate the

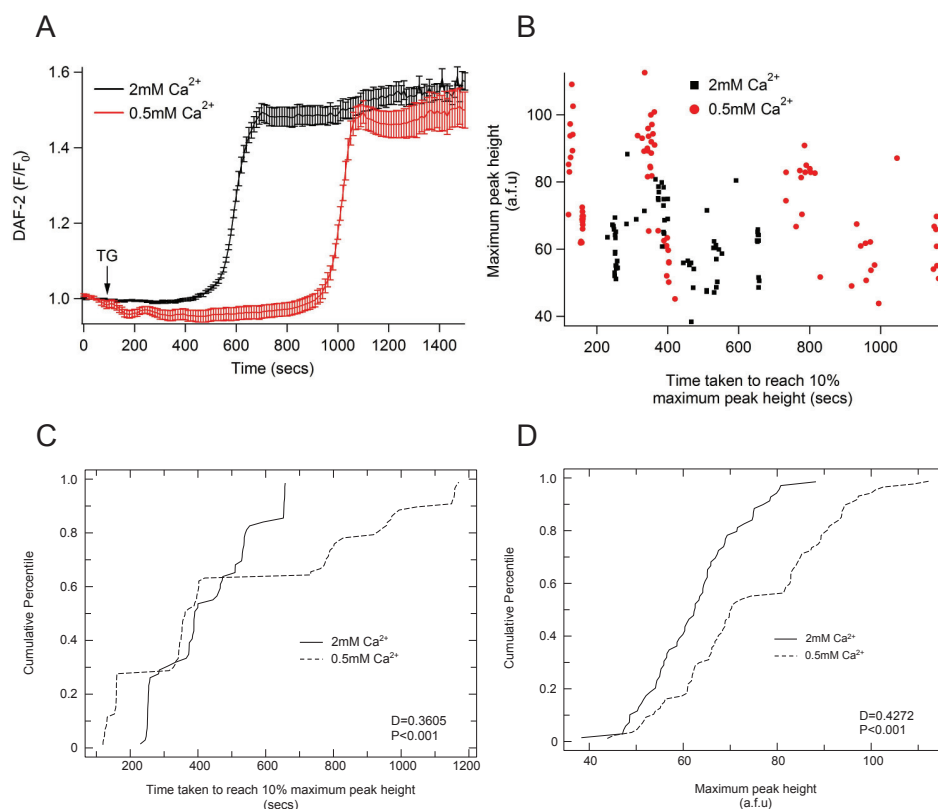
enzyme is affected by whether or not store release and  $\text{Ca}^{2+}$  entry occur contemporaneously. The significant delay in NO response time when external  $\text{Ca}^{2+}$  is reapplied after store depletion by TG indicates a potential role for either bulk  $\text{Ca}^{2+}$  rise or store release itself in the fine regulation of eNOS activity. I have therefore used a variety of methods to manipulate the bulk  $\text{Ca}^{2+}$  rise to distinguish it from local  $\text{Ca}^{2+}$  microdomains.

It has previously been shown in this thesis that reduction of external  $\text{Ca}^{2+}$  to 0.5mM reduced the rate of  $\text{Ca}^{2+}$  entry by 40%, and this was associated with a smaller bulk  $\text{Ca}^{2+}$  rise (see Fig.3.9). Maximum peak amplitude of  $\text{Ca}^{2+}$  entry was compared as a measure of bulk rise. 0.5mM external  $\text{Ca}^{2+}$  showed a  $43\% \pm 10\%$  reduction in amplitude of  $\text{Ca}^{2+}$  entry compared to 2mM  $\text{Ca}^{2+}$ . Reduced total rise in cytosolic  $\text{Ca}^{2+}$  results in quicker return to basal  $\text{Ca}^{2+}$  levels, due to extrusion by the PMCA. It is known that reducing external  $\text{Ca}^{2+}$  also reduces the spatial extent of local  $\text{Ca}^{2+}$  microdomains [257]. Lower external  $\text{Ca}^{2+}$  therefore limits both bulk and local  $\text{Ca}^{2+}$  rises. Stimulation of DAF-2 loaded sEnd.1 with TG in 0.5mM extracellular  $\text{Ca}^{2+}$  increased the spread of NO response time, compared to control replicates in 2mM  $\text{Ca}^{2+}$ . Although time taken to reach the fast phase of NO production was increased, it should be noted that some cells still responded within a similar time frame to control cells, and reducing external  $\text{Ca}^{2+}$ , therefore did not strongly shift the time taken to reach the fast stage of activation (Fig.6.6A-C). Interestingly, the range of peak sizes, used to assess total NO production was increased with reduced external  $\text{Ca}^{2+}$  (Fig.6.6D). With some cells showing large responses in 0.5mM  $\text{Ca}^{2+}$  that were similar in amplitude to those seen in 2mM  $\text{Ca}^{2+}$ . A few cells in 0.5mM  $\text{Ca}^{2+}$  showed very large response, although it is not



clear why. The effect of low extracellular  $\text{Ca}^{2+}$  should be further investigated in future work.

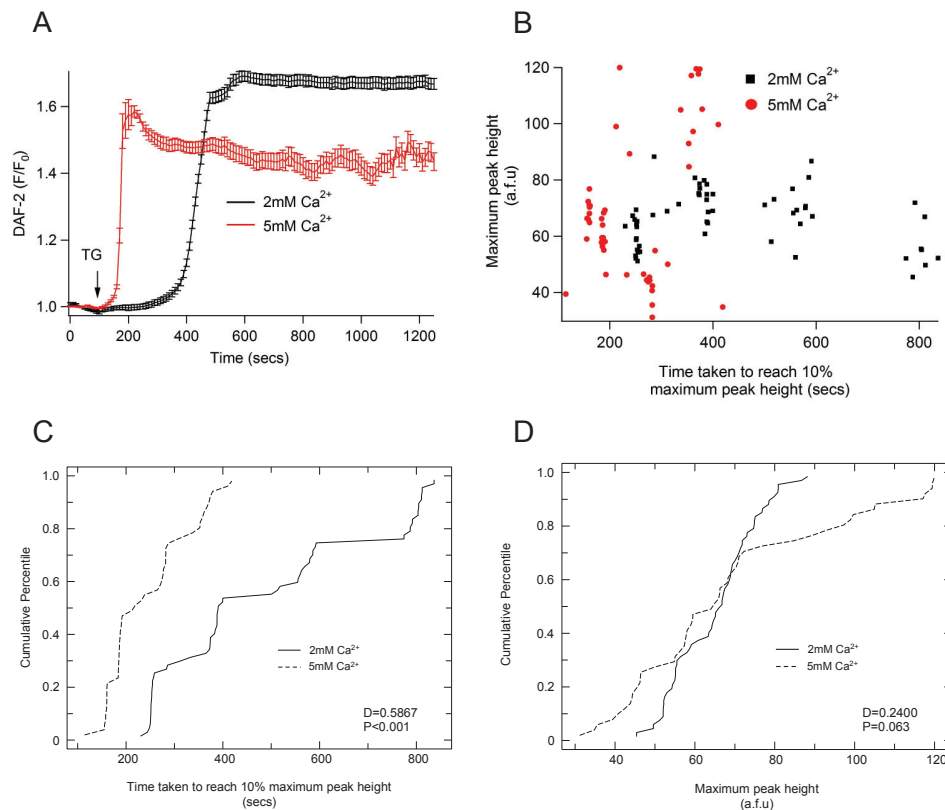
I noted a weak negative correlation between maximum peak height and time taken to reach 10% maximum peak height with both ATP treatment, and TG treatment in presence of 0.5mM  $\text{Ca}^{2+}$ . Both of these conditions have lower bulk  $\text{Ca}^{2+}$  rises. If time to activation is longer then bulk Ca will be lower during rapid NO production and is likely to cease earlier, thus a lower total NO production would correlate with a longer NO response time.



**Figure 6.6** Low extracellular  $\text{Ca}^{2+}$  increases spread of time taken to reach 10% of total NO production.

(A) A representative experiment. (B) Time taken to reach 10% maximum peak height and the maximum peak height demonstrated a weak correlation upon stimulation in presence of 0.5mM external  $\text{Ca}^{2+}$  ( $R=-0.49$ ;  $P;0.01$ ) (C) Low (0.5mM) extracellular  $\text{Ca}^{2+}$  increases the range of activation times, but crucially does not limit the ability of eNOS to activate or reduce the rate of NO production. Differences between medians is 29secs. (D) Lower external  $\text{Ca}^{2+}$  increases total NO production as indicated by maximum peak height (2mM  $\text{Ca}^{2+}$   $n=68$ ; 0.5mM  $\text{Ca}^{2+}$   $n=86$ ).

Elevated external  $\text{Ca}^{2+}$  (5mM) increased the rate of  $\text{Ca}^{2+}$  entry by 25% (see Fig.3.9). High external  $\text{Ca}^{2+}$  will additionally increase the size of lateral spread of  $\text{Ca}^{2+}$  microdomains near open CRAC channels. In contrast to low extracellular  $\text{Ca}^{2+}$ , 5mM  $\text{Ca}^{2+}$  increases both bulk and local  $\text{Ca}^{2+}$  rises. The presence of high extracellular  $\text{Ca}^{2+}$  decreased the time taken to activate eNOS by 175secs (calculated by the difference in medians) (Fig.6.7C). Although the spread of maximum peak heights appears increased in 5mM external  $\text{Ca}^{2+}$  (Fig.6.7D), this does not reach significance. Total NO production was therefore unaffected by an increase in local and bulk  $\text{Ca}^{2+}$  rises, but eNOS activation rates were increased.



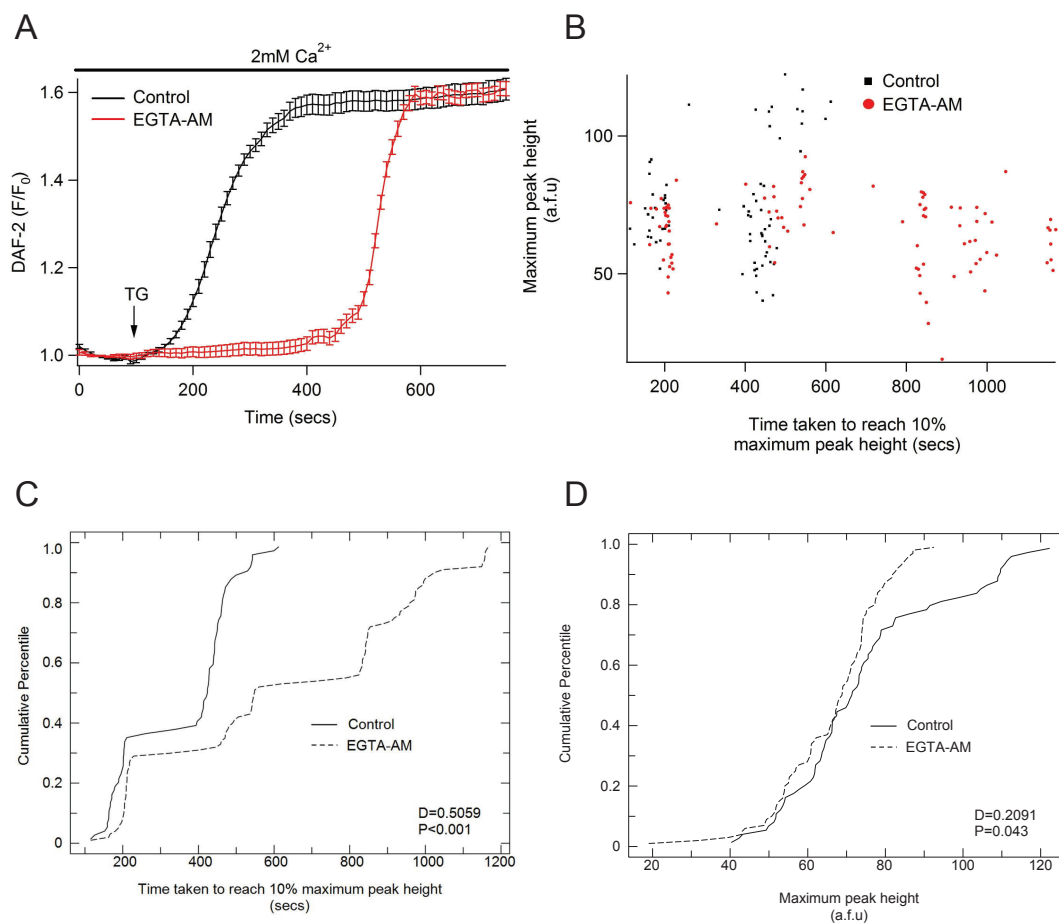
**Figure 6.7 Increased extracellular  $\text{Ca}^{2+}$  decreases time taken to reach 10% total NO production.**

(A) A representative experiment. (B) Time taken to reach 10% maximum peak height and the maximum peak height demonstrated no correlation in either group. (C) Increased extracellular  $\text{Ca}^{2+}$  (5mM  $\text{Ca}^{2+}$ ), and therefore increased entry and global  $\text{Ca}^{2+}$ , reduces time taken to reach 10% total NO production by 175secs (calculated from differences in median) (D) Supra-normal extracellular has no significant effect on total NO production (2mM  $\text{Ca}^{2+}$   $n=57$ ; 5mM  $\text{Ca}^{2+}$   $n=50$ )

EGTA-AM is a slow  $\text{Ca}^{2+}$  chelator, which is passively taken up by cells. The removal of the -AM group by intracellular esterases ensures that EGTA remains intracellularly loaded, in a similar mechanism to Fura-2-AM and DAF-2-DA loading. Intracellular EGTA chelation is too slow to effectively buffer incoming  $\text{Ca}^{2+}$  near an open channel, allowing the formation of normal local  $\text{Ca}^{2+}$  microdomains around open CRAC channels. Crucially however EGTA limits the bulk rise of  $\text{Ca}^{2+}$ , until entering  $\text{Ca}^{2+}$  saturates the pool of chelator [257]. Cells co-loaded with 25 $\mu\text{M}$  EGTA-AM and Fura-2-AM exhibited a slow increase in intracellular  $\text{Ca}^{2+}$  when stimulated with TG (2 $\mu\text{M}$ ) in the presence of 2mM extracellular  $\text{Ca}^{2+}$  (data not shown). Bulk rise reached a maximum amplitude, which was similar to that of EGTA-free control. The time taken to reach a maximum  $\text{Ca}^{2+}$  rise, however, was delayed by  $\sim 200$ secs in EGTA-AM loaded cells. Intracellular  $\text{Ca}^{2+}$  buffering by EGTA did not abolish NO production (Fig.6.8). However, the time taken to reach the fast phase of NO production was delayed consistent to the delayed bulk rise seen in  $\text{Ca}^{2+}$  (Fig.6.8C). EGTA-AM increased time taken to reach NO production by 124secs (difference between medians). A small but significant decrease in maximum peak height was also observed with EGTA. This may indicate a decrease in total NO production (Fig.6.8D).

Cumulatively, the evidence presented in this chapter strongly indicates that bulk  $\text{Ca}^{2+}$  rise contributes to the temporal regulation of the activation of eNOS and NO synthesis. A reduced bulk rise, whilst maintaining normal local  $\text{Ca}^{2+}$  microdomains near the CRAC channels was attained with either EGTA-AM loading, or dissection of store-release from  $\text{Ca}^{2+}$  entry. Although both conditions supported NO production, responses were temporally shifted compared to control. Manip-

ulation of  $\text{Ca}^{2+}$  influx through altering external  $\text{Ca}^{2+}$  with increased (5mM) and decreased (0.5mM)  $\text{Ca}^{2+}$  concentrations, decreased and increased the time taken to reach full eNOS activation respectively. Despite changes in extracellular  $\text{Ca}^{2+}$  affecting both the local  $\text{Ca}^{2+}$  microdomains and bulk  $\text{Ca}^{2+}$  rise, this data continues to support the evidence from EGTA and release-entry dissection data for a role of bulk  $\text{Ca}^{2+}$  rise in the sustained activation of eNOS. Although this work identifies bulk  $\text{Ca}^{2+}$  rise as contributing to eNOS activation particularly the kinetics, it does not contradict the previously reported role for local  $\text{Ca}^{2+}$  [197]. Instead the data may indicate a dual requirement for both bulk and local  $\text{Ca}^{2+}$ . A model for the involvement of both local and global  $\text{Ca}^{2+}$  will be discussed later in this chapter.



**Figure 6.8 Internal  $\text{Ca}^{2+}$  buffering with slow  $\text{Ca}^{2+}$  chelator EGTA increases the time taken to reach an NO response and amplitude of NO production** (A-D) 25 $\mu\text{M}$  EGTA-AM co-loaded with DAF-2-DA (30mins, 37°C), increased the spread of time taken to reach 10% maximum peak height, altered the overall level of response with no significant differences in the spread of maximum peak height. Increased spread of lag time is indicative that a global  $\text{Ca}^{2+}$  rise, rather than local  $\text{Ca}^{2+}$  microdomains, is required for eNOS activation (*control*  $n=73$ ; *EGTA*  $n=99$ ). This is supported by the effects of 0.5mM and 5mM external  $\text{Ca}^{2+}$  on time taken to reach 10% of the maximum peak height.

### 6.2.5 Phosphorylation of Ser1177 regulates the kinetics of eNOS activation

The preceding results show the time taken to initiate NO production, but not the overall amplitude attained can be altered by manipulation of global  $\text{Ca}^{2+}$ . Detection of NO synthesis is temporally dissociated from the  $\text{Ca}^{2+}$  rise in these experiments.  $\text{Ca}^{2+}$  transients at physiological temperature last  $<10$ mins (see Fig.5.4), whereas NO production can be observed over 10mins post-stimulation, consistent with the time frame of tens of minutes to hours used to detect NO in the literature [65,197]. eNOS can therefore retain activity after the increase in  $\text{Ca}^{2+}$  has terminated. eNOS sensitivity and activation is modulated not only by  $\text{Ca}^{2+}$ -CaM, but also by a wide range of additional protein-protein interactions and post-translational modifications, including denitrosylation and phosphorylation [85,216,335]. Return to basal  $\text{Ca}^{2+}$  therefore does not provide a simple on-off regulation to NO production by  $\text{Ca}^{2+}$ -CaM. Phosphorylation is critical in this process, modulating eNOS sensitivity to  $\text{Ca}^{2+}$ -CaM (as previously discussed in section 1.4.6).

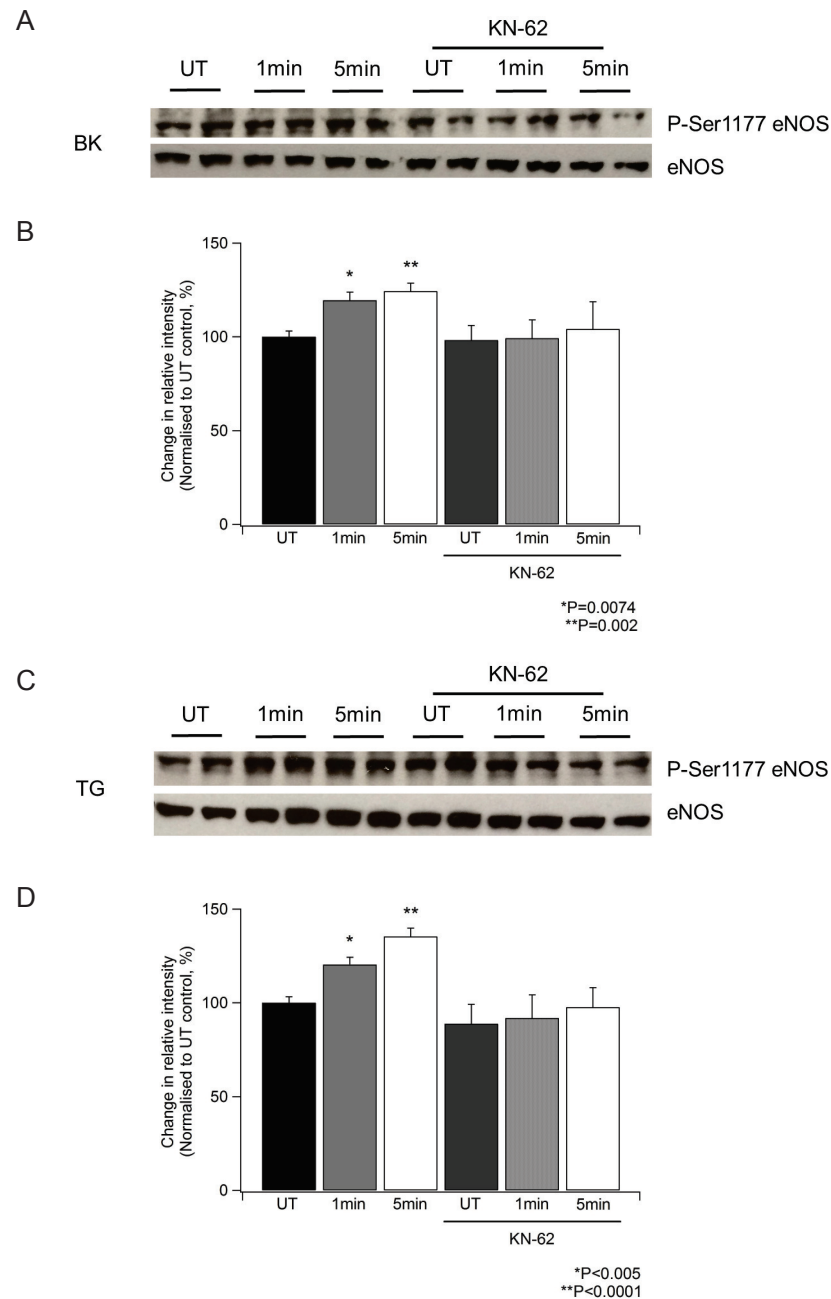
Phosphorylation of residue Ser1177 on eNOS lowers the  $\text{Ca}^{2+}$  dependency of the enzyme, increasing the level of eNOS activation [216]. Various stimulants ranging from BK to shear stress have been shown to increase phosphorylation over a timecourse of 5-10mins, returning to basal levels after 30mins [128]. The reported increase in phosphorylation over this time period correlates well with the time course of NO detection in my experiments. To determine whether eNOS phosphorylation of Ser1177 can affect NO response time, I stimulated sEnd.1 cells with TG



and BK over a 5mins time course and measured phosphorylation. Phosphorylation was determined by Western blot, utilising a phospho-Ser-1177 specific antibody. An increase in phosphorylated eNOS was determined by normalisation to total eNOS. BK has previously been established to increase Ser1177 phosphorylation, and shall be used within these experiments as a positive control for phosphorylation [128]. Although, basal phosphorylation in these cells appeared elevated compared to previous studies in the literature [128], BK (10 $\mu$ M) stimulation of sEnd.1 cells resulted in a 24% increase in phospho-Ser1177 within 5mins (Fig.6.9A-B). Furthermore, stimulation with TG also increased Ser1177 phosphorylation by 35% over 5mins (Fig.6.9C-D).

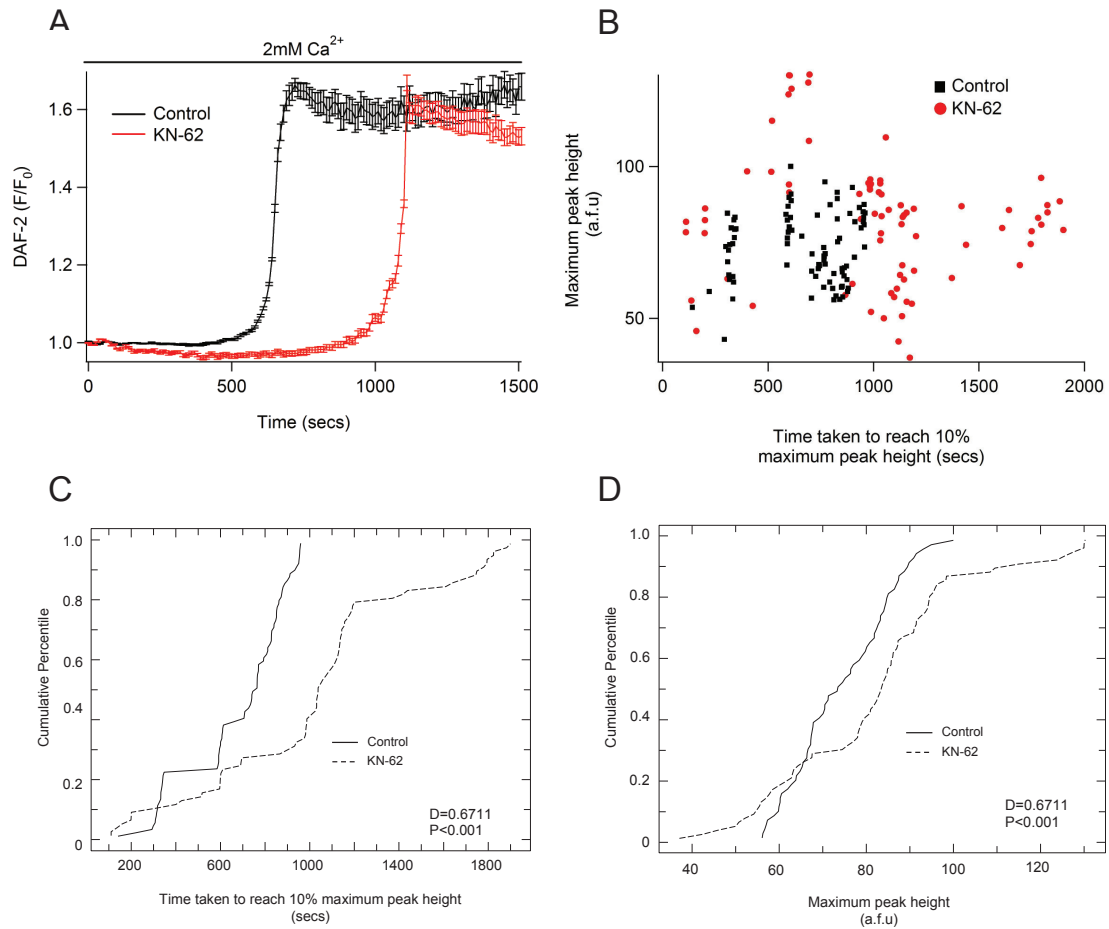
Multiple kinases have been shown to increase Ser1177 phosphorylation including Akt, AMPK, PKA and CaM kinase II (CaMKII) [50, 102, 103, 111]. There is evidence that different kinases target differentially localised eNOS populations, with Akt primarily targeting Golgi-localised eNOS [109]. Akt phosphorylation of Ser1177 is the best characterised, and is readily activated by a multitude of signals from shear stress to VEGF, some of which have been proposed to be Ca<sup>2+</sup>-independent [80, 81]. CaM kinase II is regulated by Ca<sup>2+</sup>-CaM, and its activity is thus regulated by Ca<sup>2+</sup> signalling. To investigate whether Ca<sup>2+</sup> influx through CRAC channels could effect eNOS phosphorylation, the effect of 1-[N,O-bis(1,5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenyl-piperazine (KN-62), a known selective CaM kinase II inhibitor, was investigated [136]. Pre-incubation with KN-62 (3 $\mu$ M) inhibited both agonist and TG-induced increase in Ser1177 phosphorylation. Basal levels of phosphorylation were unaffected by KN-62 treatment (Fig.6.9A-D).

In the presence of KN-62, sEnd.1 still showed eNOS activity, but an increased spread of NO response time was noted (Fig.6.10). The increase of spread demonstrates a role for eNOS phosphorylation by CaMKII in regulating kinetics but not overall extent of the NO response.



**Figure 6.9 KN-62 suppresses increased phosphorylation of eNOS Ser1177 in presence of BK and TG**

(A-B) BK (10 $\mu$ M) increases phosphorylation of Ser1177 (1min 20% $\pm$ 4% increase, 5mins 24% $\pm$ 4% increase) (C-D) TG (2 $\mu$ M) increases phosphorylation of Ser1177 (1min 20% $\pm$ 4% increase, 5mins 35% $\pm$ 4% increase) (A-D) Pre-incubation with KN-62 (3 $\mu$ M) inhibits agonist- and TG-induced phosphorylation of eNOS Ser1177.



**Figure 6.10 CaM kinase II inhibitor KN-62 increases the delay before onset of NO production**

(A-C) Pre-incubation of KN-62 (3 $\mu$ M) increases the time taken to reach 10% maximum peak height by 237secs (difference between medians). (A) A representative experiment. (B) No significant correlation exists between the maximum peak height and NO response time in either control cells or cells pre-treated with KN-62 (*control*  $r=0.1493$ ,  $p=0.23$ ; *KN-62*  $r=0.1419$ ,  $p=0.22$ ) (C) Spread of time taken to reach 10% is increased. (D) The spread of total NO production is increased in presence of inhibitor (*control*  $n=68$ ; *KN-62*  $n=75$ ).

## 6.3 Key findings of this chapter

- $\text{Ca}^{2+}$  entry through CRAC channels is sufficient to activate eNOS
- Temporal regulation of eNOS is dependent on bulk  $\text{Ca}^{2+}$  rise
- Phosphorylation of Ser1177 reduces time lag to eNOS activation

## 6.4 Discussion

Historically, store-operated  $\text{Ca}^{2+}$  entry has been shown to activate NOS-dependent NO production [197]. Subsequent to the discovery of the CRAC channel components, the molecular identity of NOS-activating SOCE was never addressed. Pharmacological inhibition and STIM-1 knockdown with siRNA reduced  $\text{Ca}^{2+}$  entry through CRAC channels and altered NOS activation by TG and ATP. NO production was significantly reduced, demonstrating that CRAC channels are required for eNOS activation.

In this chapter I establish that eNOS is activated by the store-operated CRAC channel, and that  $\text{Ca}^{2+}$  entry is more effective than store release in stimulating NO production [88, 197]. Contrary to previous reports, however, this work has identified a contributing role for bulk  $\text{Ca}^{2+}$  rise in the time course of eNOS activation [197].

Roles of local and global  $\text{Ca}^{2+}$  rises are not mutually exclusive. One can imagine a model in which rises in bulk and local  $\text{Ca}^{2+}$  act in concert to activate eNOS. It is known that CaM can bind both Orai-1 and STIM-1 resulting in fast inactivation

of the CRAC channel [238]. Close localisation of CaM to CRAC channels would facilitate the formation  $\text{Ca}^{2+}$ -CaM upon development of local  $\text{Ca}^{2+}$  microdomains. Subsequently,  $\text{Ca}^{2+}$ -CaM diffusion from the microdomain to sites of action where eNOS is located, would remove CaM from the effects of local  $\text{Ca}^{2+}$  immediately around the CRAC channel. It is known that active eNOS can exist in both the caveolae and in the Golgi [93]. Maintenance of  $\text{Ca}^{2+}$ -CaM is therefore dependent on the  $\text{Ca}^{2+}$  concentration at the sites of CaM action and thus, global intracellular  $\text{Ca}^{2+}$  rise would play a supporting role in  $\text{Ca}^{2+}$ -CaM activated processes which are distant from the  $\text{Ca}^{2+}$  channel. This could therefore explain the requirement of bulk  $\text{Ca}^{2+}$  rise in eNOS activation.

Resting  $\text{Ca}^{2+}$  levels in endothelial cells are 30-100nM, and eNOS activation has previously been shown to have a minimum threshold for  $\text{Ca}^{2+}$  of  $\sim 300\text{nM}$  [6,335,341]. Increase of intracellular  $\text{Ca}^{2+}$  above this threshold would result in a quicker activation of eNOS, with the level of activation dependent on the extent of  $\text{Ca}^{2+}$  rise. The higher the concentration of intracellular  $\text{Ca}^{2+}$ , the lower the requirement for post-translational modifications, which act to reduce the  $\text{Ca}^{2+}$  threshold of eNOS. Although CRAC channel  $\text{Ca}^{2+}$  microdomains have been reported to reach as high as  $5\mu\text{M}$  around CRAC channels, intracellular  $\text{Ca}^{2+}$  buffering rapidly reduces  $\text{Ca}^{2+}$  concentration further into the cytosol [257].  $\text{Ca}^{2+}$  concentrations lower than 300nM would fail to activate eNOS in the absence of post-translational modifications such as phosphorylation. Phosphorylation and de-phosphorylation of certain eNOS residues are known to remove auto-inhibition and often act to increase eNOS binding to  $\text{Ca}^{2+}$ -CaM. This principal is supported by my work, which shows that variations in external  $\text{Ca}^{2+}$  concentrations alter the delay seen before rapid NO

production. Increased external  $\text{Ca}^{2+}$  reduces eNOS response time, potentially circumventing the requirement of post-translational modifications by increasing both bulk and local  $\text{Ca}^{2+}$ , or increasing the rate of phosphorylation/dephosphorylation. Concurrent with this, lower external  $\text{Ca}^{2+}$  increased spread of time taken to reach rapid NO production. It could be argued that lower external  $\text{Ca}^{2+}$  is affecting time taken to reach NO production by multiple mechanisms.  $\text{Ca}^{2+}$  microdomains are reduced, which would limit the formation of  $\text{Ca}^{2+}$ -CaM itself, thereby reducing eNOS activity. Diminished  $\text{Ca}^{2+}$ -CaM formation would also delay processes which reduce the  $\text{Ca}^{2+}$ -sensitivity of eNOS, such as phosphorylation. A smaller bulk rise would not be sufficient to activate eNOS in the absence of post-translational modifications.

The binding of  $\text{Ca}^{2+}$ -CaM to eNOS is complex, not only because of numerous post-translational modifications which alter binding, but additionally for the role eNOS itself plays in formation of  $\text{Ca}^{2+}$ -CaM. CaM binds a total of 4  $\text{Ca}^{2+}$  ions, which results in a replete  $\text{Ca}^{2+}$ -CaM, that is able to facilitate eNOS activity. Partially replete  $\text{Ca}^{2+}$ -CaM does not induce NO synthesis, however is capable of binding to eNOS. The affinity of partially replete CaM for  $\text{Ca}^{2+}$  is increased upon binding to eNOS [218]. This further demonstrates the importance of a bulk rise in  $\text{Ca}^{2+}$  in eNOS activation. Not only is bulk rise important for maintaining  $\text{Ca}^{2+}$ -CaM, but may be important in its formation as well. Crucially, eNOS affinity for CaM is increased with every bound  $\text{Ca}^{2+}$  ion [218]. Release of partially replete  $\text{Ca}^{2+}$ -CaM is therefore quicker than replete. Diminished global  $\text{Ca}^{2+}$  rise and local  $\text{Ca}^{2+}$  microdomains would further perturb this process and result in delayed NO production, as seen with low external  $\text{Ca}^{2+}$ .

It has previously been shown that phosphorylation of multiple residues of eNOS act in concert to alter activity [335]. Post-translational modifications such as the de-phosphorylation of Thr495 aid  $\text{Ca}^{2+}$ -CaM binding at lower  $\text{Ca}^{2+}$  concentrations [50, 103, 266]. These processes are kinetically slow, occurring over a few minutes [81, 102, 103]. Dependency on post-translational modifications for activation would temporally delay eNOS activation, as I observed experimentally. During this period however, small amounts of  $\text{Ca}^{2+}$ -CaM would be able to bind some eNOS transiently, resulting in slow NO production and a lag phase until sufficient activation of the eNOS population is achieved to result in rapid NO production. Phosphorylation of Ser1177 on eNOS is known to increase activity of eNOS two-fold and reduce the  $\text{Ca}^{2+}$  requirements for CaM binding [216, 335]. Indeed, here it has been shown that TG is capable of increasing eNOS residue Ser1177 phosphorylation. Furthermore, inhibition of CaMKII limited phosphorylation of Ser1177 to basal levels, and delayed the onset of a rapid increase in NO production. Interestingly, I noted that basal levels of Ser1177 phosphorylation was high in sEnd.1 cells. The presence of phosphorylation may be involved in the basal production of NO by eNOS in this cell type (see Fig.5.2). The high phosphorylation at rest may be indicative of a non-linear dependence of phosphorylation on activity. Reduction of basal phosphorylation through inhibition of other kinases known to target Ser1177, such as Akt [111], may further perturb activation time, or indeed completely inhibit eNOS activation by TG. Reduction of basal Ser1177 phosphorylation could uncouple  $\text{Ca}^{2+}$  entry from eNOS. The activity of Akt after TG stimulation should be further addressed, as although not directly  $\text{Ca}^{2+}$ -dependent, it has been suggested that CaM-dependent protein kinase kinase can induce Akt activity [111]. If SOCE was further activating Akt activity this would provide even greater reg-



ulation of eNOS activity by CRAC channels. Further work is required to address this.

The involvement of phosphorylation of other eNOS residues has not been addressed in this thesis. A full analysis of TG-induced changes in the eNOS phosphorylation profile is required to completely elucidate the contribution of phosphorylation to CRAC channel-dependent eNOS activation. Phosphomimetics have previously been used in the literature to confirm the effect of phosphorylations on eNOS activity. Mutation of a threonine or serine residue to an aspartate often mimics the effect of phosphorylation, due to the sterically similar structures formed, and the negative charge of aspartate mimicking that of a phosphate group. Conversely, conversion to alanine results in no phosphorylation. Both aspartate and alanine mutants have previously been used to determine a role for eNOS residues Ser1177 and Thr495, establishing them to be critical in modulating  $\text{Ca}^{2+}$ -CaM binding to eNOS [80, 103]. Furthermore, phosphomimetics have been used to investigate downstream processes of eNOS. Endothelial cell migration can be induced by VEGF, and is dependent upon phosphorylation of Ser1177. Use of the Ser1177Asp phosphomimetic rendered migration constitutive rather than induced, and was unchanged by further stimulation of VEGF [80]. Phosphomimetics would further help to define the role of phosphorylation in the time course of eNOS activation. I would expect that inhibitory modifications on all known phosphorylated residues would ablate fast phase NO production.

Experimental results after STIM-1 knock-down provide a strong argument in favour of a model where both local and global  $\text{Ca}^{2+}$  rises are essential for efficient NO production (Fig.6.4). It is known that 2 STIM-1 proteins are required per Orai protein to form a functional CRAC channel; assuming a hexameric structure for the Orai pore [146], 12 STIM-1 proteins are therefore required [141]. Reduced STIM-1 expression results in diminished number of functional CRAC channels, however each functional channel would demonstrate a normal  $\text{Ca}^{2+}$  conductance, and local  $\text{Ca}^{2+}$  microdomains surrounding open CRAC channels would be normal. Both STIM-1 knock-down and EGTA-AM therefore should not reduce local  $\text{Ca}^{2+}$  microdomains, but yet still demonstrate changes in the kinetics of NO production, supporting a requirement for both local and global  $\text{Ca}^{2+}$  rises, which is also indicated by NO responses in 0.5mM external  $\text{Ca}^{2+}$ .

How  $\text{La}^{3+}$  blocks CRAC channels is unknown, due to the need to measure single channel conductance to elucidate the mechanisms of block. With the very low unitary conductance of CRAC channels, measurement of single channel conductance is not possible [1, 272, 379]. Lansman demonstrated that  $\text{La}^{3+}$  block of voltage-gated L-type channels is mediated by slow permeation [185].  $\text{La}^{3+}$  binds to the glutamate ring within the pore, and is slowly conducted through the L-type channel pore, being displaced by in-flowing  $\text{Ca}^{2+}$ . Free influx of  $\text{Ca}^{2+}$  occurs until another  $\text{La}^{3+}$  ion permeates the pore and again temporarily blocks the channel. Slow permeation and intermittent block limits local  $\text{Ca}^{2+}$  microdomains and the subsequent global rise in  $\text{Ca}^{2+}$ . Similarities in conduction selectivity between L-type and CRAC channels make it tempting to assume a similar mechanism occurs during block for CRAC channels [135]. However, mutations of residues in

the TM1-TM2 loop reduce block by  $\text{La}^{3+}$  however E106D which interferes with the Orai pore glutamate ring fails to alter  $\text{La}^{3+}$  block, indicating that  $\text{La}^{3+}$  may inhibit by an alternative mechanism to L-type channels [219]. This work may provide evidence to support an alternative mechanism of inhibition. STIM-1 KD and sub-maximal  $\text{La}^{3+}$  (10 $\mu\text{M}$ ) both reduce  $\text{Ca}^{2+}$  influx by approximately 50% and display similar kinetics in respect of  $\text{Ca}^{2+}$  entry (Fig.3.3, 4.3 and 4.4). Furthermore, they both display a distinctive profile with respect to eNOS activation, reducing rate of production rather than temporally shifting the response (Fig.6.4 and 6.2). The distinctive profile of both STIM-1 knock-down and  $\text{La}^{3+}$  partial block might indicate a similar mechanism.

STIM-1 knock-down reduces the number of active CRAC channels. Partial inhibition of CRAC channels with  $\text{La}^{3+}$  may result in two different outcomes: *i*) full block of some CRAC channels *ii*) partial block of all CRAC channels. A scenario where a portion of the population of open CRAC channels is completely inhibited by sub-maximal  $\text{La}^{3+}$  would result in a similar reduction in total number of channels as STIM-1. CRAC channels have been reported to have up to 1000x lower  $\text{Ca}^{2+}$  conductance than L-type channels [271]. A flickered block is facilitated by displacement of  $\text{La}^{3+}$  by  $\text{Ca}^{2+}$  permeation. Lower channel conductance would therefore limit the displacement by  $\text{Ca}^{2+}$ . This is compounded by the presence of 6 glutamates arranged as a ring in the CRAC channel pore [146]. By contrast, L-type channels have a ring of 4 glutamates, resulting in weaker  $\text{Ca}^{2+}$  binding and a higher conductivity [328]. Such limited displacement in CRAC channels could result in an almost complete block of the channel, therefore reducing total number of CRAC channels available, whilst maintaining normal conductance, and local

microdomains around channels which remain active. This is further supported by early work, which demonstrate that  $\text{La}^{3+}$  does not enter the cell within an experimental time period, when being used to block the channels, as monitored with fluorescent dyes [181]. With the model of  $\text{Ca}^{2+}$  activation of eNOS proposed in this thesis, and the similar NO production profiles of sub-maximal  $\text{La}^{3+}$  (10 $\mu\text{M}$ ) and STIM-1 knock-down demonstrated experimentally in this chapter, a complete block of some CRAC channels by  $\text{La}^{3+}$  is likely.

# Chapter 7

## General Discussion

### 7.1 The CRAC channel as an endothelial mediator of SOCE

Within this thesis, I have established that store-operated  $\text{Ca}^{2+}$  entry in the endothelial cell line sEnd.1 is mediated by CRAC channels, which is in agreement with the findings in alternative endothelial models by other groups [1,4,88,192,323]. The amplitude of  $\text{Ca}^{2+}$  entry through CRAC channels was small in sEnd.1 cells, as was also observed in the literature for other endothelial cells. This is consistent with the fact that endothelial cell  $I_{\text{CRAC}}$  current is notoriously difficult to detect with electrophysiology, requiring divalent-free solutions, which allows the conductance of a fast-inactivating inward  $\text{Na}^+$  current through the channel to be detected. The amplitude of this current was reported as  $\sim 6$  times smaller than that recorded in RBL-1 cells, and furthermore, the authors reported leaky currents after treat-

ment with channel blocker  $\text{Gd}^{3+}$  [1]. Due to difficulties in detection of  $I_{\text{CRAC}}$  in endothelia, the inability to directly detect  $\text{Ca}^{2+}$  currents, and difficulties in patching cells due to the very flat morphology of the endothelial cell, electrophysiology was not attempted in sEnd.1 .

However, the CRAC channel has been extensively profiled in the mast cell model RBL-1 with  $\text{Ca}^{2+}$  imaging and patch clamp, and can be used as a comparison of CRAC channel activity with other cell types.  $\text{Ca}^{2+}$  entry in sEnd.1 cells was sensitive to known pharmacological inhibitors of CRAC channels, including Synta-66, 2-APB,  $\text{La}^{3+}$ , and FCCP, although higher concentrations were generally required to inhibit CRAC channels in sEnd.1 compared to RBL-1 [9, 74, 77, 240]. FCCP additionally demonstrated the regulation of this store-operated  $\text{Ca}^{2+}$  entry by mitochondrial  $\text{Ca}^{2+}$  buffering, indicating that the endothelial SOC channel is sensitive to  $\text{Ca}^{2+}$ -dependent slow inactivation like the CRAC channel. Reduction of STIM-1 protein expression reduced  $\text{Ca}^{2+}$  entry, in agreement with the known gating of the CRAC channel by STIM-1.

Furthermore, CRAC channels in sEnd.1 demonstrate selectivity to  $\text{Ca}^{2+}$  over other divalent ions, including  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$ . In RBL-1 cells, CRAC channel permeability was reported as  $\text{Ca}^{2+} > \text{Sr}^{2+} > \text{Ba}^{2+}$  [142, 379]. sEnd.1 cells showed no difference between the rate of entry of  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$ , which both had significantly diminished conductance compared to  $\text{Ca}^{2+}$ . However,  $\text{Ba}^{2+}$  is insensitive to  $\text{Ca}^{2+}$  extrusion from cells [207].  $\text{Sr}^{2+}$  on the other hand is sensitive to  $\text{Ca}^{2+}$  removal mechanisms, and it was clearly evident that entering  $\text{Sr}^{2+}$  was extruded rapidly. High extrusion rates of  $\text{Sr}^{2+}$  likely limited the perceivable rate of  $\text{Sr}^{2+}$  entry. The specificity of sEnd.1 CRAC channels was therefore similar to that in RBL-1.

Characterisation of the CRAC channel in sEnd.1 has therefore created a new endothelial model system in which to investigate downstream functions of CRAC channel signalling in endothelia. Indeed this thesis has identified one such CRAC channel-activated process.

## 7.2 A model of CRAC channel activation of eNOS

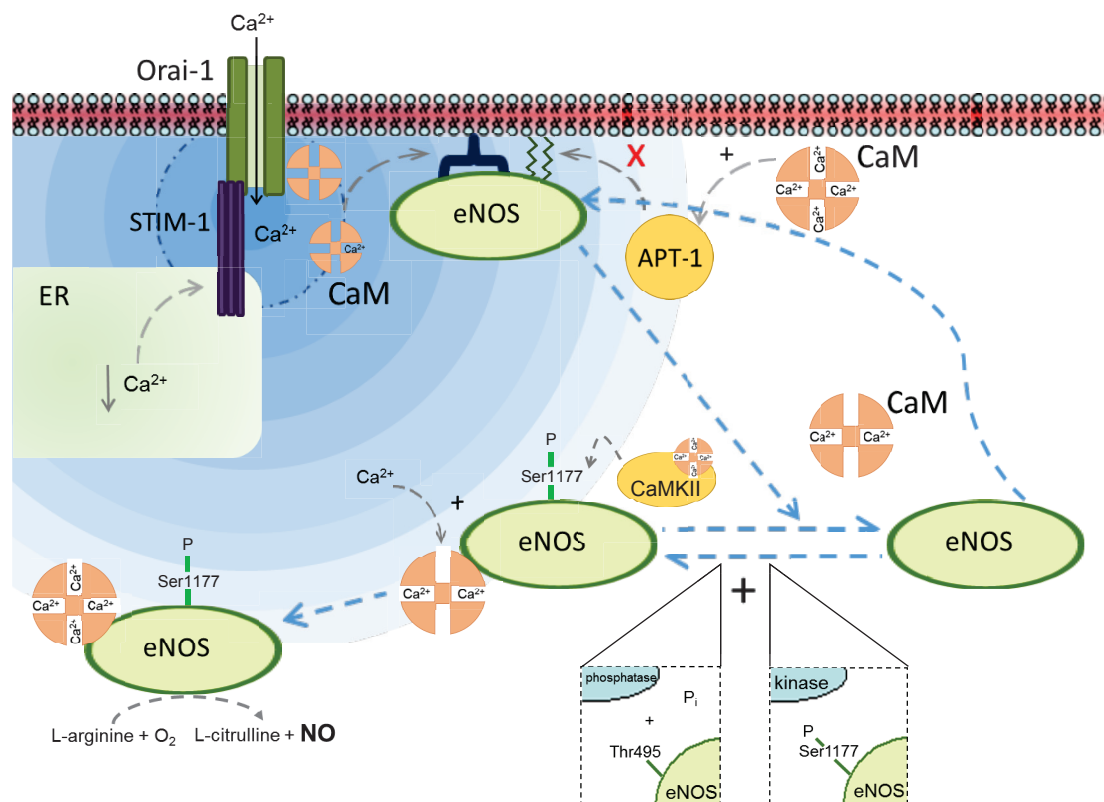
CRAC channel-dependent responses described in endothelia have been typically slow processes occurring over days, such as endothelial cell proliferation [83, 192, 202]. Here I have demonstrated a rapid response to endothelial CRAC channel opening, namely the production of NO. This rapid response may indeed be involved in slower processes, such as control of endothelial proliferation, with NO known to be pro-angiogenic [55].

The regulation of eNOS is intricate and complex, and the remit of this thesis cannot account for all aspects of regulation and protein-protein interactions. The role of  $\text{Ca}^{2+}$ , which exerts control on eNOS regulation at many levels, will be primarily discussed. This thesis has identified a role for both local  $\text{Ca}^{2+}$  microdomains around CRAC channels and a global rise in  $\text{Ca}^{2+}$  in stimulating eNOS. With the primary location of agonist-sensitive eNOS in the plasma membrane, the requirement of both local and global  $\text{Ca}^{2+}$  rises could be puzzling, with caveolae-localised eNOS likely to be within close proximity of local  $\text{Ca}^{2+}$  microdomains around CRAC channels. Indeed targeted eNOS constructs to the caveolae support higher levels of agonist-stimulated NO production than Golgi-localised or free cytosolic eNOS [51, 109]. However, it is well defined in the literature that

eNOS translocates from the caveolae to the Golgi upon agonist-stimulation and increased shear stress [97, 294]. Investigation of eNOS activity using targeted constructs may therefore not be appropriate to study eNOS regulation by subcellular localisation, as they do not recapitulate eNOS trafficking during activation. Release from the caveolae is rapid, occurring within a minute of exposure to  $\text{Ca}^{2+}$ -mobilising agonist [294]. This makes translocation unlikely to be an inhibitory mechanism, as such rapid trafficking would therefore not support sustained NO production. Activation results in dissociation of caveolin-1 and de-palmitoylation of eNOS [93]. Inhibition of palmitoylation has been shown to enhance NO production [354]. This may be due to the presence of palmitoylation enhancing eNOS interaction with caveolin-1, due to the modification directing eNOS to the caveolae [93]. The translocation of eNOS therefore may be key in removing eNOS from inhibitory mechanisms in the membrane such as re-association with the tonic eNOS inhibitor caveolin-1 [93, 126]. Furthermore, it has also been shown that translocation of eNOS facilitates the removal of further inhibitory modifications including S-nitrosylation, which acts as a negative feedback mechanism on the eNOS enzyme [86]. It has also been found that following BK stimulation the cytosolic and Golgi fraction shows markedly higher Ser1177-phosphorylated eNOS, compared to the plasma membrane, which indicates that eNOS is still likely to be active in this lower  $\text{Ca}^{2+}$  environment away from the  $\text{Ca}^{2+}$  microdomains [224]. Recent work in mesenteric arteries further support the requirement for eNOS translocation being required for sustained NO production. eNOS activation by shear stress increased intracellular  $\text{Ca}^{2+}$  and resulted in a rapid translocation to the Golgi, with concomitant increase in phosphorylated eNOS, and sustained NO production, which persisted until the removal of shear stress [97].



eNOS translocation during eNOS activation will place eNOS in multiple different cell environments with varying  $\text{Ca}^{2+}$  levels. Active eNOS will therefore be regulated by both local and global  $\text{Ca}^{2+}$  rises, as indicated within this thesis. The regulation of eNOS by both global and local  $\text{Ca}^{2+}$  can be formulated into a model, as outlined in Fig.7.1.



**Figure 7.1 A model of CRAC channel activation of eNOS** The depletion of ER  $\text{Ca}^{2+}$  stores, opens CRAC channels allowing  $\text{Ca}^{2+}$  influx into cells. Entering  $\text{Ca}^{2+}$  is subjected to internal  $\text{Ca}^{2+}$  buffering, which creates a gradient of intracellular  $\text{Ca}^{2+}$ , with high local  $\text{Ca}^{2+}$  concentrations near the CRAC channels and lower global  $\text{Ca}^{2+}$  within the cytosol [257]. With CaM localised close to CRAC channels [16, 238], high local  $\text{Ca}^{2+}$  concentrations instigate the formation of the diffusible  $\text{Ca}^{2+}$ -CaM complex.  $\text{Ca}^{2+}$ -CaM is essential to the activity of eNOS: 1)  $\text{Ca}^{2+}$ -CaM influences the release of eNOS from membrane tethering, by potentiating APT-1, which de-palmitoylates eNOS. Furthermore  $\text{Ca}^{2+}$ -CaM directly competes with the inhibitory protein caveolin-1, displacing it from its association with eNOS. 2)  $\text{Ca}^{2+}$ -CaM can influence the phosphorylation state of eNOS through increasing the activity of  $\text{Ca}^{2+}$ -dependent kinases, such as CaMKII [103]. The phosphorylation state of eNOS in part determines the binding of  $\text{Ca}^{2+}$ -CaM, and lowers the  $\text{Ca}^{2+}$ -dependency of eNOS. Lowering  $\text{Ca}^{2+}$ -dependency of eNOS is critical to maintain eNOS function away from saturating  $\text{Ca}^{2+}$  levels in the microdomains. 3) The binding of replete  $\text{Ca}^{2+}$ -CaM to eNOS facilitates the production of NO, and is the principle activating mechanism. Under sub-maximal  $\text{Ca}^{2+}$  concentration  $\text{Ca}^{2+}$  may be released from CaM limiting the activating effect of  $\text{Ca}^{2+}$ -CaM. Binding to eNOS increases CaM affinity for  $\text{Ca}^{2+}$ , prolonging eNOS activation in the cytosol when regulated by global  $\text{Ca}^{2+}$ .

We propose that  $\text{Ca}^{2+}$  local microdomains near CRAC channels are responsible for the initial formation of  $\text{Ca}^{2+}$ -CaM, with global  $\text{Ca}^{2+}$  required to maintain replete  $\text{Ca}^{2+}$ -CaM, and regulate eNOS activity away from the caveolae.  $\text{Ca}^{2+}$ -CaM is critical for many aspects of eNOS activation. Firstly, inactive eNOS in the plasma membrane requires removal of tonic inhibition by caveolin-1 and de-palmitoylation, which allows translocation.  $\text{Ca}^{2+}$ -CaM potentiates the depalmitoylation of eNOS through acyl-protein thioesterase-1 (APT-1) [364], and directly competes with caveolin-1 to bind eNOS and release tonic inhibition [126]. Secondly,  $\text{Ca}^{2+}$ -CaM binding to eNOS directly enhances eNOS activity, by facilitating electron transfer through the enzyme, with the inhibition of CaM-binding able to limit eNOS activity by up to 75% [311,315]. Thirdly,  $\text{Ca}^{2+}$ -CaM can influence the  $\text{Ca}^{2+}$ -dependency of the eNOS enzyme by enhancing phosphorylation of Ser1177 by CaMKII [103].

Ser1177 is located in one of the auto-inhibitory regions of eNOS. Phosphorylation at this site occurs upon both agonist stimulation and shear stress and can occur in the cytosolic portion of eNOS [109,224]. This autoinhibitory control element prevents eNOS activity by limiting the binding of  $\text{Ca}^{2+}$ -CaM. High levels of  $\text{Ca}^{2+}$  are required to produce sufficient  $\text{Ca}^{2+}$ -CaM to effectively bind eNOS. In the absence of Ser1177 phosphorylation,  $\text{Ca}^{2+}$ -CaM is required to both displace the autoinhibitory arm, and interact with the CaM binding domain [13]. Following Ser1177 phosphorylation, the autoinhibitory arm no longer blocks the CaM-binding site, and allows  $\text{Ca}^{2+}$ -CaM-eNOS binding at concentrations of  $\text{Ca}^{2+}$  close to basal levels [111,184]. The presence of an autoinhibitory arm is a defining feature of the  $\text{Ca}^{2+}$  dependency of eNOS. The lower  $\text{Ca}^{2+}$  requirements of iNOS, which produces NO at basal  $\text{Ca}^{2+}$  concentrations, is in part due to the lack of this region [292].

Phosphorylated Ser1177 has also been proposed to help improve electron transfer between eNOS domains [216]. Ser1177 therefore improves eNOS activity via two mechanisms.

It is clear from the time frames of the  $\text{Ca}^{2+}$  signals and NO imaging experiments within this thesis, that a return to basal  $\text{Ca}^{2+}$  concentration does not act as a simple inhibitory factor to continued eNOS activation. We have clearly shown that eNOS activity is temporally separated from SOCE. With changes in the phosphorylation state of key eNOS residues, such as Ser1177 and Thr495, mediating eNOS activity at basal  $\text{Ca}^{2+}$  concentrations, in order for eNOS activity to be halted, activatory phosphorylation changes must be reversed, e.g. dephosphorylation of Ser1177 by PKA-regulated protein phosphatase 2A, and re-phosphorylation of Thr495 by PKC [225]. Indeed Thr495 has been found to be phosphorylated above basal levels after activation, indicating that post-translational modifications may be used as a braking system to halt enzyme activity [103]. A decrease in intracellular  $\text{Ca}^{2+}$ -CaM concentration after  $\text{Ca}^{2+}$  levels have returned to basal levels may limit the formation of pro-activity modifications such as phosphorylation of Ser1177.

Although only the phosphorylation of Ser1177 has been considered within this thesis, other eNOS residues are alternatively modified with a similar time course to Ser1177 and may be contributing to the distinct kinetics observed, and the separation of  $\text{Ca}^{2+}$ -rise from rapid NO production. Thr495, conversely to Ser1177, is dephosphorylated upon eNOS activation by protein phosphatase 1, which removes electrostatic repulsion between eNOS and  $\text{Ca}^{2+}$ -CaM [266]. This occurs over a comparable time course to Ser1177 phosphorylation, and is inducible by the

same agonists. Other phosphorylation changes should also be considered, such as Ser633 which is also located in the autoinhibitory arm. Furthermore an increase in Ser633 phosphorylation occurs upon eNOS activation following stimulation with agonists such as ATP and BK, and is mediated by AMPK, which can be activated by CaM kinase kinase [17,49]. Ser633 phosphorylation also decreases eNOS  $\text{Ca}^{2+}$ -dependency, and interestingly increases over a slightly slower time period than Ser1177. It is therefore likely that phosphorylation at Ser633 contributes to eNOS activity after the peak of intracellular  $\text{Ca}^{2+}$  rise [17]. It would be interesting to investigate the changes in phosphorylation levels of both of these eNOS residues, upon CRAC channel activation. The increase in Ser633 after ATP stimulation indicates that changes may occur after  $\text{Ca}^{2+}$  entry via CRAC channels. Dissection of CRAC channel-dependent post translational modifications of eNOS may provide clarity to the global  $\text{Ca}^{2+}$  requirements of eNOS activation, and further explain the time lag to fast NO production.

We therefore propose that local  $\text{Ca}^{2+}$  signalling is critical for the initial formation of high levels of  $\text{Ca}^{2+}$ -CaM, which are able diffuse to sites of action, including the caveolae and cytosol.  $\text{Ca}^{2+}$  microdomains are crucial to ensure sufficient  $\text{Ca}^{2+}$ -CaM is formed during the highly  $\text{Ca}^{2+}$ -dependent early stages of eNOS activation. Diffusion to alternative intracellular sites means that maintenance of the  $\text{Ca}^{2+}$ -CaM complex is dependent on global  $\text{Ca}^{2+}$  rises.  $\text{Ca}^{2+}$ -CaM regulated alterations in  $\text{Ca}^{2+}$  sensitivity increases the binding of  $\text{Ca}^{2+}$ -CaM to eNOS. This in turn enhances the formation of replete  $\text{Ca}^{2+}$ -CaM in the presence of the global  $\text{Ca}^{2+}$  rise. The reduction in  $\text{Ca}^{2+}$ -CaM dependence of eNOS allows a burst of NO production, which is temporally dissociated from the peak intracellular  $\text{Ca}^{2+}$  rise.

Increased external  $\text{Ca}^{2+}$  reduces the time taken to reach maximal rates of activity by enhancing both local and global  $\text{Ca}^{2+}$ .

### **7.3 Biological relevance of CRAC channel activity in sEnd.1**

The role of CRAC channels in health and disease is becoming increasingly apparent, with CRAC channels being identified as a major player in allergies, asthma and pancreatitis [78,116,265]. With the identification of a key endothelial pathway as being activated by  $\text{Ca}^{2+}$  entry through CRAC channels, it can be postulated that CRAC channels may also provide a novel target in vascular disease settings.

Reduction in vascular NO is a well-characterised hallmark of vascular disease. With CRAC channels now being identified as required for NO production, understanding CRAC channel signalling in various vascular disease states may provide insight into the mechanisms of these diseases. It would therefore be interesting to take this work into other endothelial cell models, and further to this animal models of vascular disease, to clarify whether abrogated  $\text{Ca}^{2+}$  signalling is resulting in reduced NO production, and whether mutations in STIM-1 or Orai-1 contribute to vascular diseases.

It is well defined that ‘uncoupled’ eNOS produces  $\text{O}_2^-$  instead of NO, which occurs under conditions of oxidative stress typical of early stages of vascular disease, such as endothelial dysfunction. It is possible that endothelial CRAC channel activation by inflammatory mediators such as histamine, may further perpetuate the decline

into disease state. Furthermore,  $O_2^-$  is capable of stimulating CRAC channel-dependent  $Ca^{2+}$  oscillations [113]. Although this was shown to be controlled by NOX2 under stimulation of LPS, whether ‘uncoupled’ eNOS is capable of activating the same pathway should be considered. If  $O_2^-$  production by eNOS, further activated CRAC channel signalling then this could provide a critical feed-forward loop leading to disease. Taking activation of eNOS via CRAC channels into a disease setting could therefore reveal crucial pathways in increasing oxidative stress and dysfunction in endothelial cells.

A decrease in NO production is a hallmark of many vascular disease states, not only endothelial dysfunction. SOCE has been implicated in vascular adaptation to pregnancy, due to increased systemic production of NO. Increased NO dilates blood vessels, enhancing uterine perfusion, and furthermore increases angiogenesis in the area. A failure to increase NO production appropriately in pregnancy is associated with the onset of pre-eclampsia [31]. A role of decreased CRAC channel signalling has not to our knowledge been addressed in the pre-eclampsia setting, but could provide a useful insight.

Although there has been no report of hypertension in patients with STIM-1 mutations, or indeed vascular phenotypes, patients typically die before the age of 6 due to autoimmunity and immunodeficiency [267]. Vascular phenotypes, associated with altered blood pressure regulation, may not have had time to develop. An endothelial-specific STIM-1 knock-out mouse has recently been developed, which showed abrogated SOCE and diminished ER  $Ca^{2+}$  store content. Again no major phenotypic differences were reported, although blood pressure was not directly addressed [113,301]. Widely reported changes in endothelial migration and prolif-

eration with abrogated CRAC channel signalling were not seen, indicating compensation for SOCE-dependent processes in endothelia [1, 113]. Endothelial-specific STIM-1 KO mice are less sensitive to pro-inflammatory cytokines, and fail to facilitate leukocyte infiltration. Furthermore, they are not susceptible to inflammatory-mediator induced cell death. CRAC channels are therefore required for endothelial pathology associated with inflammatory stimuli [113]. Inflammatory mediators, including  $O_2^-$ , have been shown to induce CRAC-dependent  $Ca^{2+}$  oscillations, which are capable of supporting NFAT translocation to the nucleus [113].

It has recently been shown that dysfunctional endothelial cells have increased basal cytosolic and reduced ER store  $Ca^{2+}$ , indicating aberrant  $Ca^{2+}$  signalling [306]. Clearly, a role for CRAC channels in endothelial diseases still needs investigation, with CRAC channels providing a potential druggable target. However, with the prevalence of CRAC channels in other cell types, targeted medicines would have to be developed in order to use CRAC channels as an approach to specifically modulate endothelial disease.

It is clear that the role of CRAC channels in other endothelial cell pathways should also be considered. With evidence that a lack of CRAC channels abrogates endothelial cell responses to inflammatory mediators, it is likely that CRAC channel signalling is modulating many functional pathways in the endothelium. The role of the CRAC channel in the endothelia is therefore an exciting new area of study.



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