INTERACTIONS OF SODIUM CALCIUM EXCHANGER AND SARCO/ENDOPLASMIC RETICULUM CALCIUM ATPase IN THE PORCINE CORONARY ARTERY SMOOTH MUSCLE CELLS

INTERACTIONS OF SODIUM CALCIUM EXCHANGER (NCX) AND SARCO/ENDOPLASMIC RETICULUM CALCIUM ATPase (SERCA) IN THE PORCINE CORONARY ARTERY SMOOTH MUSCLE CELLS

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GAURI AKOLKAR

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AUTHOR: Gauri Akolkar

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ABSTRACT

Calcium (Ca^{2+}) is an important signaling molecule with tightly regulated movements across cell membranes. Ca^{2+} transporters play a key role in this regulation. The sarco/endoplasmic reticulum (SER) Ca^{2+} pump (SERCA) and plasma membrane Ca^{2+} pump (PMCA) keep cytosolic Ca^{2+} levels $[Ca^{2+}]_i$ low. The sodium calcium exchanger (NCX) is bidirectional and may work to expel Ca^{2+} or bring it into the cell depending on the electrochemical gradient. NCX has been proposed to play a role in refilling the SER Ca^{2+} pool.

The overall objective of this thesis was to determine the effect of SER Ca^{2+} depletion on NCX- SERCA interactions in pig coronary artery smooth muscle by monitoring changes in $[Ca^{2+}]_i$ and the co-localization between NCX and SERCA. NCX mediated increase in $[Ca^{2+}]_i$ was observed in Na^+ loaded smooth muscle cells. The effect of SER depletion by thapsigargin was examined on the NCX mediated increase in $[Ca^{2+}]_i$. A decrease in the NCX mediated increase in $[Ca^{2+}]_i$ was observed upon inhibition of SERCA pump with thapsigargin. The effect of SER depletion on proximity of NCX and SERCA proteins was also investigated using immunofluorescence confocal microscopy using anti-NCX1 and anti-SERCA2 antibodies and fluorescence labeled secondary antibodies. Na^+ loaded smooth muscle cells in NMG buffer were incubated with or without SERCA inhibitor thapsigargin. Resulting image stacks were analyzed for the co-localization of NCX1 and SERCA2 in the areas near the plasma membrane. SER depletion with thapsigargin increased the co-localization between NCX1 and SERCA2 near the plasma membrane of smooth muscle cells. Thus, SER Ca^{2+} depletion moves plasma membrane protein NCX and SER protein SERCA closer to each other and decreases the NCX mediated Ca^{2+} entry in the cell. These interactions may be crucial to smooth muscle Ca^{2+} regulation and

has the ability to be a potential therapeutic target during oxidative stress. However, the nature of these interactions needs to be explored.

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ABBREVIATIONS

$[Ca^{2+}]i$ – intracellular calcium

ATP- Adenosine triphosphate

BSA- Bovine serum albumin

CBD – Calcium binding domain

CPA- Cyclopiazonic acid

DBHQ - 2,5-di(t-butyl) hydroquinone

DMEM- Dulbecco's modified eagle medium

EC- Endothelial cells

SER- Sarco/Endoplasmic reticulum

IP_3 – Inositol trisphosphate

IP_3R- Inositol trisphosphate receptors

NCKX - Na^+ - Ca^{2+} - K^+ Exchanger (Sodium calcium potassium exchanger)

NCX – Na^+ Ca^{2+} Exchanger (Sodium calcium exchanger)

NMG- N-methyl glucamine

PBS- Phosphate buffered saline

PKA- Protein kinase A

PLB- Phospholamban

PLM- Phospholemann

PM – Plasma membrane

PMCA – Plasma membrane calcium ATPase

ROCC- receptor operated calcium channel

ROS – Reactive oxygen species

RyR- Ryanodine receptor

SER – Sarcoendoplasmic reticulum

SERCA – Sarco/endoplasmic reticulum calcium ATPase

SLN- Sarcolipin

SMC- Smooth muscle cell

STIM- Stromal interacting molecule

TRPC – Transient receptor protein channel

VOCC- voltage operated calcium channel

XIP – exchanger inhibitory peptide

1.0 INTRODUCTION

Calcium is an essential regulator of physiological responses in living cells. In resting smooth muscle cells (SMC), intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) is maintained in nanomolar range, which is almost 10,000 times lower than that of extracellular concentration. A low level of $[Ca^{2+}]i$ is maintained and in order to provide a transient signal, it is altered by various pathways of Ca^{2+}entry, Ca^{2+}exit, release of sequestered Ca^{2+} and/or Ca^{2+} binding to various proteins. This signaling uses a variety of Ca^{2+} regulatory mechanisms including various channels, pumps, exchangers and binding proteins. Plasma membrane calcium ATPase (PMCA), Na^{+} Ca^{2+} exchanger (NCX) and sarco/endoplasmic reticulum calcium ATPase (SERCA) play important roles in regulation of $[Ca^{2+}]i$. Any defects in these processes may have dire pathological consequences.

Previous studies performed in our lab focused on the roles of SERCA, NCX and PMCA in $[Ca^{2+}]_i$ regulation in smooth muscle. Our initial results showed diminished NCX mediated entry of radioactive Ca^{2+} ($^{45}Ca^{2+}$) on inhibition of SERCA. This indicated a functional linkage between NCX and SERCA. My study concerns the role of NCX and its interactions with SERCA on $[Ca^{2+}]i$ changes in the pig coronary artery smooth muscle cells.

1.1 Coronary Artery

The coronary artery delivers oxygenated blood and nutrients to the heart (Cox and Vatner, 1982; Walia *et al.*, 2000; Grover and Samson, 1992). Any damage to the artery or any obstruction in blood flow may cause serious physiological effects, such as development of various cardiovascular diseases and can finally lead to cardiac arrests (Grover and Samson, 1992).

Coronary artery is composed of three different layers: intima, media and adventitia (Malhotra *et al.*, 2003). The innermost layer, intima, is made up of a monolayer of endothelial cells, which acts as a barricade between blood and the underlying vascular tissue (Walia *et al.*, 2000; Malhotra *et al.*, 2003). The media is made up of smooth muscle cells with elastic fibres which allow the coronary artery to stretch without any mechanical damage. Surrounding the media is the outermost layer, adventitia, which is made up of connective tissue and functions as mechanical support to the coronary artery (Malhotra *et al.*, 2003). Both endothelial and smooth muscle cells play important roles in maintaining vascular tone for regulation of proper blood flow. The following section focuses on smooth muscle and endothelial cells of the coronary artery.

1.1.1 Endothelium

Endothelium is located in the inner lining of the arteries and is composed of a monolayer of cells. Endothelium has three discrete cell surfaces; luminal, abluminal and lateral. Each of these surfaces plays an important role in arterial function. The luminal surface is in direct contact with blood and has receptors and adhesion molecules. The abluminal surface secretes many substances like nitric oxide and endothelin to smooth muscle cells and hence functions as a regulator of vascular tone (Ghitescu *et al.*, 2002). The lateral surface joins neighbouring cells through intercellular junctions.

1.1.2 Smooth muscle cells

Smooth muscle cells are mononucleated, spindle shaped and non-striated. Each cell has bundles of thick and thin filaments of myosin and actin respectively. Contraction of smooth muscle occurs via cross bridges between actin and myosin upon increase in cytosolic Ca^{2+}. Contraction can also occur by Ca^{2+} independent pathways (Lodish, 2000). Ca^{2+} plays a pivotal

role in controlling vascular tone, making it a significant contributor to the regulation of systemic blood pressure and supply of blood to all organs. Vascular contraction is induced by an increase in the concentration of free $[Ca^{2+}]i$ and its subsequent lowering leads to relaxation of vascular smooth muscle cells (Doong *et al.*, 2006). Hence the concentration of $[Ca^{2+}]i$ needs to be regulated precisely. The following sections will discuss the Ca^{2+} regulation and various mechanisms involved in increasing and decreasing $[Ca^{2+}]i$ and the key transporters involved in these mechanisms.

1.2 Calcium homeostasis

Ca^{2+} is an important ion which is involved in the initiation and maintenance of numerous physiological responses in all cell types (Zheng and Wang, 2007; Blauestein *et al.*, 1999). It is an ubiquitous second messenger involved in regulating diverse functions such as fertilization, electrical signaling, contraction, secretion, memory, gene transcription and cell death (Bers, 2008; Fameli *et al.*, 2007; Zhang *et al.*, 2004; Carafoli, 2009; Clapham, 2007). In resting smooth muscle cells, $[Ca^{2+}]i$ is maintained around 100 to 200 nM whereas extracellular calcium concentration is maintained around 2 mM (McCarron , 2006 ; Blauestein *et al.*, 1999). Ca^{2+} is 10,000 times less concentrated inside the cell than outside and hence a small increase in $[Ca^{2+}]i$ leads to a large background to signal ratio leading to a large influence on the modulation of Ca^{2+} targets. Therefore $[Ca^{2+}]i$ needs to be precisely regulated by concerted action of various entry and exit processes. The Ca^{2+} levels should be able to increase and decrease rapidly to provide transient signals (Blauestein *et al.*, 1999; Carafoli, 1991). $[Ca^{2+}]i$ dynamics are regulated by a variety of Ca^{2+} regulatory mechanisms including various Ca^{2+} channels and pumps. $[Ca^{2+}]i$ can also be buffered by binding to various Ca^{2+} binding proteins and other transcription factors, thus

decreasing the free $[Ca^{2+}]i$ (Hirota *et al.*, 2007; Bers *et al.*, 2008). Any defects in these processes may have dire consequences. Abnormal Ca^{2+} regulation in coronary artery can lead to serious cardiovascular pathologies.

1.2.1 Mechanisms of decreasing $[Ca^{2+}]_i$

Elevated $[Ca^{2+}]i$ is required for only brief periods after which it is decreased to the basal levels. The decrease in $[Ca^{2+}]i$ is brought about by Ca^{2+} extrusion by PMCA and NCX or by Ca^{2+}sequestration into sarco/endoplasmic reticulum (SER) by the SERCA pump (Clapham, 2007; Misquitta *et al.*, 1999; Strehler *et al.*, 2007). SERCA and NCX play a crucial role in the removal of $[Ca^{2+}]i$ in smooth muscle cells; 92 % of $[Ca^{2+}]i$ is removed by SERCA, 7% by NCX and 1% by PMCA and mitochondria (Shin *et al.*, 2008). $[Ca^{2+}]i$ is also lowered by binding to Ca^{2+} binding proteins such as calsequesterin, calreticulin and calnexin. Calsequesterin and calreticulin in the SER bind Ca^{2+}. They increase the Ca^{2+} storage capacity of SER and modulate the function of SERCA (Lee and Michalak, 2010). Ca^{2+} is stored close to Ca^{2+} release channels for quick release of Ca^{2+} into the cytosol upon stimulation. (Frank *et al*, 2003).

1.2.2 Mechanisms of increasing $[Ca^{2+}]i$

An increase in $[Ca^{2+}]i$ can occur either by Ca^{2+} influx from extracellular fluid through channels in PM or by release of Ca^{2+} sequestered in the SER or other organelles (Clapham, 2007). Voltage or receptor operated calcium channels (VOCC and ROCC respectively) may allow entry of extracellular Ca^{2+}. In addition to these channels, NCX can operate in Ca^{2+} entry mode and hence increase the $[Ca^{2+}]i$. $[Ca^{2+}]i$ can also be increased by the release of Ca^{2+} from the SER through Ca^{2+} channels activated by inositol 1, 4, 5- trisphosphate (IP3) or Ca^{2+} (or the plant

alkaloid ryanodine). Depletion of Ca^{2+} from intracellular stores activates Ca^{2+} entry from the extracellular space for refilling of the SER by store deletion dependent or store operated calcium entry (SOCE) (Cahalan, 2009; Hewavitharana *et al.*, 2007; Parekh *et al.*, 2010). Inwardly rectifying Ca^{2+}current (I_{CRAC}) is a form of SOCE and has been associated with two families of proteins: the SER resident stromal interaction molecules (Stim) and the Orai proteins in the PM. In addition to Stim and Orai, members of transient receptor protein (TRP) family may be involved in SOCE. In contrast to the Orai channels, the TRP channels are weakly selective for Ca^{2+}. Other than SOCE function, some members of TRP family can increase $[Ca^{2+}]i$ by receptor induced Ca^{2+} entry (Ambudkar *et al.*, 2007; Ng *et al.*, 2009; Worley *et al*, 2007). NCX, Stim, Orai and TRP will be discussed further.

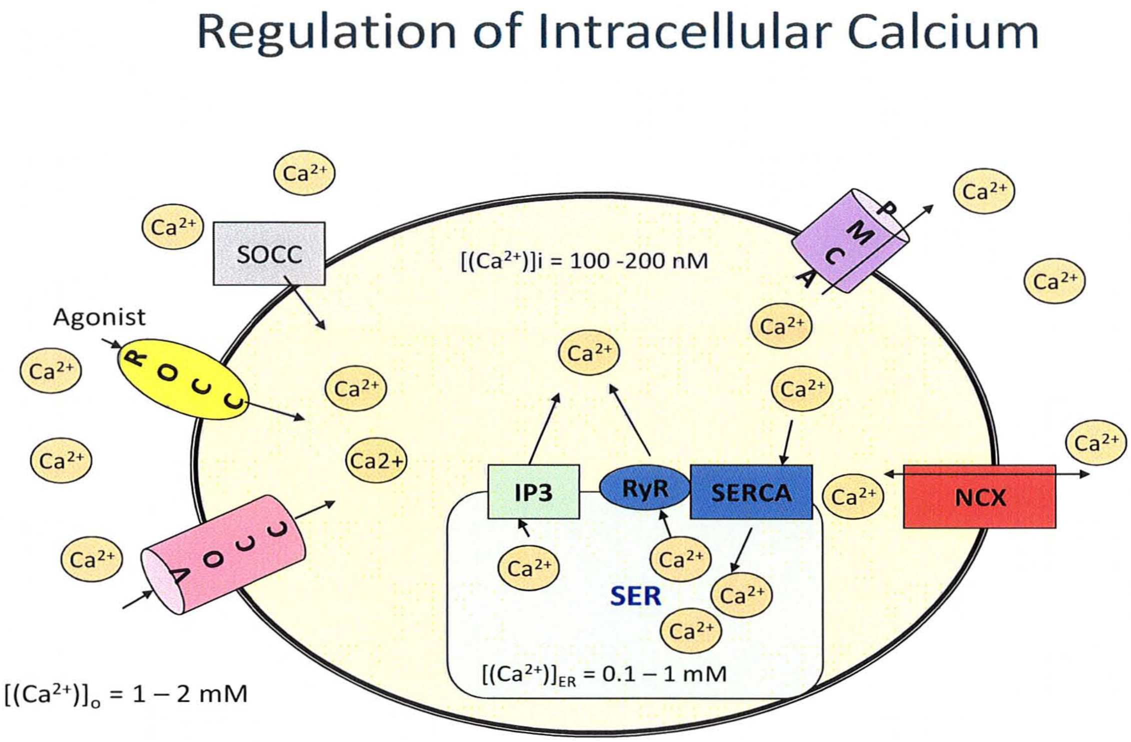

Figure 1. Overview of Calcium Homeostasis. $[Ca^{2+}]$i is maintained at around 100 to 200 nM meanwhile extracellular Ca^{2+} is around 1-2 mM, thus Ca^{2+} must be highly regulated inside the cell. Inside the cell two organelles are responsible for storage of calcium: the SER and the mitochondria (the mitochondrial Ca^{2+} storage will not be elaborated). Mechanisms to raise $[Ca^{2+}]$i which are located in the PM are: the voltage operated (VOCC), the store operated (SOCC) and the receptor operated calcium channels (ROCC). Mechanisms to release Ca^{2+} from the SER into the cytosol are the: ryanodine receptors (RyR) and inositol trisphospate receptors (IP_3R). Mechanisms to reduce $[Ca^{2+}]$i are through PMCA, SERCA and NCX.

1.3 Calcium pumps

Three types of Ca^{2+}ATPase pumps are found in cells of higher animals, PMCA, Secretory pathway calcium ATPase (SPCA) and SERCA. These pumps are involved in maintaining low $[Ca^{2+}]i$. PMCA pumps achieve this by extrusion of cytosolic Ca^{2+}, while SPCA and SERCA pumps maintain the $[Ca^{2+}]i$ by sequestering Ca^{2+} in intracellular organelles like Golgi and SER respectively (Birini and Carafoli, 2009; Wuytack *et al.*, 2002). They belong to family of P-type ATPases which are characterized by conservation of ATP energy in the form of phosphorylated enzyme intermediate (Carafoli, 2009). They are considered as high affinity, low capacity calcium pumps (Carafoli, 2002; Carafoli, 2009; Guerini, 1998; Pande, 2005). SERCA pump is one of the main focuses of this thesis and will be discussed in detail in the following section.

1.3.1 SERCA

SER stores contain most of the $[Ca^{2+}]i$, while the cytosol contains only a small fraction as free Ca^{2+}. SER membrane has the Ca^{2+} pump, SERCA, which plays an important role in accumulating Ca^{2+} in SER stores. SERCA is a pivotal molecule for maintaining a balanced concentration of intracellular Ca^{2+}. It is localized in the SR membrane. The pump serves dual functions: to cause muscle relaxation by lowering cytosolic calcium and to restore the SER calcium store necessary for muscle contraction (Birini and Carafoli., 2009; Floyd and Wray, 2007). It belongs to family of cation transporters termed P-type ATPases, which include plasma membrane Ca^{2+} ATPase (PMCA) and Na^{+}/K^{+} ATPase. A notable feature of P-type ATPases is the transfer of terminal phosphate from ATP to an aspartate/glutamate residue in the catalytic domain, which results in a reversible conformational change (Floyd and Wray, 2007) . SERCA pump couples the hydrolysis of ATP to the movement of Ca^{2+} across the membrane (Lee and

East., 2001; Wuytack *et al.*, 2002). It transports two Ca^{2+} ions from the cytosol into the SER against the concentration gradient using one ATP molecule. Defects in SER Ca^{2+} uptake function is one of the major contributing factors to the progression of heart failure. (Frank *et al*, 2003; Birini and Carafoli., 2009)

1.3.1.1 Structure and Function

SERCA pumps constitute single polypeptides with a molecular mass of approximately 97-115 kDa. The general structure for all SERCA pumps includes 10 transmembrane helices M1 to M10 and three distinct cytoplasmic domains; A- domain (actuator or anchor domain), a P-domain (phosphorylation domain), and an ATP binding N- domain (Lee and East., 2001; Wuytack *et al.*, 2002; Birini and Carafoli, 2009). The A -domain is located between transmembrane helices M2 and M3, whereas the P and N- domains are located between transmembrane helices M4 and M5. The ATP binds to N- domain adjacent to the P- domain. On binding of ATP there is an energy transfer from N -domain to P- domain through calcium binding sites (Lee and East., 2001; Wuytack *et al.*, 2002). Calcium binding sites lie side by side near the cytoplasmic surface of the lipid bilayer and are accessible only from the cytoplasm and not from the SER lumen. These sites are near the center of the transmembrane domain spanning helices M4, M5, M6 and M8 (Lee and East., 2001; Wuytack *et al.*, 2002). In addition to the Ca^{2+} and ATP binding, the cytoplasmic domain also has sites for interaction with SERCA inhibitor phospholamban (Wuytack *et al.*, 2002).

The SERCA pump is observed in two conformational states E1 and E2. Ca^{2+} binds to the high affinity site on the cytoplasmic face of the SERCA pump in E1 state. This triggers the binding of ATP on the N- domain of the pump (Lee and East., 2001; Wuytack *et al.*, 2002). Transfer of phosphate from N- domain to the P- domain leads to hydrolysis of ATP and

phosphorylation of the enzyme. This initiates a series of conformational changes in the enzyme and the calcium ions are released to the luminal side of the membrane following the conversion of the pump to the low affinity E2 conformation. Dephosphorylation follows and the pump returns to the E1 state (Lee and East., 2001; Wuytack *et al.*, 2002; Birini and Carafoli, 2009). Reuptake of Ca^{2+} into the SER takes only a few milliseconds due to the high Ca^{2+} affinity of SERCA pumps.

1.3.1.2 Isoforms

In vertebrates, three distinct genes encode for SERCA pump, SERCA1, 2 and 3. Alternative splicing of these genes produces more than 10 isoforms of the SERCA pump. All the SERCA isoforms are predicted to have essentially identical transmembrane topologies and tertiary structures. SERCA1 is expressed in fast-twitch skeletal muscle and is alternatively spliced producing two splice variants SERCA1a and 1b. SERCA1a is mainly expressed in fast-twitch skeletal muscle, while SERCA1b is abundant in fetal and neonatal stages (Frank *et al.*, 2003; Periasamy and Huke., 2001; Misquitta *et al.*, 1999; Floyd and Wray, 2007). Four splice variants encode for the SERCA2 isoforms, variant 1; SERCA2a is the primary isoform expressed in cardiac and slow-twitch skeletal muscle tissue and smooth muscle (Floyd and Wray, 2007) whereas variants 2–4 encode for SERCA2b, which is the predominant isoform in nonmuscle (variants 2 and 3) and neuronal cells (variant 4). SERCA2b is expressed ubiquitously at low levels and is considered to be the house-keeping calcium pump (Floyd and Wray, 2007; Frank *et al.*, 2003; Periasamy and Huke., 2001; Misquitta *et al.*, 1999). SERCA 2b has higher affinity for calcium compared to SERCA 2a, whereas SERCA 2a has a higher turnover rate than SERCA 2b (Periasamy *et al.*, 2007; Misquitta *et al.*, 1999). The structural differences in the C-terminal

region of the two isoforms have shown to contribute to the functional differences between them. Recently, a third isoform SERCA 2c, has been reported in cardiac muscle (Periasamy *et al.*, 2007). SERCA3 isoforms are expressed in several non-muscle tissues but appear to be a minor form in muscle. SERCA3 isoforms are expressed at high levels in the hematopoietic cell lineages, platelets, epithelial cells, fibroblasts, and endothelial cells (Birini and Carafoli, 2009; Misquitta *et al.*, 1999; Periasamy *et al.*, 2007; East M., 2000). In humans, SERCA3 is known to encode for six isoforms 3a–3f, which are expressed in multiple tissues and cell types at mRNA level, whereas, there are data only for the 3a, b and c isoforms for expression at protein level (Periasamy *et al.*, 2007).

1.3.1.3 Regulation

Function of Ca^{2+} regulation by SERCA pump can be modulated by several indirect and direct factors like phosphoprotein, protein kinases, thyroid hormone, insulin, reactive oxygen species (ROS), redox agents and nitric oxide.

The most predominant indirect mechanism of modulation of SERCA is by the phosphoprotein phospholamban (PLB). It is an integral SER membrane protein containing 52 amino acids and expressed predominantly in cardiac muscle and in small amounts in slow twitch skeletal muscle, smooth muscles and endothelial cells. PLB inhibits SERCA2a, SERCA2b, and SERCA1a pumps equally, but not the SERCA3 pump. SERCA 3 lacks the putative interacting domain for PLB (Periasamy *et al.*, 2007; Misquitta *et al.*, 1999). PLB is the key regulator of SERCA2 and inhibits its activity in a reversible manner. The inhibitory function of PLB is modulated by phosphorylation/ dephosphorylation and by an increase in $[Ca^{2+}]i$ (Periasamy *et al.*, 2007; East M., 2000; Misquitta *et al.*, 1999). PLB inhibits the apparent affinity of SERCA2

for Ca^{2+} in its dephosphorylated form. Upon phosphorylation through stimulation and enhanced cAMP dependent protein kinase A (PKA) activity, the inhibition of phospholamban on SERCA2 is relieved. Phospholamban can be phosphorylated at two distinct sites; at serine 16 by cAMP-dependent PKA, and at threonine 17 by Ca^{2+}-calmodulin–dependent protein kinase (CaMKII) during β-adrenergic stimulation (Frank *et al.*, 2003). Phosphorylation disrupts the physical interaction of PLB with SERCA2 and thus stimulates SER Ca^{2+} transport by increasing the affinity of the SERCA2 for Ca^{2+}, without a significant change in maximal velocity (Vmax) (Frank *et al.*, 2003; Bhupathy *et al.*, 2007). Phosphorylation by each kinase increases the apparent affinity of SER Ca^{2+}ATPase for Ca^{2+} and thereby increases the rate of Ca^{2+} sequestration in SER. Inhibitory effects of PLB are restored through dephosphorylation by an SER associated phosphatase, type 1 phosphatase (pp1) (East., 2000; Periasamy *et al.*, 2001; Bhupathy *et al.*, 2007)).

An alteration in the ratio of PLB and SERCA can affect SER Ca^{2+} transport. Absence of PLB enhanced SER calcium uptake and increased the rates of contraction and relaxation. This is associated with an increase in SERCA2 affinity for calcium (Bhupathy *et al.*, 2007). On the other hand, over-expression of PLB in the heart results in a decrease in SER Ca^{2+} uptake and depressed cardiac contractile performance *in vivo*. A shift in the ratio of PLB to SERCA leads to a corresponding shift in SERCA affinity for Ca^{2+}, such that an increase in this ratio leads to decreased Ca^{2+} affinity (Periasamy and Huke., 2001; Bhupathy *et al.*, 2007) .

Another small molecular weight protein, sarcolipin (SLN) is also involved in the regulation of SR Ca^{2+} ATPase activity. The mechanism of regulation of SERCA2 activity by SLN can be either by its inhibitory function mediated through PLB or through its direct interaction with SERCA2. SLN forms a ternary complex by binding with both PLB and

SERCA2 (Bhupathy *et al.*, 2007). Furthermore, SLN can also mediate its inhibitory effect on SERCA independent of PLB. In contrast to PLB, the inhibitory effect of SLN on SERCA2 is observed even at high Ca^{2+}. The C-terminus of SLN could be involved in the Ca^{2+} independent inhibition of SERCA2 and could contribute to the inhibitory function of SLN at high calcium concentrations. Whereas, overexpression of SLN showed reduced affinity of SERCA2 for Ca^{2+} (Bhupathy *et al.*, 2007; East J, 2000).

Other than indirect modulation of SERCA through PLB, the direct modulation of SERCA 2 is mediated by a calmodulin dependent protein kinase (Frank *et al.*, 2003). Phosphorylation of SERCA2 by calmodulin kinase modulates the Vmax of the Ca^{2+} pump without changing the apparent affinity of the pump (EC_{50}). This phosphorylation is seen in cardiac and smooth muscle SR, but not in the skeletal SR (Frank *et al.*, 2003; East *et al.*, 2000).

Hormones have been shown to cause alterations in SERCA activity (Frank *et al.*, 2003; Bhupathy *et al.*, 2007). For example, thyroid hormone levels have been shown to modulate SER function by altering the expression of SERCA and its regulators. In hyperthyroidism, a decrease in levels of both the inhibitors, PLB and SLN is seen relative to SERCA2a levels. In hypothyroidic hearts, however, SERCA levels are decreased, without changing PLB and SLN levels (Bhupathy *et al.*, 2007; Periasamy *et al.*, 2007). Alterations in the expression of SERCA and PLB are shown to be the major contributors for the altered calcium homeostasis (Periasamy and Huke., 2001).

Nitric oxide (NO) has been identified as a potential modulator for stimulation of SERCA function in platelets and coronary artery smooth muscle cells. It is thought that this effect is mediated through the actions of PLB in smooth muscle cells, but details of the signalling pathway by which nitric oxide exerts its influence over SERCA pumps are incomplete (East.

2000). There is also evidence that nitric oxide may result in the covalent modification of SERCA pumps. Metabolism of nitric oxide produces peroxynitrite radicals which can oxidize Cys and Arg residues in SERCAs (East. 2000).

Redox agents play an important role in modulating the activity of SERCA pump. The cysteine residue at the catalytic domain is susceptible to thiol (SH) group modification (Zima and Blatter, 2006). The redox state of these SH groups are known to modulate SERCA activity (Grover and Samson, 1988; Grover *et al.*, 1995). Oxidizing and reducing agents differ in their modes of action; oxidizing agents like 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) oxidize thiol group and inhibit pump activity, while reducing agents like dithiothreitol and glutatione protect SERCA from this inhibition (Grover and Samson, 1988; Grover *et al.*, 1995). Similar to redox agents the activity of SERCA pump is also sensitive to oxidative stress. O_2^- and H_2O_2/OH^-, inhibit the Ca^{2+} uptake in the SER by interfering directly with ATP hydrolysis. Furthermore ROS can uncouple Ca^{2+} uptake activity from ATP hydrolysis, leading to inhibition of SERCA function. In addition, ROS can cause peroxidation of membrane phospholipids and thus inhibit the activity of membrane bound enzymes (Reeve., 1985). Different isoforms of SERCA exhibit different sensitivities to ROS. The SERCA3 isoform is more resistant than SERCA2b to peroxides (Barnes *et al.*, 2000). Cardiac SERCA2a has been found to be more sensitive to hydrogen peroxide/ hydroxyl radical compared to O_2^- (Misquitta *et al.*, 1999). Nitric oxide does not appear to affect SERCA Ca^{2+} pump activity by S-nitrosylation of cysteine residues directly; however peroxynitrite was found to increase activity of SERCA2 (East J, 2000).

1.3.1.4 Inhibitors

Various plant and chemical compounds like thapsigargin, cyclopiazonic acid (CPA) and hydroquinones inhibit specifically SERCA. The most specific and potent of these inhibitors is thapsigargin (TG), a sesquiterpene lactone which is extracted from the root of the umbelliferous plant *Thapsia garganica*. Although, thapsigargin is specific inhibitor for all the isoforms of SERCA, the sensitivity to it is different for the various SERCA isoforms (apparent Ki values being 0.21, 1.3, and 12 nM for SERCA1b, SERCA2b, and SERCA3a, respectively) (Wootton and Michelangeli., 2006) . Thapsigargin binds SERCA with nanomolar affinity in the Ca^{2+} free enzyme state and forms a dead-end inhibitory complex. In contrast it had no effect on other ATPases like PMCA or Na^+K^+ATPase (Treiman *et al.*, 1998; Inesi and Sagara., 1994; Birini and Carafoli, 2009).

Another SERCA inhibitor cyclopiazonic acid is one of the toxic metabolites produced by the molds *Pencillium cyclopium* and *Aspergillusflavus*. Cyclopiazonic acid is a potent inhibitor of Ca^{2+} sequestration and ATPase activity in SER of rat skeletal muscle. It has lower affinity for SERCA compared to thapsigargin, it is effective in micromolar concentrations (Birini and Carafoli, 2009). The interaction of cyclopiazonic acid with SERCA is favoured by low ATP concentrations (Wootton and Michelangeli., 2006; Inesi and Sagara., 1994).

Chemical compounds like 2,5-di(t-butyl) hydroquinone (DBHQ) , 2-Aminoethoxydiphenyl borate (2-APB) and curcumin have been used to modulate changes in the Ca^{2+} signalling through Ca^{2+} transporters. DBHQ was found to inhibit ATP-dependent Ca^{2+} transport by liver microsomes, while it was ineffective in blocking Ca^{2+} transport across mitochondrial or plasma membranes. Similar to cyclopiazonic acid, DBHQ has an affinity for

SERCA at micromolar range whereas its mechanism of action is similar to that of thapsigargin (Birini and Carafoli, 2009; Wootton and Michelangeli., 2006).

1.3.1.5Pathophysiology and effect of ROS

The effects of ROS on SERCA have been examined in endothelium (SERCA2 and 3) and smooth muscle (SERCA2) of the coronary artery (Walia *et al.*, 2003; Grover *et al.*, 1988; Grover *et al.*, 2003). The effects of peroxide, superoxide and peroxynitrite on SERCA in this artery have been examined at various organizational levels: arterial contractility, changes in $[Ca^{2+}]i$, and pump activity in isolated membranes and permeabilized cells. All three species of ROS irreversibly damage SERCA2 dependent Ca^{2+} transport in the isolated membranes but the effect on SERCA3 is less severe. The mechanism of the damage involves inhibition of an initial step in the reaction cycle of SERCA2 which involves the formation of a high energy intermediate acylphosphate, which is inhibited by all the ROS examined. A functional consequence of the SERCA2 damage in this tissue is the decrease in contraction in response to the SERCA2 inhibitors cyclopiazonic acid and thapsigargin (Grover *et al.*, 1995). The force produced in response to the actions of angiotensin II and endothelin B is also decreased, possibly because these agents depend on the Ca^{2+} stores in the SER. The smooth muscle cells cultured from this tissue produce an increase in $[Ca^{2+}]i$ with substances such as angiotensin II, cyclopiazonic acid and thapsigargin. Exposure to ROS decreases these responses. An interesting observation is the heterogeneity of the effects of peroxide and superoxide in the permeabilized cells. Peroxide is equipotent in inhibiting loading into the IP3-sensitive and -insensitive Ca^{2+} pools. In contrast, superoxide pretreatment inhibits loading into the IP3-sensitive pool but not into the IP3-

insensitive pool (Elmoselhi *et al.*, 1996). The exact significance of this observation remains to be determined.

1.3.2 Plasma membrane Ca^{2+}-ATPase (PMCA)

Plasma membrane Ca^{2+} ATPase (PMCA) is a P-type Ca^{2+} ATPase found in the plasma membrane of almost all cells. PMCA is a low capacity, high affinity Ca^{2+} transporter (Carafoli, 2002; Floyd and Wray, 2007). PMCA of smooth muscle and other cells is regulated by calmodulin. PMCA also counter-transports a proton, which may lead to pH changes around smooth muscle cells, affecting PMCA activity and intracellular pH. The structure of PMCA is similar to SERCA with 10 membrane-spanning regions, however, the primary difference between the two ATPase lies in their carboxy terminal tail region. PMCA has a longer terminal tail region compared to SERCA. This tail region is responsible for regulation of PMCA by calmodulin (Guerini D., 1998; Strehler *et al.*, 1990). In smooth muscle, there is evidence that PMCA activity is regulated by cGMP and PKC as well as calmodulin (Ruknudin and Lakatta, 2007). There are four different isoforms of the PMCA gene, PMCA 1–4. These isoforms share 80–90% amino acid sequence homology (Floyd and Wray, 2007). The isoforms of PMCA differ in their basal activity and stimulation by calmodulin which plays an important role in shaping Ca^{2+} signals. PMCA-1 has been described as a 'house keeping' Ca^{2+} pump, whereas PMCA-4 has the lowest basal activity and greatest stimulation by calmodulin and a role in shaping Ca^{2+} signals has been suggested. PMCA-2 has been implicated in hearing loss. PMCA-3 has the most restricted distribution and has only been reported in neuronal tissues (Guerini D., 1998; Strehler *et al.*, 1990). In smooth muscle there is good evidence of PMCA-1 and 4 being expressed; indeed these two isoforms may be present in all cells (Szewczyk *et al.*, 2007). Lanthanum and vanadate

are non-specific inhibitors of all PMCA isoforms that bind to the cytosolic domain of the pump (Carafoli E, 1991; Pande *et al.*, 2005). Lack of a specific inhibitor hinders the understanding of physiological roles of PMCA in Ca^{2+} homeostasis and smooth muscle function. Recently, specific inhibitors of PMCA called caloxins have been developed. Furthermore these caloxins have been shown to demonstrate isoform specificity (Pande *et al.*, 2006).

1.4 Ca^{2+} Exchangers

Two families of plasma membrane calcium exchanger proteins are known in mammalian tissues, Na^+-Ca^{2+} exchanger (NCX) and Na^+-Ca^{2+}-K^+ exchanger (NCKX). In smooth muscle cells, the exchanger mainly involved in the exchange of Ca^{2+} and Na^+ is NCX and hence will be discussed in further detail. The following sections discuss the kinetics and structure of NCX, its regulation in smooth muscle cells, specific inhibitors developed for NCX and its pathophysiological implications.

1.4.1 NCX

NCX is a ubiquitously expressed transmembrane protein which catalyzes the electrogenic exchange of Na^+ and Ca^{2+} across the plasma membrane. Movement of one Ca^{2+} ion is coupled to reciprocal movement of three Na^+ ions (Philipson and Nicoll, 1992; Shigekawa and Iwamoto, 2001; Blaustein and Leaderer, 1999; Hryshko and Philipson, 1997). Depending on the electrochemical gradients of the substrate ions it can operate in either the Ca^{2+} efflux (forward) or Ca^{2+} influx (reverse) mode (Shigekawa and Iwamoto, 2001; Matsuda *et al.*, 1997). Although NCX is expressed in almost all tissues, its activity differs among different species and different tissues. NCX extrudes approximately 30% of Ca^{2+} in rabbit, guinea pig and human ventricles

while it extrudes a very small portion in rat and mouse ventricles (Shigekawa and Iwamoto, 2001). The rate of transport of ions depends on the number of NCX proteins expressed in the tissue, the concentration of the substrate ion in the cell, the binding affinity of the substrate to the exchanger as well as the other regulatory factors described later. NCX is a high capacity, low Ca^{2+} affinity transporter. (Carafoli E, 1991; Shigekawa and iwamoto,2001; Blaustein and Leaderer, 1999; Birini and Carafoli, 2009)

1.4.1.1 Structure

The NCX super-family includes mammalian NCX along with the homologues from squid, *Drosophila and Arabidopsis*. Members of the NCX superfamily are defined by the presence of sequence motifs known as α repeats. These are the regions of intramolecular homology within the transmembrane segments of the proteins. Outside of α repeat region, very limited sequence similarity exists between the various NCX proteins of different species (Philipson *et al.*, 2002; Hryshko and Philipson, 1997). Mammalian NCX protein has 3 isoforms: NCX1, 2 and 3 (Matsuda *et al.*, 1997; Philipson *et al.*, 2002). These isoforms are very similar in structure and share 70% amino acid identity in their overall molecular structure (Matsuda *et al.*, 1997). NCX1 is ubiquitously expressed, however high expression is observed in cardiac muscle, kidney and brain and lower expression is observed in many other tissues. NCX 2 and 3 expression is limited to tissues such as brain (Shigekawa and Iwamoto, 2001; Philipson *et al.*, 2002; Blaustein and Leaderer, 1999). Since coronary artery smooth muscle cells contain predominantly NCX1, the following sections will focus on it.

NCX1 is composed of 970 amino acids with a molecular mass of approximately 110 kDa (Shigekawa and Iwamoto, 2001; Lytton J, 2007). Further alternative splicing generates a minor

variation of 5-10 kDa among different isoforms (Lytton J, 2007). During biosynthesis, a 32 amino acid long signal sequence is cleaved off from the N-terminus to yield a mature protein. Approximately half of the NCX1 protein constitutes a transmembrane domain and the remaining half forms a large cytoplasmic domain (f- loop) (Matsuda *et al.*, 1997; Shigekawa and Iwamoto, 2001). Mature NCX1 protein comprises 9 transmembrane domains, two loops formed by α repeats, a large hydrophilic cytoplasmic loop with an internal C- terminius and external N-terminus (Shigekawa and Iwamoto, 2001; Philipson *et al.*, 2002; Lytton J, 2007). The N-terminal and C-terminal halves of the transmembrane contain 2 internally conserved repeat sequences of 40 amino acids designated as α1 and α2 (Matsuda *et al.*, 1997; Ruknudin and Lakatta, 2007; Hryshko and Philipson, 1997). These repeats are found on the opposite sides of membrane. The α1 repeat is formed by portions of trans-membrane segment 2 and 3, whereas α2 repeat is formed of portion between trans-membrane segment7 and 8 (Shigekawa and Iwamoto, 2001; Ruknudin and Lakatta, 2007; Hryshko and Philipson, 1997). It is hypothesized that the hydrophilic faces of α repeats form a portion of the ion translocation pathway. The putative loop regions of α repeats contain 3 conserved aspartic acids which are important for affinity of the substrate. Threonine 103 at the cytoplasmic portion of TMS2 α1 plays a very important role in the ionic selectivity of the exchanger (Shigekawa and Iwamoto, 2001; Lytton J, 2007).

The cytosolic domain is long with about 550 amino acids located between trans-membrane region 5 and 6 (Hryshko and Philipson, 1997). It is highly hydrophilic and can undergo dramatic conformational changes. It contains two Ca^{2+} binding regulatory sites, an inactivation region known as the exchanger inhibitory peptide (XIP) and sites for alternative splicing (Shigekawa and Iwamoto, 2001; Philipson *et al.*, 2002; Lytton J, 2007). The XIP region is a 20 amino acid segment at the N-terminal end of the cytoplasmic loop, near the membrane

lipid interface, it is rich in basic and hydrophobic residues. The XIP region functions as an autoinhibitory domain and plays a central role in the activation and inactivation of NCX activity. This region has been implicated in regulation of NCX1 function by both Na^+ and acidic phospholipids (Philipson *et al.*, 2002).

On the other hand, C- terminal to the XIP region has 2 conserved clusters of 135 acidic amino acids. These regions bind to Ca^{2+} with high affinity and act as Ca^{2+} regulatory sites. Calcium binding domain 1 binds four Ca^{2+} ions with high affinity at one end of the β repeats, this causes a conformational change which is thought to be responsible for the regulatory effect of Ca^{2+} binding on NCX1 (Hryshko and Philipson, 1997). Calcium binding domain 2 forms a structure homologous to the first, but it binds to Ca^{2+} with a lower affinity and without a large conformational change (Shigekawa and Iwamoto, 2001; Lytton J, 2007). Mutating Ca^{2+}-binding Asp residues within the calcium binding domain decreased Ca^{2+} affinity (D447V or D498I). On either side of the calcium binding domain there is a conserved internal repeat motif designated as β repeats. β1 overlaps the N-terminal portion of calcium binding domain, while β2 is located on the C-terminal side. The calcium binding domain and the β repeat form a folded structure for binding of Ca^{2+}. The structure is similar for all the isoforms of NCX (Lytton J, 2007).

NCX1 undergoes extensive splicing within the large intracellular loop. Splice variants are generated from the gene in a region corresponding to the carboxy terminus of intracellular loop in a tissue specific manner. Six cassette exons; A, B, C, D, E and F, code for these sites. These exons are expressed in different combinations in different tissues. Exons A and B are mutually exclusive thus maintaining an open reading frame. Exon A is present in excitable tissues while exon B is present in non excitable tissues. Heart contains NCX 1.1 while vascular tissue contains NCX 1.3 and 1.7 (Lytton J, 2007).

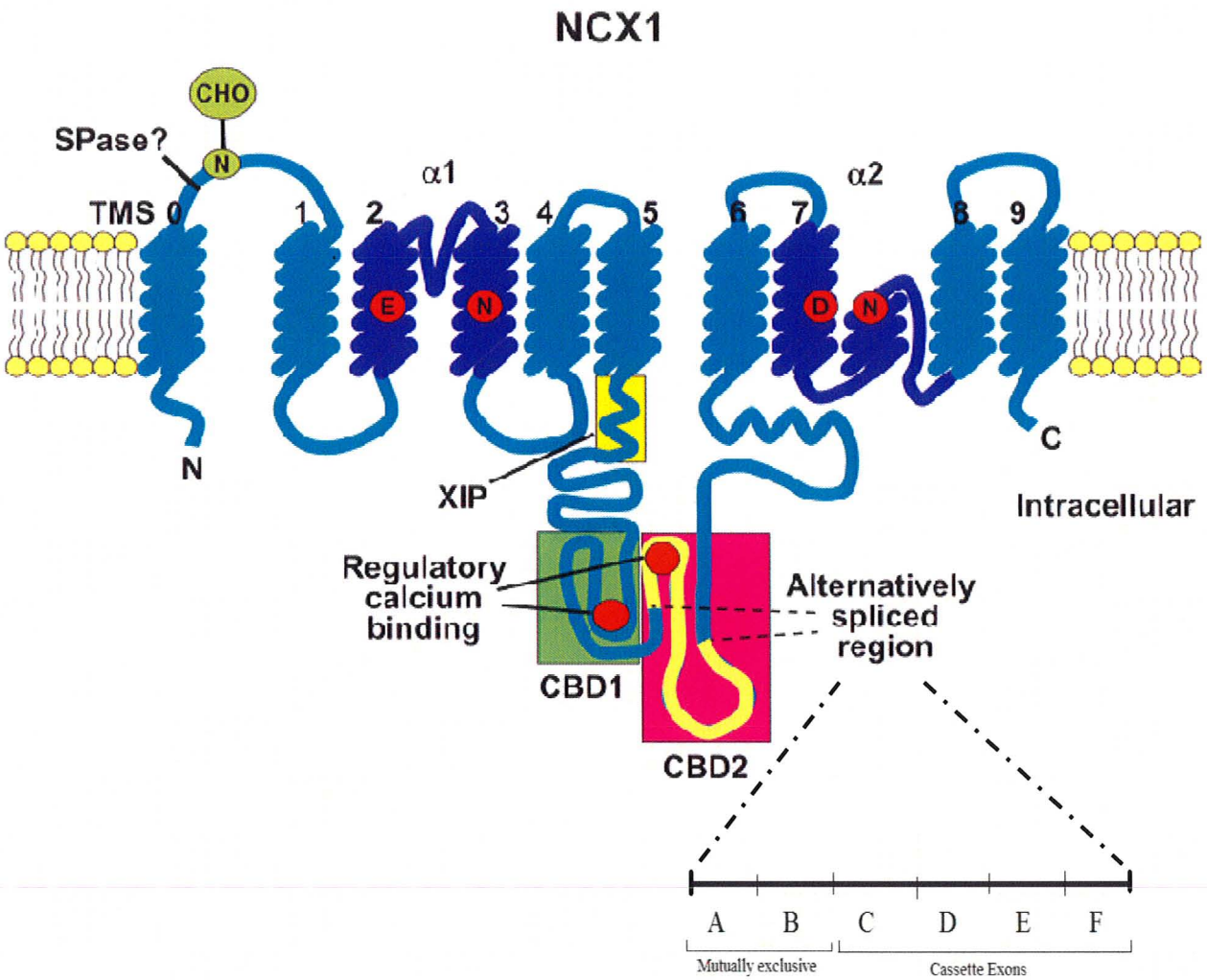

Figure 2: Topological model for NCX1

NCX1 has 9 transmembrane spanning segments (TMS). The cytosolic loop contains two calcium binding domains (CBD), a regulatory calcium binding site and a regulatory site (XIP). It also has a site for alternative splicing. *(Modified from Lytton J, 2007)*

1.4.1.2 Regulation

The rate of transport of Ca^{2+} by NCX depends on various factors like kinetic factors, amount of exchange protein present, occupancy of ion binding sites and the regulation of activity. NCX1 function is regulated by a variety of intracellular and extracellular factors. Several factors involved in regulation of NCX activity include intracellular concentrations of Na^+ and Ca^{2+}, intracellular pH, metabolic compounds like ATP, PIP2, phosphoarginine, various kinases and phosphatases, phospholemman, redox agents and nitric oxide (Shigekawa and Iwamoto, 2001; Lytton J, 2007; Blaustein and Leaderer, 1999).

NCX is subject to two forms of intrinsic regulation by the transport substrates Na^+ and Ca^{2+} designated as I1 and I2 respectively. An increase in intracellular Na^+ (25–100 mM) inactivates NCX and is termed as the Na^+ dependent inactivation. An increase in cytoplasmic Ca^{2+} in sub-micromolar concentration (0.2 – 1 μM) promotes NCX activity and reverts Na^+ dependent inactivation. Ca^{2+} binds to the acidic clusters in Ca^{2+} regulatory sites in the central region on the large cytosolic loop of NCX with high affinity at a site distinct from the Ca^{2+} transport site (Haworth and Goknur, 1997; Matsuda *et al.*, 1997; Shigekawa and Iwamoto, 2001; Lytton J, 2007). Ca^{2+} bound to the regulatory site is not transported. Binding of regulatory Ca^{2+}activates NCX activity with an apparent *K*d of 0.2–0.4 mM. Unlike Na^+, Ca^{2+} has two distinct binding sites, one for Ca^{2+} transport and another for Ca^{2+} regulation (Dipolo and Beauge, 2006; Shigekawa and Iwamoto, 2001). No exchanger activity is seen in the absence of regulatory calcium. The intracellular Ca^{2+}-dependent activation might be a protective mechanism that only allows NCX to be turned on when $[Ca^{2+}]i$ is high and off when $[Ca^{2+}]i$ is low. Na^+ dependent inactivation is enhanced in an acidic environment and attenuated by Ca^{2+}, ATP and PIP2 (Shigekawa and Iwamoto, 2001; Matsuda *et al.*, 1997).

In addition to substrate ions Na^+ and Ca^{2+}, NCX activity is also dependent on the intracellular pH. Na^+ dependent inactivation is enhanced by high intracellular proton concentration (low pHi), while high pHi increases the exchange activity (Matsuda *et al.*, 1997; Doering and Lederer, 1993). The hydrophilic cytosolic loop of NCX is the target for proton action. The inhibitory action depends on the concentration of $[Na^+]I$ (Shigekawa and Iwamoto, 2001).

ATP is another important activator of NCX. Cardiac NCX activity is intrinsically regulated by ATP dependent mechanisms (Blaustein and Leaderer, 1999; Dipolo and Beauge, 2006). High ATP concentrations stimulate NCX activity by acting as a phosphoryl donor molecule (Haworth and Goknur, 1997; Shigekawa and Iwamoto, 2001; Matsuda *et al.*, 1997). Depletion of ATP in adult cardiomyocytes diminished NCX activity by 80%. Cellular ATP depletion inhibits NCX1 and 2, but does not affect NCX3 activity. ATP depletion affects many aspects of cell metabolism, reduced phosphorylation of proteins and alteration of cytoskeleton structure. ATP depletion may inhibit NCX activity by changing its membrane anchorage due to disruption of the cytoskeleton (Shigekawa and Iwamoto, 2001). ATP does not affect the transport rate at saturating substrate concentrations, suggesting that it acts as a catalyst for binding and not as a source of energy (Blaustein and Leaderer, 1999). The second mode of action of ATP involves production of phosphorylated phospholipid, PIP2. PIP2 stimulates exchange activity by removing Na^+ dependent inactivation. PIP2 plays a constitutive regulatory role in cardiomyocytes and maintains a high NCX activity. Changes to PIP2 and other phospholipids in response to external stimuli contribute to regulation of NCX activity (Shigekawa and Iwamoto, 2001; Matsuda *et al.*, 1997; Lytton J, 2007).

The XIP region at the N-terminus of the cytosolic domain has a major influence on Na^+ dependent inactivation. Na^+ interacts with the regulatory site (XIP) other than the transport site during the Na^+ dependent inactivation process (Lytton J, 2007). The XIP region is also involved in PIP2 activation of NCX activity (Shigekawa and Iwamoto, 2001).

A number of other proteins associate with NCX1 on the intracellular loop and regulate its transport function. Various kinases like PKA and PKC and phosphatases such as PP1 and PP2A are known to interact with NCX1. These proteins regulate the phosphorylation of NCX1 (Ruknudin and Lakatta, 2007; Shigekawa and Iwamoto, 2001). PKC and PKA cause phosphorylation of NCX leading to stimulation of exchange activity (Lytton J, 2007). Extracellular signals activate NCX activity by mechanisms involving PKC activation. Another phosphatase, calcineurin (PP2B), associates with all three isoforms of NCX. It interacts with the β1 repeat of the intracellular loop region of NCX1 and decreases its activity (Ruknudin and Lakatta, 2007; Shigekawa and Iwamoto, 2001; Lytton J, 2007).

Another important regulator of NCX is phospholemman, a 15kDa protein with a single transmembrane domain (Lytton J, 2007; Cheung *et al.*, 2007; Ruknudin and Lakatta, 2007). It is a member of a family of transport regulators and shares sequence homology with the SERCA regulator phospholamban (Cheung *et al.*, 2007). Phospholemman, which is expressed abundantly in the heart, is an endogenous inhibitor of NCX activity. The effects of phosphorylation of phospholemman by PKC and PKA can influence NCX activity directly or indirectly (Lytton J, 2007; Cheung *et al.*, 2007). Phosphorylation at Ser68 by PKA causes the C-terminal cytosolic tail of phospholemman to bind to two regions of the N-terminal region of the intracellular loop and inhibit NCX function (Ruknudin and Lakatta, 2007). Phospholemman inhibited I_{NaCa} by interacting with regions encompassing residues 238–270 and 300–328 of NCX1. Its cytoplasmic

tail associates with the proximal linker domain of NCX1 at the cytoplasmic surface of the plasma membrane . In a similar way, sorcin is also a small phosphoprotein and is thought to influence the activity of NCX (Ruknudin and Lakatta, 2007).

Activity of NCX is sensitive to different combinations of reducing or oxidizing (redox) reagents. Redox agents like dithiothreitol, glutathione, $FeSO_3$, hydrogen peroxide and superoxide result in marked stimulation of NCX activity by attenuating the intracellular Na^+-dependent inactivation (Reeves *et al.*, 1986). Hydrogen peroxide as well as the superoxide generated from the xanthine - xanthine oxidase reaction, substantially enhanced NCX-mediated Ca^{2+} fluxes, while a strong oxidant such as hypochlorus acid (HOCl) inhibited NCX mediated Ca^{2+} influx (Zima and Blatter, 2006). Oxidants like $FeSO_4$ and dithiothreitol caused redox modification of NCX leading to removal of Na^+ dependent inactivation of the exchanger (Shigekawa and Iwamoto, 2001). Stimulation of the exchanger activity requires a combination of reducing agents like dithiothreitol, glutathione or Fe^{2+} along with oxidizing agents like hydrogen peroxide, or glutathione disulfide (Zima and Blatter, 2006). The sensitivity of the exchanger to ROS affects the exchange of substrate ions Na^+ and Ca^{2+} across plasma membrane under the conditions of oxidative stress. Redox stimulation appears to be substrate-dependent as it is promoted by the presence of Na^+ and is antagonized by Ca^{2+}. Redox agents seem to act directly on the Na^+- Ca^{2+} exchange carrier, perhaps by promoting thiol-disulfide exchange. Metal ions may mediate the transfer of electrons between the redox reagents and the appropriate thioldisulfide groups in the exchange carrier and increase the transport activity (Reeves *et al.*, 1986).

Phosphoarginine is another metabolic regulator of NCX which increases the activity of NCX in calcium removal mode (Blaustein and Leaderer, 1999). The mechanism of action of phosphoarginine requires intracellular Mg^{2+} and Ca^{2+}. Unlike ATP, it does not affect the

affinities for the transported ions. However, similar to ATP, activation by phosphoarginine also appears to involve phosphorylation – dephosphorylation reactions (Blaustein and Leaderer, 1999).

Nitric oxide (NO) is an important modulator of vascular function. The release of NO from the vascular endothelium causes vasorelaxation in vascular smooth muscle through the activation of the guanylate cyclase/cGMP/PKG system. NCX-mediated vasoconstriction is regulated by the endothelium via guanylate cyclase/NO in the rat aorta (Zhao and Majewski, 2008). The underlying mechanisms probably involve the enhancement of NO release from the endothelium and do not involve a direct effect of NO on NCX in the smooth muscle (Zhao and Majewski, 2008). This finding may be important under pathological conditions where NCX operates in reverse mode, such as during ischaemia. In ischaemia, NO release from the endothelium is diminished and this would unmask the effects of NCX, which under ischaemic conditions operates in reverse mode increasing the $[(Ca^{2+})_i]$ and therefore mediates vasoconstriction (Schulz *et al.*, 2004). In this way, NCX may exacerbate overall vasoconstriction in ischaemic conditions.

1.4.1.3 Inhibitors

NCX inhibitors are crucial in understanding the physiological role of NCX and for application in pathological conditions (Iwamoto and Kita, 2004). There are different types of inhibitors such as inorganic ions, synthetic peptides and organic compounds. These inhibitors are poorly specific to NCX and block other ion transporters and channels at low doses (Shigekawa and Iwamoto, 2001; Blaustein and Leaderer, 1999). They also exert blocking effects on other cell systems sometimes showing cardiovascular activities, which limits their use as specific blockers

of NCX activity. In addition, these NCX inhibitors also lack the specificity to inhibit NCX in a particular mode of action.

A variety of divalent and trivalent cations with high density of positive charge like Ni^{2+}, Mn^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, La^{3+} and Gd^{3+} often bind to Ca^{2+} binding sites, and so have been employed as substitutes or inhibitors for various Ca^{2+} dependent processes. Cations like La^{3+}, Ni^{2+}, Mn^{2+} and Cd^{2+} can enter cells, as a surrogate for Ca^{2+} on the Na^{+}/Ca^{2+} exchanger. These metal ions inhibit Na^{+}/Ca^{2+} exchange, but none of these ions are specific inhibitors of NCX (Iwamoto T, 2004; Shigekawa and Iwamoto, 2001; Blaustein and Leaderer, 1999).

Numerous structurally distinct organic molecules have been tested for their ability to inhibit the NCX. Some of the most effective and potent compounds identified to date are certain amiloride analogs. Amiloride, an acylguanidine diuretic, is, itself, a weak inhibitor of the exchanger (IC_{50} ~1 mM), but it is a much more potent blocker of certain epithelial Na^{+} channels (Blaustein and Leaderer, 1999). Amilorides are potent inhibitors of Na^{+}-H^{+}-exchanger (NHE) compared to NCX (Pedersen *et al.*, 2007). Hydrophobic substitutions at the acylguanidinium nitrogen with a benzyl group or at the 5-position amino group of the pyrazine rings, substantially increase the potency to block NCX. Unfortunately, many of these compounds have very limited solubility in aqueous solutions. The major problem with amiloride derivaties is their lack of specificity for NCX (Pedersen *et al.*, 2007; Watanabe *et al.*, 2006). Several antiarrhythmic agents such as quinacrine and bepridil (a substituted pyrrolidine ethanamine) also inhibit the NCX (Shigekawa and Iwamoto, 2001). These molecules, too, are nonselective; they inhibit other transport processes as well, at concentrations similar to those needed inhibit the exchanger (Watanabe *et al.*, 2006). A number of other molecules such as ascorbic acid and other

antioxidants are weak inhibitors of the NCX (Watanabe *et al.*, 2006). Again, however, these molecules all have other actions that can be expected to overshadow their effects on NCX.

XIP, an intrinsic regulator of NCX is the most potent and selective inhibitor of NCX activity. The XIP derived from the primary sequence of cardiac NCX1 decreases the Vmax of NCX activity (Matsuoka *et al.*, 1997). It inhibits NCX activity with an IC_{50} value of about 1.5 μM (Shigekawa and Iwamoto, 2001). It is significantly less potent in inhibiting Na^+-dependent Ca^{2+} efflux from sarcolemmal vesicles than in inhibiting the reverse reaction. XIP has little effect on Na^+,K^+-ATPase, SR Ca^{2+}-ATPase, or L-type Ca^{2+} currents, and it does not increase membrane conductance when applied to the intracellular surface by use of the excised-patch technique (Matsuda *et al.*, 2005). Synthetic forms of XIP are effective NCX inhibitors but the major problem is their inability to cross the cell membranes. Thus, its usefulness as an NCX inhibitor is limited because it acts only from the cytoplasmic side (Blaustein and Leaderer, 1999; Shigekawa and Iwamoto, 2001). Furthermore, it may bind calmodulin and could interfere with the function of calmodulin- binding proteins and thus it is difficult to interpret the physiological consequences of XIP (Matsuda *et al.*, 2005).

In addition to XIP and other organic compounds, other peptides, such as the molluscan cardioexcitatory tetrapeptide Phe-Met-Arg-Phe-NH2 (FMRFa) and its analogues and the cyclic hexapeptide Phe-Arg-Cys-Arg-Cys- Phe-CONH2 (FRCRCFa), have been reported to inhibit NCX activity (Shigekawa and Iwamoto, 2001; Blaustein and Leaderer, 1999).

Benzyloxyphenyl derivatives KB-R7943, SEA 0400 and SN-6 are more potent and selective inhibitors for NCX. KB-R7943 (2-[2-[4-(4 nitrobenzyloxy) phenyl]- ethyl]isothiourea methanesulfonate) has an isothiourea group in the molecule which is an important characteristic of NCX inhibition. KB-R7943 inhibits the Ca^{2+} influx through NCX in cultured cardiomyocytes

and vascular smooth muscle cells at micromolar concentrations (IC_{50} = 1.2–2.4 μM) (Iwamoto and Kita, 2004; Shigekawa and Iwamoto, 2001). However, surprisingly, KB-R7943 scarcely inhibits the Ca^{2+} efflux through NCX (IC50 = ~ 30 μM). In fact, KB-R7943 suppresses the outward current of NCX in cardiomyocytes over 60 times more strongly than the inward current (Shigekawa and Iwamoto, 2001). In other words, KB-R7943 is an agent that inhibits selectively the Ca^{2+} influx mode of NCX. It has little effect on other ion transporters like Na^+/H^+ exchanger, Na^+, K^+-ATPase, and Ca^{2+}-ATPases up to 10 μM (Iwamoto and Kita, 2004; Iwamoto T, 2004; Matsuda *et al.*, 2001; Watanabe *et al.*, 2006). While at higher concentrations it significantly suppresses various cation channels. KB-R7943 has isoform selectivity; it inhibits NCX3 approximately 3 times more strongly than NCX1 or NCX2. The C-terminal side of the repetitive sequence in the molecules (the α-2 region) was the important site of action of the agent. The ion transport pathway of NCX consists of the membrane loops of the α-1 and α-2 regions that face each other and transmembrane regions at both sides of two membrane loops (Shigekawa and iwamoto, 2001). All the important amino acids associated with the affinity of KB-R7943 exist on the membrane loop of the α-2 region. It is thought that KB-R7943 inhibits ion transport by blocking pores formed in a region from the outside of the cell (Iwamoto and Kita, 2004; Watanabe *et al.*, 2006).

SEA 0400 (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline), is a stronger and more specific inhibitor than KB-R7943 (Matsuda *et al.*, 2001; Matsuda *et al.*, 2005). The inhibitory action of SEA 0400 is approximately 100 times stronger than that of KB-R7943. SEA 0400 and KB-R7943 have a common benzyloxyphenyl structure (Iwamoto and Kita, 2004; Amran *et al.*, 2003; Matsuda *et al.*, 2005; Watanabe *et al.*, 2006; Shigekawa and Iwamoto, 2001). This inhibitor is highly specific to NCX as it scarcely inhibits other receptors, channels

and transporters at the concentrations that the agent inhibits NCX. SEA 0400 inhibits dose-dependently the Ca^{2+} influx through NCX in cultured nerve cells (IC50 = 5–33 nM) and myocardial cells (IC50 = 92 nM). SEA0400 at 3 μM does not affect L-type Ca^{2+} channels, N-type Ca^{2+} channels, Na^{+} channels or other ion channels and exchangers including Na^{+}-H^{+} exchanger, K^{+} channel or SOCC (Iwamoto and Kita., 2004; Matsuda *et al.*, 2005).

Another benzyloxyphenyl derivative SN-6 showed inhibitory potency for NCX1 similar to KB-R7943, but was more specific for NCX1 than KB-R7943 (Watanabe *et al.*, 2006). SN-6 predominantly works as a blocker of Ca^{2+} overload via NCX under hypoxic/ ischemic conditions. This inhibitor preferentially acts on NCX under ATP-depleted conditions (Iwamoto, 2004; Akabas , 2004).

Even though NCX isoforms share 70% homology, the interaction domain of each benzyloxyphenyl derivative seems to differ among isoforms. Three benzyloxyphenyl derivatives NCX inhibitors differ in their potency and isoform specificity (Matsuda *et al.*, 2005; Shigekawa and Iwamoto, 2001). These inhibitors showed differences in their IC_{50} values when tested on NCX1 in fibroblasts (SEA 0400, IC_{50} = 0.056 μM; KB-R7943, IC_{50} = 4.3 μM; SN-6, IC_{50} =2.9 μM for NCX1) (Iwamoto T, 2004). These inhibitors have different isoform selectivities: KB-R7943 is 3-fold more selective for NCX3 than for NCX1 and NCX2, whereas SEA 0400 predominantly blocks NCX1, it can mildly block NCX2, and exerts almost no influence upon NCX3. SN-6 is selectively inhibits NCX13- to 5-fold more than NCX2 and NCX3 (Iwamoto T, 2004; Shigekawa and Iwamoto, 2001). Accordingly, these benzyloxyphenyl derivatives should be properly used depending on the target organs, which express specific NCX isoforms. Interestingly, all benzyloxyphenyl derivatives inhibit the Ca^{2+} influx mode by NCX1 much more effectively than the Ca^{2+} efflux mode, although its mechanism is unknown at present. The

inhibitory effects of benzyloxyphenyl derivatives are related to the kinetics of I1 inactivation. In KB-R7943, the α2 repeat region seems to be almost exclusively responsible for the differential drug responses between NCX1 and NCX3. On the other hand, in SEA0400 and SN-6, the first intracellular loop and the fifth transmembrane span (and part of the XIP region) are mostly responsible for the difference in the drug responses. Site-directed mutagenesis within these regions reveal Glycine - 833 and Asn-839 in NCX1 are common molecular determinants required for inhibition by all benzyloxyphenyl derivatives (Iwamoto, 2004; Shigekawa and Iwamoto, 2001).

1.4.1.4 Physiological role of NCX

The NCX in the plasma membrane operates in parallel with several other Ca^{2+} transport systems (e.g., the PMCA pump and Ca^{2+} channels) and in series with other Ca^{2+} transport systems that are located in organellar membranes (e.g., SER Ca^{2+} pumps, mitochondrial Ca^{2+} transport) and plays an important role in regulation of cellular Ca^{2+} (Carafoli, 2002; Carafoli, 2009; Guerini, 1998; Shin *et al.*, 2008; Clapham, 2007; Shigekawa and Iwamoto., 2001; Blauestein and Leaderer, 1999). Different aspects like its localization, bidirectional mode of action and control of SER Ca^{2+} content shed light on the physiological role of NCX.

Immunocytochemical studies for some cell types have revealed the specific localization of NCX. In smooth muscle cells (both freshly isolated and cultured), and in cultured astroglial cells and neurons, NCX appears to be confined to regions of the PM that are closely opposed to underlying junctional SER . This distribution of NCX contrasts with the uniform distribution of PMCA (Floyd and Wray, 2007; Blauestein and Leaderer, 1999). This difference in distribution of the two Ca^{2+} transport systems suggests differences in their functional basis. PMCA is a high

affinity Ca^{2+} transporter involved in regulation of low Ca^{2+} levels- it has a housekeeping role; whereas NCX is a high capacity exchanger, which plays an important role in lowering the high cytosolic Ca^{2+} (Guerini, 1998; Shin *et al.*, 2008; Clapham, 2007; Shigekawa and Iwamoto., 2001; Blauestein and Leaderer, 1999). At junctional spaces underlying SER it plays an indirect role in modulating the Ca^{2+} stores. NCX is abundant in the presynaptic nerve terminal for the modulation of Ca^{2+} dependent release of neurotransmitters relative to other areas of the neuron, while PMCA is close to the synaptic vesicle docking site (Blauestein and Leaderer, 1999). The cell body shows distribution of the exchanger in a reticular pattern over the surface similar to smooth muscle and astrocytes. In contrast to the focal distribution in smooth muscle cells, cardiac myocytes have a uniform distribution of NCX and it is involved in cardiac excitation – contraction coupling. In skeletal muscle, the exchanger may be absent from the peripheral (surface) PM and may be confined to transverse tubule (t-tubule) membrane. It is suggested that NCX, PMCA pump isoform 1 (PMCA1), and the dyhydropyridine receptor (i.e., voltage-gated Ca^{2+} channel) may all be confined to junctional regions of the t-tubule membrane (Blauestein and Leaderer, 1999).

NCX is a bidirectional exchanger and can mediate both entry and exit of Ca^{2+} in the cell. The direction of transport is determined by the electrochemical gradient, while the concentration of the substrate ions and other regulatory factors (as mentioned in section on regulation of NCX) determine the rate of transport (Guerini, 1998; Shin *et al.*, 2008; Clapham DE., 2007; Shigekawa and Iwamoto., 2001; Blauestein and Leaderer, 1999). NCX plays an important role in the extrusion of Ca^{2+} in many types of cells, following the periods of stimulation of the cell (i.e., when membrane potential is more negative than Na^{+}/Ca^{2+} potential) and $[Ca^{2+}]i$ is elevated. The exchanger has capacity to extrude large amounts of intracellular Ca^{2+} as compared to other Ca^{2+}

transport systems. It has 10-fold higher turnover rate than that of the PMCA pump (Wray and Floyd, 2007; Shigekawa and Iwamoto, 2001; Dipolo and Beauge, 2002, Guerini, 1998; Shin *et al.*, 2008; Clapham, 2007; Blauestein and Leaderer, 1999). In addition to the Ca^{2+} extrusion, NCX mediated Ca^{2+} influx plays an important physiological role in certain cell types and some pathological conditions. NCX operates primarily in Ca^{2+} influx mode in the erythrocytes of dogs, and some rodents and other carnivores. Furthermore, the exchanger plays an important role in cardiac myocytes in Ca^{2+} influx mode. The increase in Ca^{2+} flux due to NCX is involved in excitation- contraction coupling (Blauestein and Leaderer, 1999). Similarly, the high density of NCX at the presynaptic nerve terminals are involved in excitation – secretion coupling due to NCX mediated Ca^{2+} entry. Purinergically stimulated rat aortic smooth muscle cells showed increase in Ca^{2+} influx primarily by the NCX. Stimulation caused Na^{+} entry through nonspecific cation channels (NSCCs), increasing $[Na^{+}]_i$. This could drive NCX in Ca^{2+} entry (reverse) mode in localized regions of the plasma membrane (Lemos *et al.*, 2007). Small increases in the cytosolic Ca^{2+} occur in microdomains. This increase in Ca^{2+} in microdomains can be due to Ca^{2+} influx or efflux, however due to lack of selective inhibition it is difficult to resolve this definitively. In smooth muscle cells the Ca^{2+} entry mode of NCX is involved in refilling of SERCA (Fameli *et al.*, 2007; Nazer and van Breeman, 1998; Hirota, Pertens, and Janseen, 2007; Blaustein and Lederer, 1999; Matsuda *et al.,* 2006). NCX may influence the $[Ca^{2+}]_i$ and indirectly modulate SER Ca^{2+} load. It can also trigger Ca^{2+} release from SER. An increase in the cytosolic Ca^{2+} due to operation of NCX in reverse mode is involved in pathological conditions such as hypertension (Iwamoto, 2004) , which is discussed later in the pathophysiology section.

Localization of the NCX in the plasma membrane that overlies junctional SER may provide a clue about one of the functions of the exchanger (Nazer and Van Breemen, 1998). This

localization of the exchanger implies that it probably plays a role in loading and unloading the Ca^{2+} stores or at least in modulating the fractional saturation of the stores and influences overall cell activity. NCX are localized in the lipid raft microdomains of plasma membrane known as caveolae along with other transporter proteins which leads to formation of specialized regulation pockets (Kuszczak *et al.*, 2010; Pani *et al.*, 2008; Kuszczak *et al.*, 2011). Na^+K^+ATPase are colocalized with NCX in the PM regions that overlie junctional SER (Floyd and Wray, 2007) Inhibition of the Na^+ pump with an endogenous ouabain-like compound or change in the membrane potential in the microdomain, influences NCX to operate in its Ca^{2+} influx mode. The increase in Ca^{2+} in the restricted cytosolic space between PM and SER consequently increases the amount of Ca^{2+} stored in SER (Nazer and Van Breemen, 1998; Blauestein and Leaderer, 1999). Transient receptor potential channel 6 (TRPC6) are also localized close to NCX in the microdomains (Pani *et al.*, 2008). Na^+ entry mediated byTRPC6 is responsible for peripherally localized $[Na^+]i$ transient (LNat) elevation. These LNats are restricted to subsarcolemmal cytoplasmic microdomains and facilitate Ca^{2+} entry via NCX (Lemos *et al.*, 2006).

1.4.1.5 Pathophysiology and effects of ROS

Ca^{2+} influx through plasma membrane channels plays an important role in regulating myogenic tone and vasoconstriction. Vascular myogenic tone and an increase in cytosolic Ca^{2+} contribute to the increased peripheral vascular resistance in hypertension (Pulina *et al.*, 2010). NCX has been implicated in a variety of vascular pathologies including oxidative stress, hypertension, hypoxia and ischemia reperfusion. Other than in the cardiovascular system; NCX also plays a role in degeneration of white mater after spinal cord injury, optic nerve injury and

brain trauma (Blauestein and Leaderer, 1999; Toshihide, 2009; Diedrichs *et al.*, 2007; Zhang *et al.*, 2010).

The effects of ROS on NCX vary from tissue to tissue. The effects of ROS have been associated with oxidative modification of thiols on both SERCA and NCX (Kuster *et al.*, 2010). The effects on NCX may depend on the splice variants and hence the effects may vary between tissues. In a patch clamp study, hydrogen peroxide irreversibly increased the NCX-mediated Ca^{2+}-entry in cells overexpressing NCX1.1 (cardiac variant) without affecting Ca^{2+}-efflux. In direct contrast, hydrogen peroxide decreased the Ca^{2+}- entry by NCX1.3 (vascular variant) (Soliman *et al.*, 2009). In rabbit ventricular cardiomyocytes, ROS induced an elevation in NCX1, a mechanism likely contributing to myocyte dysfunction and death in heart failure. The NCX modulation by ROS may be complicated further due to the interactions with other pathways. In guinea pig ventricular myocytes, hydrogen peroxide increased the NCX current via two signal transduction pathways: one involves NHE and PI3K-dependent mechanism while the other acts via activation of a Src family tyrosine kinase (Hinata *et al.*, 2007). The effects may also vary with the types of ROS. In brain synaptic vesicles and transfected CHO-K1 cells, hydrogen peroxide (up to 800 μM) had no effect on NCX, whereas, peroxyl radicals and peroxynitrite caused oligomerization and fragmentation of NCX leading to its inhibition (Huschenbett *et al.*, 1998). In addition to alteration of NCX activity, ROS may also cause regulation of NCX at the mRNA level.

Hypertension is associated with changes in the NCX activity in various arterial beds. Salt sensitive hypertension is triggered by calcium entry through NCX1 in arterial smooth muscle cells (Zhang *et al.*, 2007). Oxidative stress induced by hydrogen peroxide and peroxynitrites impairs NCX in salt sensitive hypertension. Functional activity and expression of Ca^{2+} regulatory

mechanism is significantly altered during heart failure (Shin *et al.*, 2008). Under pathological conditions such as cardiac ischemia/reperfusion injury and tachycardia, the exchanger protein levels are significantly increased causing Ca^{2+} overload due to elevated levels of intracellular Na^{+} (Iwamoto *et al.*, 2004; Shin *et al.*, 2008; Watanabe *et al.*, 2006). NCX may be one of the targets for improvement of Ca^{2+} mediated pathology. NCX specific inhibitors are investigated as a pharmaceutical tool to help combat hypertension and ischemia/reperfusion (Iwamoto T., 2004; Matsuda *et al.*, 2005).

Hypoxia has been proposed to inhibit NCX activity, however, recently Meng *et al* (2008) found that hypoxia induces elevation in $[Ca^{2+}]_i$ by NCX in reverse mode.

Cardiac arrhythmia is the leading cause of death in cardiovascular disease. NCX1 contributes to the development of cardiac arrhythmia by reduced extrusion of Ca^{2+} while it shows an increased Ca^{2+} influx by reverse mode. This shifts the Ca^{2+} extrusion / sequestration balance leading to Ca^{2+} overload. Upregulation of NCX1 in chronic hypertension or heart damage eventually leads to heart failure (Iwamoto *et al.*, 2004;).

Other than the cardiovascular system, pathology due to the action of NCX is observed in the brain. Excitotoxic stimulation during ischemia changes the membrane potential and ion gradient which causes entry of Ca^{2+} by reverse mode of NCX. This leads to unregulated Ca^{2+} entry and activation of a number of Ca^{2+} sensitive degradative enzymes leading to neuronal degeneration.

1.4.2 Na^{+}-Ca^{2+}-K^{+} exchanger

Similar to NCX, Na^+-Ca^{2+}-K^+ exchanger (NCKX) is also electrogenic and bidirectional. Calcium exchange is dependent on a Na^+ and K^+ gradient. Both these exchangers can operate to bring in or to remove calcium from the cytosol depending on the gradient and membrane potential (Dong *et al.*, 2006; Guerini D., 1998). NCKX primarily co-transports Ca^{2+} and K^+ from the cytosol to the extracellular space and allows Na^+ to flow into the cell. It exchanges four Na^+ for one of each of K^+ and Ca^{2+} (Dong *et al.*, 2006; Guerini D., 1998). NCKX does not play a major role in smooth muscle cell and therefore will not be discussed in further detail.

1.5 SER Refilling

1.5.1 SOCE – Stim, orai and TRPC

Ca^{2+} depletion in the SER acts as a trigger for Ca^{2+} entry to refill the SER by a pathway termed store operated calcium entry (SOCE). It is suggested that the activation of SOCE may be due to altered interactions between SER and PM. Two families of proteins play an important role in this process: Stim and Orai (Hewavitharana *et al.*, 2007; Abdullaev *et al.*, 2008; Bird *et al.*, 2008; Cahalan *et al.*, 2007). These proteins mediate three important steps in SOCE; sensing Ca^{2+} levels in the SER lumen, transduction of the signal to the plasma membrane and then opening of highly selective Ca^{2+} channels in the plasma membrane. After the SOCE mediated increase in cytosolic Ca^{2+}, it is sequestered into the SER by SERCA protein.

Stim1 protein is found in tubular SER and to some degree in the plasma membrane. Its role in SOCE has been shown by suppression of Stim1 expression that prevents SOCE (Aubart *et al.*, 2009; Lu *et al.*, 2009). Stim1 is N- glycosylated at its luminal side and acts a sensor of SER luminal Ca^{2+} concentration (Cahalan *et al.*, 2007, Dziadek *et al.*, 2007). When SER luminal Ca^{2+} concentration decreases below 100-200 μM, Stim1 undergoes a conformational change. In the basal state when SER Ca^{2+} stores are filled, Stim1 is a dimer stabilized by C-

terminal coiled - coil interaction. The C- terminal portion forms dimers, whereas the N- terminal ER domain is monomeric at basal SER Ca^{2+} concentrations. When the SER Ca^{2+} store is depleted, Stim1 oligomerization occurs and triggers its translocation towards the plasma membrane. Total internal reflection microscopy (TIRF) measurements indicate that the aggregated Stim1 in the SER approaches as close as 10-20 nm to the PM and interacts directly with Orai1 and possibly with other plasma membrane proteins (Walker *et al.*, 2008). Other members of the Stim family may also play a role but currently the nature of this role is not clear.

Orai is an integral plasma membrane protein and forms the channel component of SOCE (Hewavitharana *et al.*, 2007; Parekh *et al.*, 2010; Cahalan *et al.*, 2007). Three different Orai proteins (1, 2 and 3) display notable differences in their function. Orai 1 is the most potent in reconstituting Ca^{2+} influx in most cells, and it is highly selective for Ca^{2+}. The working model is that Stim1 clustering and translocation begins when SER luminal Ca^{2+} concentration falls below 300 μM and results in reorganization of Orai subunits in the PM to form Ca^{2+} channels. Part of the evidence is that expression of Orai 1 and 2 alone decreases the SOCE, while their coexpression with Stim1 leads to an increase in this activity. Another piece of evidence is the movement of Stim1 towards PM (Cahalan *et al.*, 2007). Finally, an increase in FRET between Stim1 and Orai1 has been demonstrated under the conditions that activate SOCE (Calloway *et al.*, 2009).

Another protein family known to play an important role in SOCE is the transient receptor potential (TRP) family. These proteins are non selective cation channels and have been divided into several subfamilies: TRPC, TRPM and TRPV. TRPC is the classical or the canonical protein family with the members TRPC 1-7. In some smooth muscles TRPC1 is abundant and plays an important role in SOCE, other members of TRPC have also been implicated in SOCE

(Ambudkar *et al.*, 2007; Worley *et al.*, 2007; Poburko *et al.*, 2008; Adebiyi *et al.*, 2010). TRPC have been shown to act as ROCC since they may be activated by metabolites such as diacylglycerol produced upon receptor activation or by direct linkage to IP3 activated channels in the SER (Alicia *et al.*, 2008). The ER sensor Stim1 can also bind to the TRPC proteins. Association between TRPC1 and Stim 1 has been reported in platelets and vascular smooth muscle cells (Ambudkar *et al.*, 2007; Ng *et al.*, 2009; Worley *et al.*, 2007). This Stim1-TRPC channel complex may lead to SOCE. The role of TRPC in SOCE is controversial with some studies supporting this role while others challenging it. In the A549 endothelial cell line, store operated and thapsigargin induced Ca^{2+} entry decreased by 50% and 25% respectively, when TRPC1 expression was suppressed selectively by antisense oligonucleotides directed against this protein (Brough *et al.*, 2001). In contrast, TRPC 1 is not obligatory for SOCE as vascular smooth muscle cells from TRPC1 null mice showed similar SOCE induced as those from wild-type mice (Dietrich *et al.*, 2007). To complicate matters, TRPC are also seen to physically and functionally interact with Orai 1 protein (Liao *et al.*, 2009) . TRPC1 may also form a ternary complex with Orai1 to perform the SOCE function. The role of TRPC in SOCE may be complicated by their ability to allow influx of Na^{+}. Hence, in actuality the mechanism of SOCE may be far more complex. In one model, TRPC, NCX and SERCA may form a signal linkage. The non-selective cation transport through TRPC would locally modulate Na^{+}-gradients (LNats), thereby altering the NCX mediated Ca^{2+} entry (Poburko *et al.*, 2008). In addition, the TRPM family has also been implicated in SOCE (Hecquet *et al.*, 2010).

1.5.2 NCX and SER refilling

A linkage between NCX and SERCA has been reported in blood vessels. The following is the experimental evidence for a *functional linkage* between NCX and SERCA in pig coronary artery smooth muscle cells. Immunofluorescence microscopy and biochemical experiments also suggest proximity between NCX and SERCA in smooth muscle cells cultured from this artery.

NCX plays an important role in SOCE and refilling of SER. Distribution of the Ca^{2+} transporter in membrane microdomains or positioning them in close proximity to cellular organelles, would make their effect reach beyond calcium homeostasis. Results from our laboratory demonstrate diminished Ca^{2+} entry via NCX in response to inhibition of SERCA with thapsigargin. Na^{+}-loaded smooth muscle or endothelial cells show NCX mediated Ca^{2+}-entry when placed in a Na^{+}-free solution. In smooth muscle, but not in endothelial cells, this Ca^{2+} entry is decreased by inhibition of SERCA with thapsigargin (Davis *et al.*, 2009). This indicates a functional linkage of SERCA and NCX. Other studies based on measurement of $[Ca^{2+}]$ i showed increase in Ca^{2+} entry via the reverse mode of NCX following depletion of SER calcium store. Van Breemen and colleagues found other evidence to support the linkage between NCX and SERCA by measuring $[Ca^{2+}]$i. In this study they observed release of Ca^{2+} from the SER in the absence of extracellular Ca^{2+} and inhibition of NCX activity. NCX seems to play an important role in SER refilling and they appear to be linked functionally. Due to their functional linkage, the proteins might be in close proximity to each other for efficient calcium homeostasis. Several models have been proposed to explain the NCX-SERCA relationship in various cells. One of them is a "junctional cytoplasmic space model" in which NCX and SERCA occur in close complex within a narrow cytoplasmic space between subsurface SER and the plasma membrane (Poburko *et al.*, 2008).

The functional studies of linkage of NCX and SERCA are supported by structural evidence from immunocytochemistry and co-localization studies. Immunocytochemical studies indicate that the exchanger in vascular smooth muscle cells appears to be restricted primarily to plasma membrane regions that are adjacent to SER (Kuszczak *et al.*, 2010). Localization of the proteins close to each other separated by a narrow space is characterized by restricted diffusion of calcium ions and can be explained by a "limited space diffusion model" to increase local Ca^{2+} to prevent further Ca^{2+} entry. Our lab carried out co-localization studies for the two proteins NCX and SERCA using fluorescence microscopy. When the coefficient of co-localization was calculated, NCX1 showed perfect co-localization with SERCA, while this was not always true for SERCA indicating a partial co-localization relation between the two proteins. The exchanger appears to be confined to plasma membrane regions that are closely apposed to underlying junctional SER. This structural evidence of co-localization implies that NCX plays an important role in loading of Ca^{2+} stores.

1.6 Measurement of intracellular calcium

Three different techniques are commonly employed for the measurement of intracellular calcium fluxes: (1) radioactive tracer analysis (2) patch clamp electrophysiology and (3) fluorescent Ca^{2+} indicators. Each technique has several advantages and disadvantages and involves measuring different parameters. Electrophysiology is an invaluable tool; this technique is capable of detecting tiny single channel currents. NCX activity can be stimulated by changes in voltage or changes in extracellular Na^{+}. Inhibitors are used to block the ion current or changes in $[Ca^{2+}]i$ to monitor NCX activity. These experiments yield a large amount of information about

NCX regulation in different cell or tissue types, however it is unable to detect electro-neutral transport.

Another method of detection using radioactive trace would measure the cellular uptake of radioactive $^{45}Ca^{2+}$. Reversal of Na^+ gradient can drive NCX in reverse mode leading to entry of $^{45}Ca^{2+}$. However the temporal resolution is relatively poor and therefore sub-optimal for measurement of stimulated Ca^{2+} fluxes. Also the background signal is generated by non specific adsorption of $^{45}Ca^{2+}$ within extracellular space. The other disadvantage associated with this technique is the use of radioactivity and safety concerns.

The third technique of using intracellular calcium indicators has recently become very popular owing to its safe, efficient and easy method of use. There are a large number of different calcium probes available for detection of intracellular calcium. Fluorescent Ca^{2+} chelators are introduced in cells by passive diffusion using the esterified form and are blocked inside by removal of ester moiety. Being relatively non polar, ester diffuses freely into the cells across plasma membrane. Cytosolic esterases cleave the ester group of the chelator and render it hydrophilic. This method has several advantages, importantly it gives a better insight into the relation between Ca^{2+} changes during excitation and contraction in addition to the relationship between organelle and membrane microdomains. Fluorescence microscopy can be useful to detect $[Ca^{2+}]i$ concentration at different domains within cells and the properties of Ca^{2+} wave generation. These fluorescent probes have become the method of choice.

1.7 Previous lab results

In smooth muscle cells, changes in the levels of Ca^{2+} cause contraction and relaxation of muscle cells and regulate the blood flow in the arteries. Changes in the $[Ca^{2+}]i$ levels affect the

arterial tone, therefore its levels have to be regulated tightly. Ca^{2+} transporters play a key role in this regulation. The high affinity pumps SERCA and PMCA keep $[Ca^{2+}]i$ levels low. NCX may work to extrude Ca^{2+} or bring it into the cell. Previous studies done in our lab focused on two proteins, NCX and SERCA, involved in regulation of $[Ca^{2+}]i$. These studies accessed Na^{+} dependent NCX mediated Ca^{2+} entry in pig coronary artery smooth muscle cells and endothelial cells. Endothelial cells had a 5 fold higher NCX activity compared to SMC (figure 3). The functional association between NCX and SERCA were determined by monitoring the changes in NCX mediated $^{45}Ca^{2+}$ entry upon alteration in SERCA activity. The SERCA pump was inhibited using thapsigargin. This inhibition of SERCA caused a decreased NCX mediated $^{45}Ca^{2+}$entry. This inhibition was not observed in SMC pretreated with the Ca^{2+} chelator BAPTA. BAPTA acts as a sink for Ca^{2+} and reduces the $[Ca^{2+}]_i$ this creates a gradient for NCX activity and there is a marked increase in NCX mediated Ca^{2+} entry (figure 4). These studies done in our lab showed that NCX and SERCA are functionally linked in pig coronary artery smooth muscle (Davis *et al.*, 2008). This functional linkage might be crucial for regulation of Ca^{2+} in arterial smooth muscle cells; however the nature of the linkage has yet to be identified.

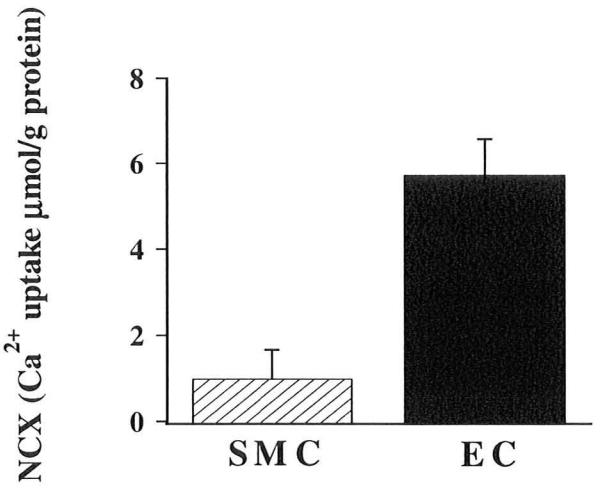

Figure 3: NCX mediated Ca^{2+} uptake in BAPTA loaded EC and SMC.

Ca^{2+} uptake was significantly greater in EC compared to SMC loaded with BAPTA ($p<0.05$).

(Modified from Davis *et al*, 2008)

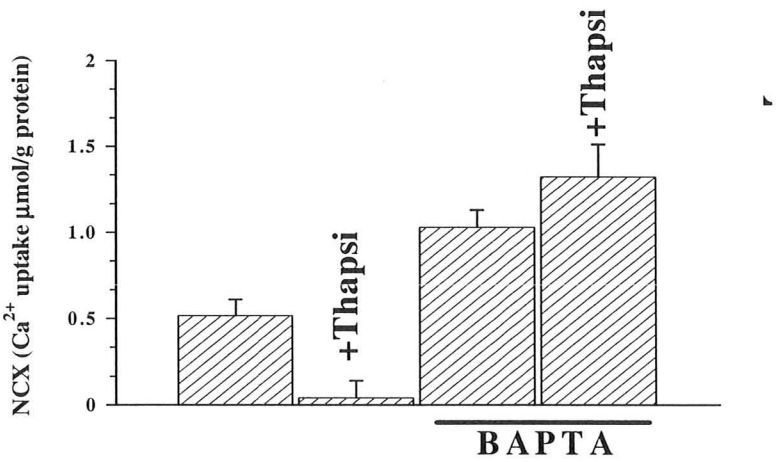

Figure 4: Effect of thapsigargin on the NCX mediated Ca^{2+} uptake in control and BAPTA loaded cultured smooth muscle cells. Smooth muscle cells were either preloaded with or without BAPTA prior to Na^{+} loading. SERCA inhibitor thapsigargin or DMEM is added. BAPTA loaded cells showed greater NCX mediated Ca^{2+} uptake ($p<0.05$). Thapsigargin

significantly inhibited NCX mediated Ca^{2+} uptake in the cells not loaded with BAPTA ($p<0.05$) but not in the cells loaded with BAPTA ($p>0.05$). (Modified from Davis *et al.*, 2008).

1.8 Overall objective

NCX plays an important role in regulation of $[Ca^{2+}]_i$. In coronary artery smooth muscle cells, NCX and SERCA are functionally associated to maintain $[Ca^{2+}]_i$ homeostasis. NCX is also thought to be involved in SER refilling. In order to achieve this functional linkage NCX and SERCA proteins could be physically linked or in close proximity of each other. The main objective of my thesis is to understand the influence of interactions between NCX and SERCA on changes in $[Ca^{2+}]_i$ in pig coronary artery smooth muscle cells.

Aim 1 is to determine the effect of Na^+ dependent NCX mediated entry on $[Ca^{2+}]_i$.

Rationale : The previous Na^+ dependent NCX mediated Ca^{2+} entry experiments determined the NCX activity level in SMC and EC. Unfortunately, these experiments did not indicate the actual concentration of Ca^{2+} change in the cell. Using Ca^{2+} sensitive fluorescent dyes aim 1 will monitor the changes in $[Ca^{2+}]_i$ upon Na^+ dependent Ca^{2+} entry through NCX.

Aim 2 is to verify the effect of SERCA inhibition on NCX mediated changes in cytosolic $[Ca^{2+}]$ levels.

Rationale: Previous experiments on cultured smooth muscle cells showed diminished Na^+ dependent $^{45}Ca^{2+}$ entry through NCX on inhibition of the SERCA pump with thapsigargin. This showed a functional coupling of NCX and SERCA for regulation of cytosolic Ca^{2+}. The effect of this linkage on cytosolic Ca^{2+} needs to be verified.

Aim 3 is to assess changes in the physical linkage or co-localization of NCX and SERCA proteins in response to SERCA inhibition and SER Ca^{2+} depletion.

Rationale: Previous experiments showed a functional linkage between NCX and SERCA. Inhibition of SERCA with thapsigargin would block the SER Ca^{2+} sequesteration, this caused diminished Ca^{2+} entry through NCX. The two proteins NCX and SERCA might therefore, be physically close to each other. Here, I would like to determine if the localization of NCX and subsurface SERCA are altered in the experiments conducted for Aim 2.

Pig coronary artery is used as an experimental model, since the anatomy and physiology of pigs is similar to that of humans. Our lab has done extensive research using pig hearts, especially in calcium related studies. In addition, large quantities of fresh hearts can be obtained on a routine basis from a large abattoir.

2.0 MATERIAL AND METHODS

2.1 Materials

Bovine serum albumin,, ouabain, nystatin, probenecid and thapsigargin were purchased from Sigma Aldrich (St Louis, Missouri, USA). KB-R7943 was purchased from Tocris Biosciences (Ellisville, Missouri, USA). Nitrendipine was purchased from Miles Pharmaceuticals (West Haven, Connecticut, USA). 4-Br A23187 was purchased from Teflabs (Austin, Texas, USA). Dulbecco's modified Eagle's medium (DMEM), Fluo 4AM, goat anti mouse alexa 488 and 568, goat anti rabbit alexa 488 and 568 were purchased from Invitrogen (Burlington, Ontario, Canada). SEA 0400 was synthesized by Dr. Lorand Kiss at the University of Szeged, Hungary. Rabbit polyclonal NCX 1 antibodyPi11-13 was obtained from Swant Laboratories (Switzerland) while mouse IID8 was purchased from Affinity Bio Reagents (CO, USA). Anti fade/mounting medium vectashield was purchased from Vector laboratories (Burlington, Ontario).

2.2 Methods

2.2.1 Smooth muscle cell isolation and cell culture

Fresh pig hearts were obtained and immediately kept in ice cold PSS containing (in mM): NaCl 138, $CaCl_2$ 2, Glucose 10, HEPES 10, KCl 5 and $MgCl_2$ 1.The left anterior descending (LAD) branch of the coronary artery was dissected in a Krebs buffer composed of the following (in mM): 120 NaCl, 22 $NaHCO_3$ 4.6 KCl, 1.1 K_2HPO_4, 1.1$MgSO_4$, 7.8 glucose, and 1.7 $CaCl_2$,, 0.03 EDTA. The pH was maintained by bubbling with 5% CO_2 / 95% O_2. Connective tissue, fat, and cardiac muscle were carefully dissected leaving the artery intact. The artery was pinned at the ends and de-endothelized gently by using a cotton swap. A layer of smooth muscle tissue was gently cut off from the artery leaving the adventitia tissue. Smooth muscle tissue was cut into small pieces, and placed in a tube containing DMEM. The tissue was immediately digested using digestion buffer made of the following (in mg/ml): collagenase type-1 5.8 , elastase 0.5, Bovine serum albumin 1, trypsin soyabean inhibitor 1, DNAase 0.15 and ATP 2.4 for an hour at 37 °C deg in DMEM in an incubator. The digestion buffer was changed and the tissue was incubated in fresh digestion buffer for 1 hour at 37 °C in an incubator. To disperse the cells, the tissue was then triturated with a serological pipette. The digestion buffer was passed through 300 micron mesh and was suspended on a cushion of fetal bovine serum (FBS) which was then centrifuged for 5 min at 200 rpm. The cells were removed from suspension and passed through a 40 micron mesh to obtain single isolated SMC. The resulting dispersed SMCs were plated on a cover glass in 35 mm petri-plate at 37°C in DMEM containing 10% FBS and placed in the incubator and used in experiments.

Smooth muscle cells were isolated from left coronary artery as described in above protocol and cultured as previously described (Grover *et al.*, 1985). The cells were grown to confluence and then replated into a larger flask. The culture media, DMEM, were supplemented with 0.5 mM HEPES, 2 mM glutamine, 50 mg/l gentamicin, 0.125 mg/l amphotericin B, and 10% fetal bovine serum (pH 7.4). After the second passage, the cells were frozen in aliquots of 2 million cells/ml. Confluent cells from passage 3 were plated on costar flat bottom 96 well plate for Ca^{2+} fluorescence experiments, for immunofluorescence experiments they were grown on 35 millimeter petri dishes which had a hole drilled through the bottom and a coverglass glued over the hole. These cells were used on day 7 of growth. The cells were characterized to ensure the purity of the batch. These cells reacted positively to anti-smooth muscle α-actin which is found in SMC and negatively to anti-eNOS and anti-von Willebrand factor, which are found in EC. This confirms the presence of SMC and the EC are absent in the cultured cell batch. This was carried out by our research assistant Sue Samson.

2.2.2 Measurement of intracellular Ca^{2+} for cultured smooth muscle cells

Sub confluent cells from passage 4 were used for experiments. Cells were plated in a 96 well plate. Wells were washed twice with Makhlouf's Buffer (MB) (containing in mM): NaCl, 115; HEPES, 25; Glucose, 12; KCl, 5.8; KH_2PO_4, 2.2; $MgCl_2$, 0.6; $CaCl_2$ 2] to remove DMEM and then were loaded with the calcium sensitive fluorescent dye Fluo-4 AM (4 μM), pluronic acid (0.02%) and probenecid (2 mM) in buffer and then incubated for 45 min in the dark at room temperature (22°C). Wells were washed twice with buffer to remove extracellular dye and then again placed in dark for 20 minutes for digestion of the dye in the ester form. Later cells were sodium loaded with calcium free buffer containing MOPS Tris (20 mM), oubain 1 mM, nystatin

25 μM, nitrendipine 10 μM and EGTA 100 μM for 20 min in dark. The cells were then washed twice with Na^+ free MOPS buffer containing MOPS Tris, nitrendipine and EGTA (as mentioned in buffer composition table 1) and then calcium free buffer containing 140 mM Na^+ or 140 mM NMG^+ buffer (Na^+ minimal buffer) was added. Background fluorescence was recorded and then 0.3 mM calcium was added to all the wells. Fluorescence was recorded on a fluorescence plate reader (Tecan Safire excitation 485 nm, emission 525 nm) for 10 minutes at 37°C. Each well was calibrated for Fmin using ionophore 4Br – A 23187 (6 μM) and 1 mM EGTA and then Fmax was obtained by adding excess calcium (1.5 mM) . For experiments in which inhibitors were added, the plate was divided in four sections: wells containing (a) Na^+ no inhibitor (b) NMG^+ no inhibitor (c) Na^+ with inhibitor (d) NMG^+ with inhibitor.

For SER depletion experiments, thapsigargin was added along with 140 mM Na^+ or 140 mM NMG^+ buffer (Na^+ minimal buffer), incubated for five minutes and then 0.3 mM calcium was added. This was followed by calibration with Fmin and Fmax.

2.2.3 For Primary culture smooth muscle cell

Isolated smooth muscle cells were plated on collagen coated cover glass and used on 7^{th} day of isolation. Experimental procedure was same as that of cultured smooth muscle cells. Briefly, cells on the cover glass were loaded with fluo-4AM for 45 minutes in MB in dark. Washed twice and then incubated for 20 minutes in dark. Then after sodium loading for 20 minutes, the cover glass was mounted on the microscope mounting plate. Fluorescence of single cells was recorded using a z-stack on a spinning disk confocal microscope.

2.2.4 Immunofluorescence Experiments

Immunofluorescence experiments were carried out on cultured smooth muscle cells. Each experiment was carried out over a period of two days and involved fixing and permeabilizing of cells, then overnight incubation with primary antibody followed by washing and labeling with secondary antibody the following day.

2.2.4.1 Fixing and permeabilization of cultured SMC

Cultured smooth muscle cells from passage 4 were grown on 35 mm tissue culture dish which had a hole drilled through the bottom and a coverglass glued over the hole.. Cells were washed thrice with phosphate buffer saline (PBS) containing (in mM) NaCl 137, KCl 2.7, Na_2HPO_4 8 and KH_2PO_4 1.5 (pH 7.4) (buffer composition table #) and then fixed for 10 minutes with 2% paraformaldehyde in PBS. Following fixation the cells were incubated with 0.1 M glycine in PBS to quench the effect of paraformaldehyde. Cells were washed with PBS for 10 minutes and then dehydrated using ice cold methanol for 15 minutes at -20^0C. Later on the cells were rehydrated by washing thrice with PBS for 5 minutes. All the non specific sites were blocked by adding 10% goat serum in PBS for one hour at room temperature. For the experiments carried out using thapsigargin, prior to fixing, cells were washed thrice with PBS and then Na^+ loaded using MOPS tris buffer containing ouabain, nystatin and nitrendipine as mentioned in table # for 15 minutes. The cells were then incubated for 5 minutes in NMG buffer containing thapsigargin (3μM) or DMSO (vehicle) and EGTA (0.5mM). Following this 0.3 mM Ca^{2+} was added and cells were incubated for 5 minutes. The cells were then fixed and permeabilized with the same protocol as above.

2.2.4.2 Labelling with Antibodies

After fixing with paraformaldehyde and blocking non specific sites, cells were incubated overnight with primary antibodies for NCX1 or SERCA2 or both together at 4°C. Antibodies were diluted in PBS containing 10% goat serum. Incubation without any primary antibody served as a negative control. Next morning cells were washed twice with PBS-tween-20 (0.5%) to remove excess unbound primary antibody. The cells were fixed again with 2% paraformaldehyde for 10 mins followed by washing with 0.1 M glycine in PBS. Then the cells were washed with PBS- tween 20 every 10 min for 40 minutes followed by incubation with appropriate alexa conjugated secondary antibody. The plates were incubated for 45 minutes in the dark at room temperature (22° C) with secondary antibody described in table 2. Following incubation, the cells were washed in the dark every 10 min for one hour with PBS–tween 20 to remove excess unbound secondary antibody. Finally 25 µl of vectashield mounting medium was added to the coverslip as an mounting/anti-fade reagent for prolonged storage of samples in addition to preventing photobleaching. The cells were then viewed under *Leica DMI 6000 B* SP5 microscope at 63X magnification. Fluorescent images were obtained from top to the bottom of cell with the z step of 0.25 micron. These images were obtained using sequential scanning with excitation wavelengths, 488 nm (argon laser) with emission of 510-545 nm and 568 nm (HeNe laser) with emission of 600 - 645 nm. These images were further analyzed for co-localization as described in data analysis section.

Table 1: Buffer Compositions

BUFFER	FINAL COMPOSITIONS (mM)
Makhlouf's Buffer	115 NaCl, 25 HEPES, 12 Glucose, 5.8 KCl, 2.2 KH_2PO_4, 0.6 $MgCl_2$ and 1 $CaCl_2$ pH 7.4 with NaOH
PSS	134 NaCl, 6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose, pH = 6.4 at 25°C
PBS	137 NaCl, 2.7 KCl, 8 Na_2HPO_4 and 1.5 KH_2PO_4 pH 7.4
MOPS Tris buffer	140 NaCl, 0.8 $MgCl_2$, 20 MOPS pH 7.4 with Tris
MOPS Tris Washing Buffer	20 MOPS, 20 MgCl2.6H_2O (pH 7.4 with Tris), to this add 0.01Nitrendipine, 0.1 EGTA
Krebs	7.8 Glucose, 22 $NaHCO_3$, 115 NaCl, 4.6 KCl, 1 $MgSO_4$, 1.1 KH_2PO_4, 0.03 EDTA, 1.7 $CaCl_2$
Na^+ loading buffer	MOPS Tris buffer, 1 Oubain, 0.025 Nystatin, 0.01 Nitrendipine and 0.1 EGTA

Table 2: Primary and secondary antibodies concentration

Primary antibody	Species	Dilution	Secondary Antibody	Dilution
NCX – п 11-13	rabbit	20x	goat anti rabbit alexa 488 goat anti rabbit alexa 568 goat anti rabbit alexa 633	600x 600x 600x
SERCA – IID8	mouse	20x	goat anti mouse alexa 488 goat anti mouse alexa 568	600 x 600x

2.3 Data Analysis

2.3.1 Intracellular Ca^{2+} measurements

A change in intracellular calcium was reported in terms of change in fluorescence intensity. The highest fluorescence value obtained on addition of high Ca^{2+} in the presence of the calcium ionophore 4Br- A 23187 was considered as Fmax. Similarly the lowest value obtained on addition of EGTA and 4Br-A23187 was considered as Fmin. These values were obtained for each well with cells on 96 well micro-titre plate. The change in fluorescence was calculated for each well using the respective Fmin and Fmax obtained for that well. The percent increase in fluorescence intensity was calculated as $\Delta F = 100 * (F - F\ min) / (F\ max - F\ min)$. Slopes were calculated at five minutes. Results from 3 different experiments were pooled and values were presented as a mean ± SEM of the specific number of replicates. Statistical analysis was performed using Instat computer software (San Diego, USA). For analysis of 2 groups an unpaired t-test was used while an ANOVA test was used for 3 or more groups. This was followed by a Tukey-Kramer multiple comparison to test the null hypothesis. P-values <0.05 were considered significant to negate the null hypothesis.

2.3.2 Co-localization analysis

The images obtained on Leica SP5 microscope were analyzed using the program Image J (NIH, USA). The image was opened by splitting into its two component channels. Background was substracted from each channel using background substraction from region of interest. Four slices from the middle of the stack of images were chosen as the slices of interest from both the channels and saved as a separate image. These slices have to be same for both the channels. The remaining slices were not used. This was done for the image obtained by both the channels for

excitation, 488 and 568 nm. Then the 4 median stacks from both the channels are combined together using stack interleaver. Since NCX is localized in the plasma membrane, small areas near the plasma membrane were selected and cropped. Each area of interest was rotated in such a way that the plasma membrane was parallel to X-axis. From this selected and rotated area, a thin area of 10 – 15 μm length (parallel to the X axis) and 1- 1.5 μm height (along the Y axis from PM to the interior of cell) was selected as a region of interest and was cropped further. From each cell 20 to 30 areas near the PM were cropped to be analyzed further. For each cropped area the stack was then de-interleaved into two substacks of different excitation channels using substack maker. The two substacks obtained are further analysed for co-localization using Just another Co-localization Plugin (JACoP). Mander's coefficient between two proteins, NCX and SERCA was calculated. Pearson's coefficient and Costes randomization were also analyzed. The values obtained from different images were pooled and presented as a mean ± SEM of the specific number of images. Statistical analysis was performed using Instat.

3.0 RESULTS

This thesis focuses on examining the changes in $[Ca^{2+}]i$ due to NCX mediated Ca^{2+} entry in cultured smooth muscle cells. The effect of interactions between NCX and SERCA on cytosolic Ca^{2+} is determined. Furthermore the effect of these interactions is examined on the localization and proximity of these two proteins, NCX and SERCA. The results discussed in this section are based on studies done on $[Ca^{2+}]i$ measurements and structural studies for co-localization of NCX and SERCA using immunofluorescence.

3.1. $[Ca^{2+}]i$ studies

Hypothesis: The increase in cytosolic Ca^{2+} mediated by NCX is sequestered in SER by the SERCA pump. Inhibition of the SERCA pump with thapsigargin would also inhibit NCX mediated increase in cytosolic Ca^{2+}.

Validation of method used, characterization of NCX activity and the effect of SERCA inhibition on NCX activity and cytosolic Ca^{2+} levels are discussed in this section.

3.1.1 Characterization of NCX mediated increase in cytosolic Ca^{2+}

3.1.1.1 Time course for Na^+ dependent increase in cytosolic Ca^{2+}

NCX mediated increase in cytosolic Ca^{2+} was examined in pig coronary artery cultured smooth muscle cells. The cells were Na^+ loaded and then transferred into calcium free Na^+ containing or NMG^+ (Na^+ minimal) buffer as described in methods. The cells were incubated with 0.1mM EGTA to chelate intracellular Ca^{2+}, and deplete $[Ca^{2+}]i$. Finally 0.3 mM Ca^{2+} was

added and fluorescence was recorded. A time course analysis of changes in cytosolic Ca^{2+} was carried out. Initially the values obtained for fluorescence intensity were used to calculate change in $[Ca^{2+}]i$, but there was a lot of variability and fluctuations in the values obtained for $[Ca^{2+}]i$. The increase in the fluorescence intensity of a calcium sensitive fluorescent dye is a function of the increase in cytosolic Ca^{2+}, therefore $[Ca^{2+}]i$ was expressed in terms of the change in fluorescence intensity. This was calculated as a percent increase in fluorescence intensity as described in data analysis section. The increase in fluorescence intensity was measured and calculated for cells in buffer containing Na^+ and NMG^+ (Figure 5A). The difference in intensity obtained between the Na^+ and NMG^+ groups was considered as NCX mediated increase in fluorescence intensity (Figure 5B). The increase in fluorescence intensity due to NCX was observed to be linear for the first 5 minutes. After 5 minutes, the increase in fluorescence intensity gradually starts to plateau. Consequently, a 5 minute time interval was chosen for subsequent experiments.

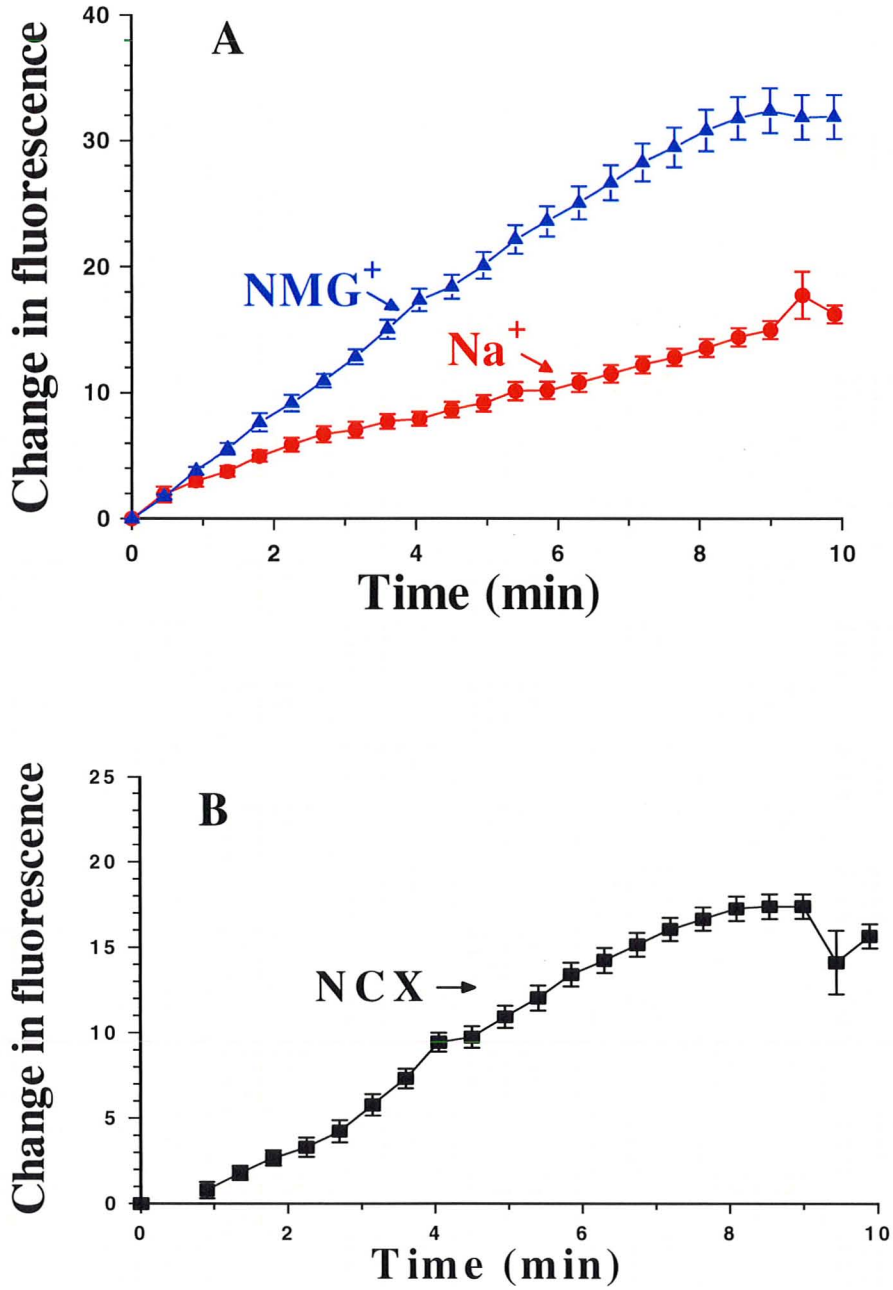

Figure 5: Time course of change in fluorescence intensity mediated by NCX dependent increase in cytosolic Ca^{2+} in cultured SMC. A. Increase in fluorescence intensity in buffer containing Na^+ or NMG^+. **B.** The difference between the fluorescence intensity obtained for NMG^+ and Na^+ represents the increase in fluorescence intensity due to NCX mediated increase in

cytosolic Ca^{2+}. Results were pooled from 8 different experiments, n= 109 for Na^+ and n = 118 for NMG^+)

3.1.1.2 Pharmacological validation of NCX activity by NCX inhibitors SEA 0400 and KB-R7943

NCX specific inhibitors SEA 0400 and KB-R7943 were used to validate that the differences in fluorescence intensity obtained for cells in buffer containing Na^+ and NMG^+ were due to an NCX mediated increase in cytosolic Ca^{2+}.

NCX mediated increase in cytosolic Ca^{2+} was examined using 10 μM KB-R7943 as seen in Figure 6. The inhibitor had no significant effect on the change in fluorescence intensity in the Na^+ containing buffer. Whereas significant inhibition ($p< 0.01$) of NCX in reverse mode (calcium entry mode) was seen in the presence of 10 μM KB-R7943 in NMG^+ containing buffer (2.423 ± 0.359) compared to NMG DMSO (vehicle) group(4.07 ± 0.442). Figure 7 shows that the NMG group treated with 3 μM SEA 0400 showed a significant decrease in the calculated slope value (2.23 ± 0.27) for the first 5 minutes compared to the NMG DMSO group (4.22 ± 0.44) ($P < 0.001$). SEA 0400 did not affect the change in cytosolic calcium levels in the Na^+ buffer containing cells. Similar results were obtained using both the inhibitors, KB-R7943 and SEA 0400. This implicates the involvement of NCX in Na^+ dependent increase in cytosolic Ca^{2+} in the cells.

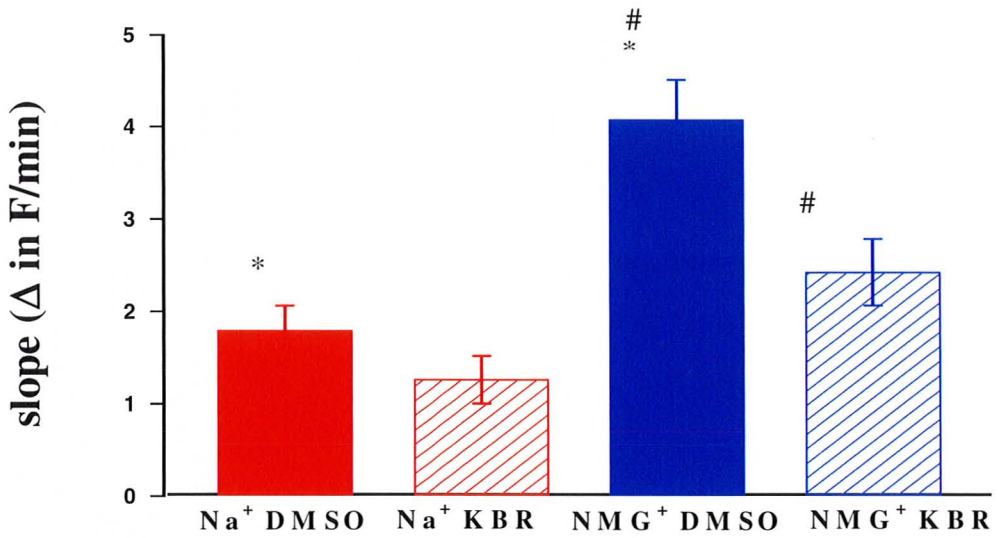

Figure 6: Effect of KB-R7943 on NCX mediated increase in cytosolic Ca^{2+} in cultured SMC. KB-R7943 significantly lowered NCX mediated increase in cytosolic Ca^{2+} in SMC in NMG^+ containing buffer (2.423 ± 0.176) compared to NMG DMSO (4.07 ± 0.442) buffer without inhibitor ($p < 0.01$). No significant difference ($p > 0.05$) was seen in SMC with Na^+ containing buffer with (Na^+ KBR) (1.26 ± 0.257) or without inhibitor (Na^+ DMSO) (1.79 ± 0.273). Results were obtained from 4 different experiments performed on different days with n= 34. (* indicates significant difference between Na^+ DMSO and NMG^+ DMSO group; # indicates significant difference between NMG^+ DMSO and NMG^+ KBR group).

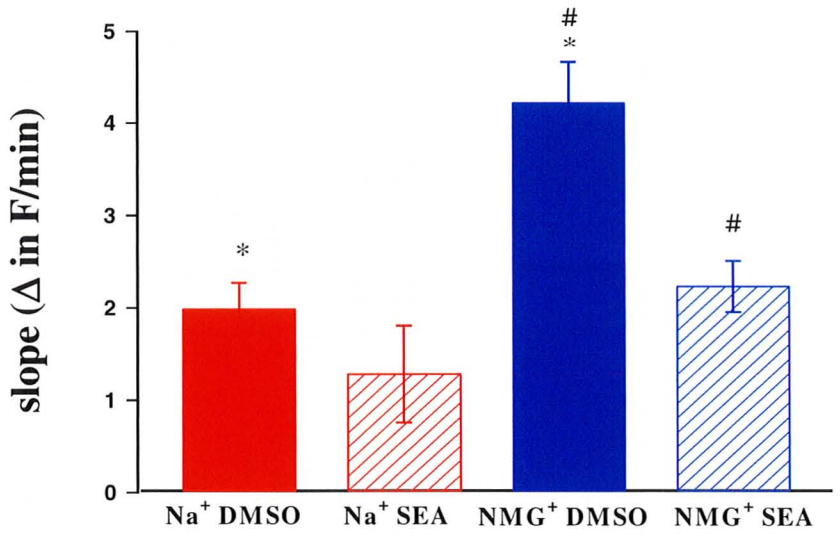

Figure 7: Effect of SEA 0400 on NCX mediated increase in cytosolic Ca^{2+} in cultured SMC. SEA 0400 significantly lowered NCX mediated increase in cytosolic Ca^{2+} in SMC in NMG^+ containing buffer (2.23 ± 0.276) compared to NMG DMSO (4.22 ± 0.44) buffer without inhibitor ($p < 0.001$). No significant difference ($p > 0.05$) was seen in SMC with Na^+ containing buffer with (Na^+ SEA) (1.28 ± 0.524) or without inhibitor (Na^+ DMSO) (1.98 ± 0.279). Results were obtained from 4 different experiments performed on different days with n= 33. (* indicates significant difference between Na^+ DMSO and NMG^+ DMSO group; # indicates significant difference between NMG^+ DMSO and NMG^+ SEA group).

3.1.2. Effect of SERCA inhibitors (thapsigargin and CPA) on cytosolic Ca^{2+}

Thapsigargin and CPA are selective inhibitors of the SERCA pump. The initial experiment was done to monitor their effects on the changes in cytosolic Ca^{2+} in SMC placed in Makhlouf's Buffer (MB) without any Na^+ loading (Figure 8). The cells in the buffer which did not have any SERCA inhibitors showed a steady state fluorescent intensity. In comparison, the cells exposed to the SERCA inhibitors gave a transient increase in the fluorescence intensity

which indicates an increase in cytosolic Ca^{2+} levels. The fluorescence intensity then gradually decreased and returned to resting level at about three minutes.

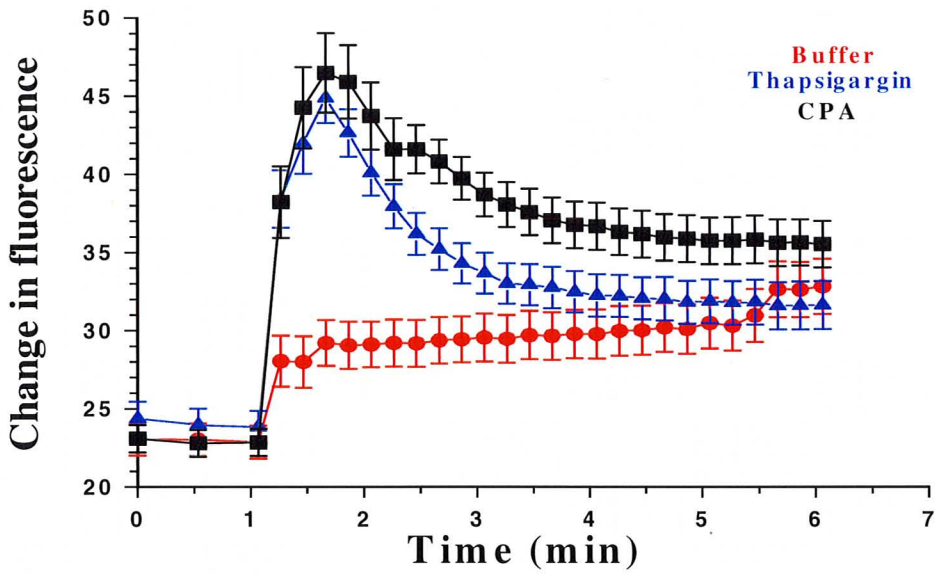

Figure 8: The effect of SERCA inhibitors on intracellular Ca^{2+} levels in cultured SMC. Significant increase ($p< 0.001$) in cytosolic Ca^{2+} was seen in SMC containing SERCA inhibitor CPA or thapsigargin compared to SMC with vehicle only.

3.1.3Effect of Thapsigargin on NCX mediated increase in cytosolic Ca^{2+}

Cultured smooth muscle cells were sodium loaded and then transferred to Na^+ containing or Na^+ free (NMG^+) buffer. The SERCA inhibitor thapsigargin was added to some cells to investigate the effect of SERCA inhibition on NCX activity (see methods for detail). SMC exposed to NMG^+ DMSO buffer showed an increased fluorescence intensity (4.807 ± 0.248) as compared to the cells in Na^+ DMSO buffer (2.170 ± 0.149) ($p < 0.001$). This was due to NCX mediated calcium entry. When thapsigargin (1uM) was added to the cells in NMG buffer, the

NCX mediated increase in fluorescence intensity was decreased (2.709 ± 0.167) ($p < 0.001$) and was similar to cells in Na^+ DMSO buffer (Figure 9). To understand the influence of SERCA inhibition of NCX, the localization of two proteins was examined further by immunofluorescence experiments under this condition.

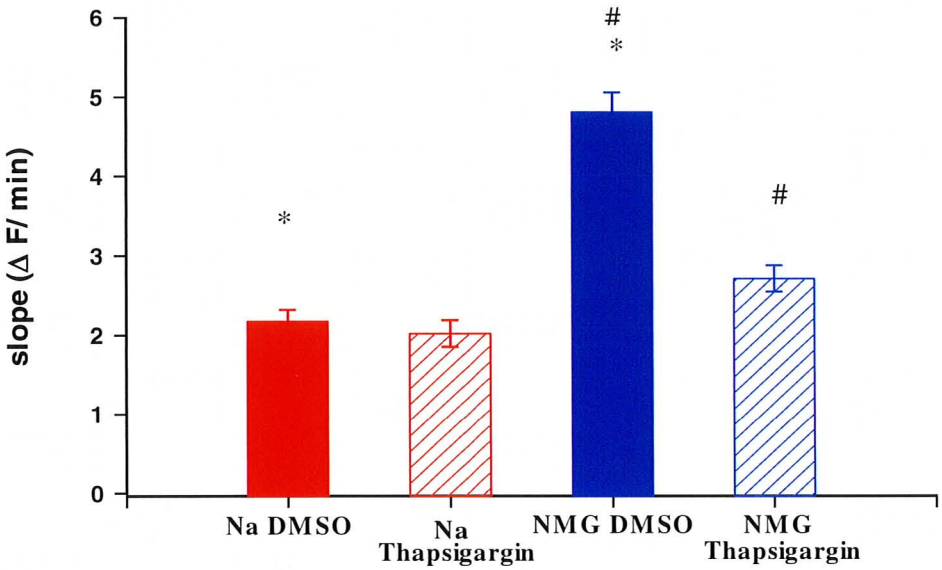

Figure 9: Effect of Thapsigargin on NCX mediated increase in cytosolic Ca^{2+}. SERCA inhibitor thapsigargin caused a significant decrease in NCX mediated increase in cytosolic Ca^{2+} in SMC containing NMG buffer (NMG Thapsigargin 2.709 ± 0.167) compared to SMC in NMG DMSO (4.807 ± 0.248) without any inhibitor ($p < 0.001$). There was no significant difference in SMC loaded with Na^+ containing buffer with (2.017 ± 0.167) or without thapsigargin (2.170 ± 0.149) ($p > 0.05$) n= 54. Results were obtained from 4 different experiments carried out on different days. (* indicates significant difference between Na^+ DMSO and NMG^+ DMSO group; # indicates significant difference between NMG^+ DMSO and NMG^+ Thapsigargin group).

3.2. Structural studies

Hypothesis: Inhibition of SERCA pump with thapsigargin would alter the colocalization of NCX and SERCA.

Antibodies against NCX1 and SERCA 2 were used to perform immunofluorescence studies to observe the colocalization between these proteins. The primary antibody was bound to a secondary antibody conjugated with an alexa fluorophore which can be excited at a specific wavelength. This was observed under a confocal microscope. Antibodies against NCX1 and SERCA 2 were bound to alexa conjugated secondary antibodies with different excitation and emission wavelengths. Optimization of the protocol for immunofluorescence experiment and antibody concentration was carried out to examine the colocalization of NCX1 and SERCA2 protein under different conditions.

3.2.1 Optimization of protocol

The mouse monoclonal antibody IID8 was used against the SERCA2 protein and a rabbit polyclonal п11-13 antibody was used to target the NCX1 protein. Fixing the cells a second time with 2% paraformaldehyde after overnight incubation with primary antibody resulted in better staining of antibody. After fixing with primary antibody, the cells were washed using detergent tween-20 (PBS- tween20) to block non specific binding. Optimization of both the primary antibodies was done at different dilutions such as 20 and 40 times dilution. A twenty fold dilution was found to work best for both antibodies. Alexa conjugated goat anti-mouse IgG or goat-anti rabbit IgG was used as a secondary antibody for IID8 and п11-13 antibody respectively. Alexa conjugated secondary antibodies with excitation wavelengths of 488, 568 and 633 were used against both primary antibodies. Optimization of the dilution of secondary

antibody was done using different dilutions such as 200, 400 and 600 x. It was observed that 600 x dilution of secondary antibody showed best staining for both primary antibodies. No primary antibody was used as a negative control. Figure 10 shows images of staining with secondary antibody goat anti rabbit alexa 568 and goat anti mouse alexa 488 in the absence of any primary antibody. Figure 11 shows representative images for staining with primary antibodies for NCX1 and SERCA2 (rabbit π11-13 and mouse IID8 respectively). Figure 11 A shows the staining obtained for rabbit π11-13 (20x dilution) at using excitation wavelength of 561 nm. Figure 11 B shows the staining obtained for mouse IID8 (20x dilution) using an excitation wavelength of 488 nm for the same cells. The overlap of π11-13 and IID8 for the same cell is seen in figure 11 C. The overlap between the two proteins is seen as a white colour.

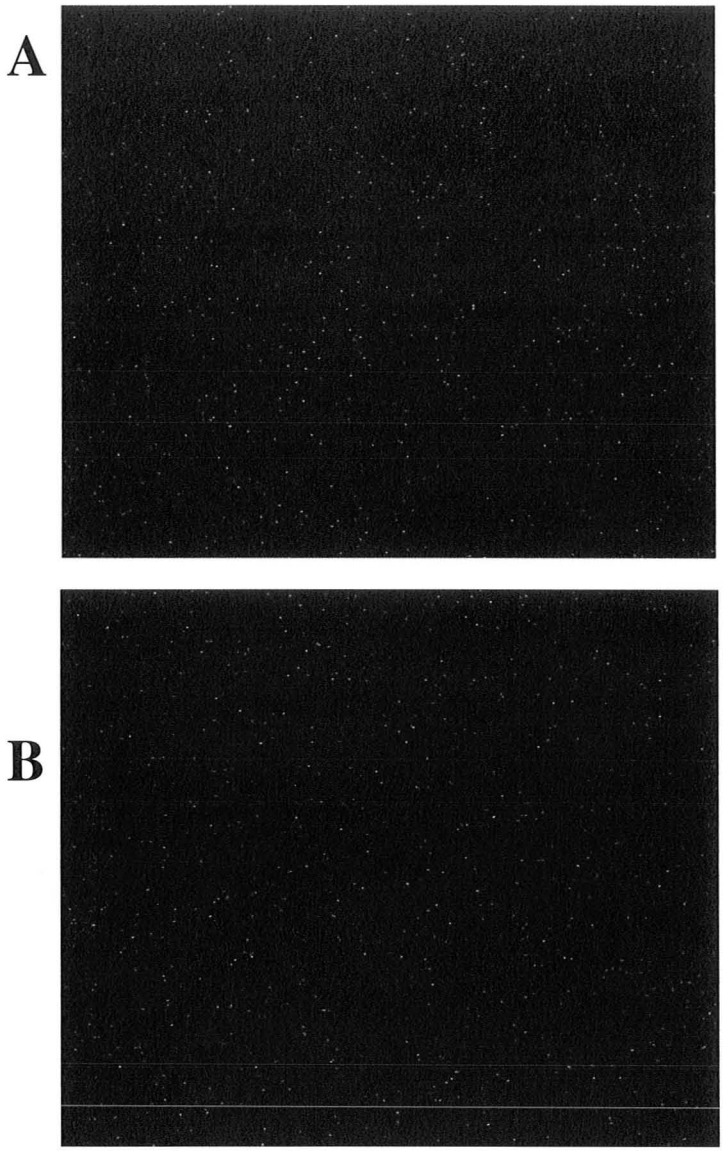

Figure 10: Image from the middle of a stack of SMC without primary antibodies for NCX1 and SERCA2 (rabbit π11-13 and mouse IID8 respectively).

A. Image obtained with secondary antibody goat anti rabbit alexa 561 (600x dilution).

B. Image obtained with secondary antibody goat anti mouse alexa 488 (600x dilution).

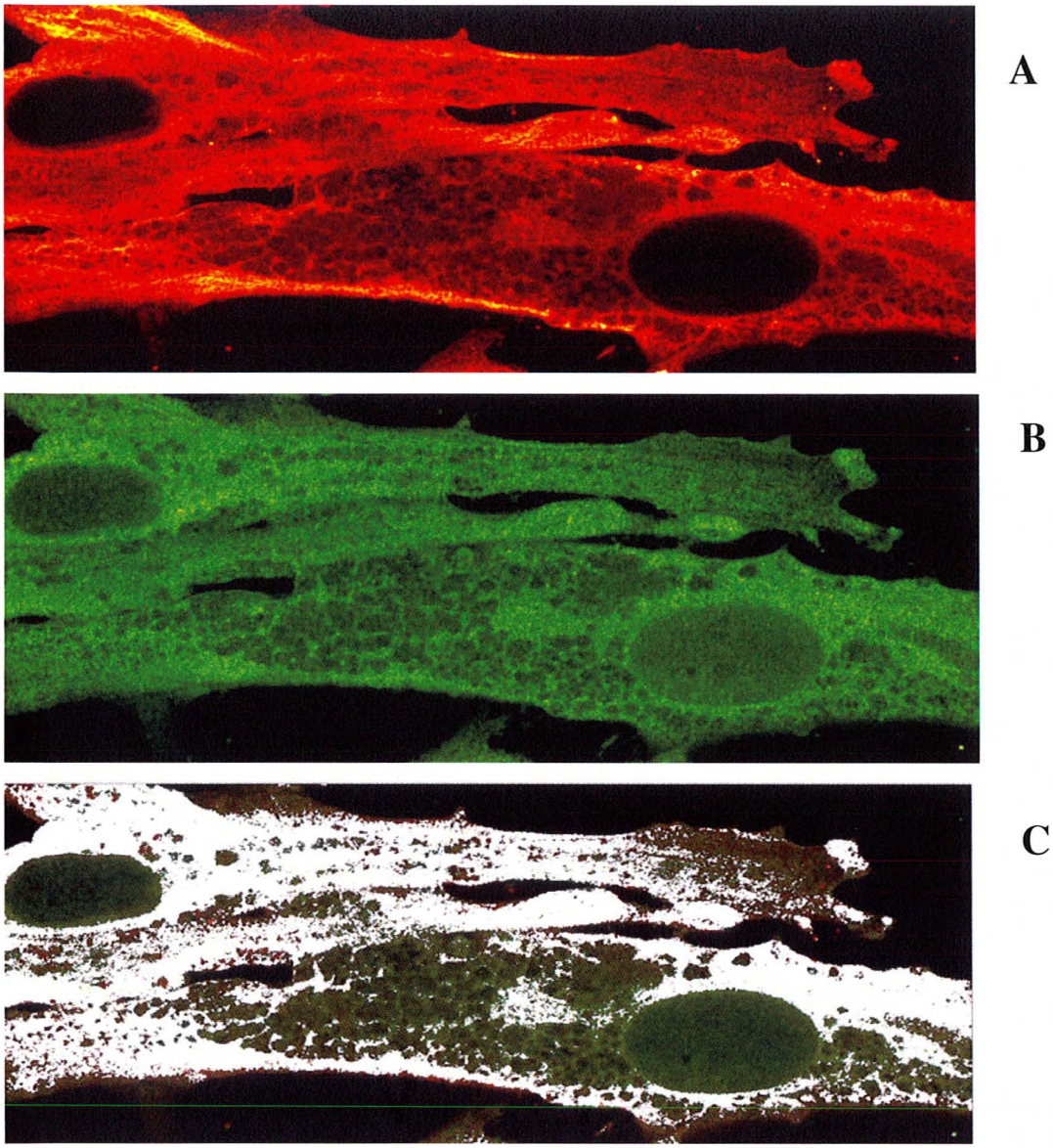

Figure 11: Images of middle stack of SMC with anti NCX1 and SERCA2 antibodies. **A**. SMC labeled with anti NCX1 (π11-13, 20x dilution) and secondary antibody goat anti rabbit alexa 568 (600 x dilution). **B**. Same cell labeled with anti SERCA2 (IID8, 20x dilution) with secondary antibody goat anti mouse alexa 488 (600x dilution). **C**. Overlap of NCX1 and SERCA1. Overlap between two proteins is seen as white colour.

3.2.2 Effect of Thapsigargin on co-localization of NCX1 and SERCA2

The effect of inhibition of SERCA with thapsigarin was examined on localization of NCX1 and SERCA2. SMC were Na^+ loaded and then placed in NMG^+ (Na^+ minimal) buffer with or without thapsigargin (3μM). SMC were fixed and then incubated overnight with antibodies against NCX1 (rabbit π11-13) and SERCA2 (mouse IID8) (20 x dilution for both the antibodies) as described in detail in methods section. The primary antibody then bound to a secondary antibody conjugated with fluorescent dye. Figure 12 shows the representative images for Na^+ loaded SMC in NMG buffer. Figure 12 A shows the staining obtained for rabbit π11-13 at an excitation wavelength of 561 nm. Figure 12 B shows the staining obtained for mouse IID8 at an excitation wavelength of 488 nm for the same cells. The overlap of π11-13 and IID8 for the same cells is seen in figure 12 C. The overlap between the two proteins is seen as white colour. Group 1 shows images for Na^+ loaded SMC in NMG buffer without thapsigargin, while group 2 shows images for Na^+ loaded SMC in NMG buffer with thapsigargin

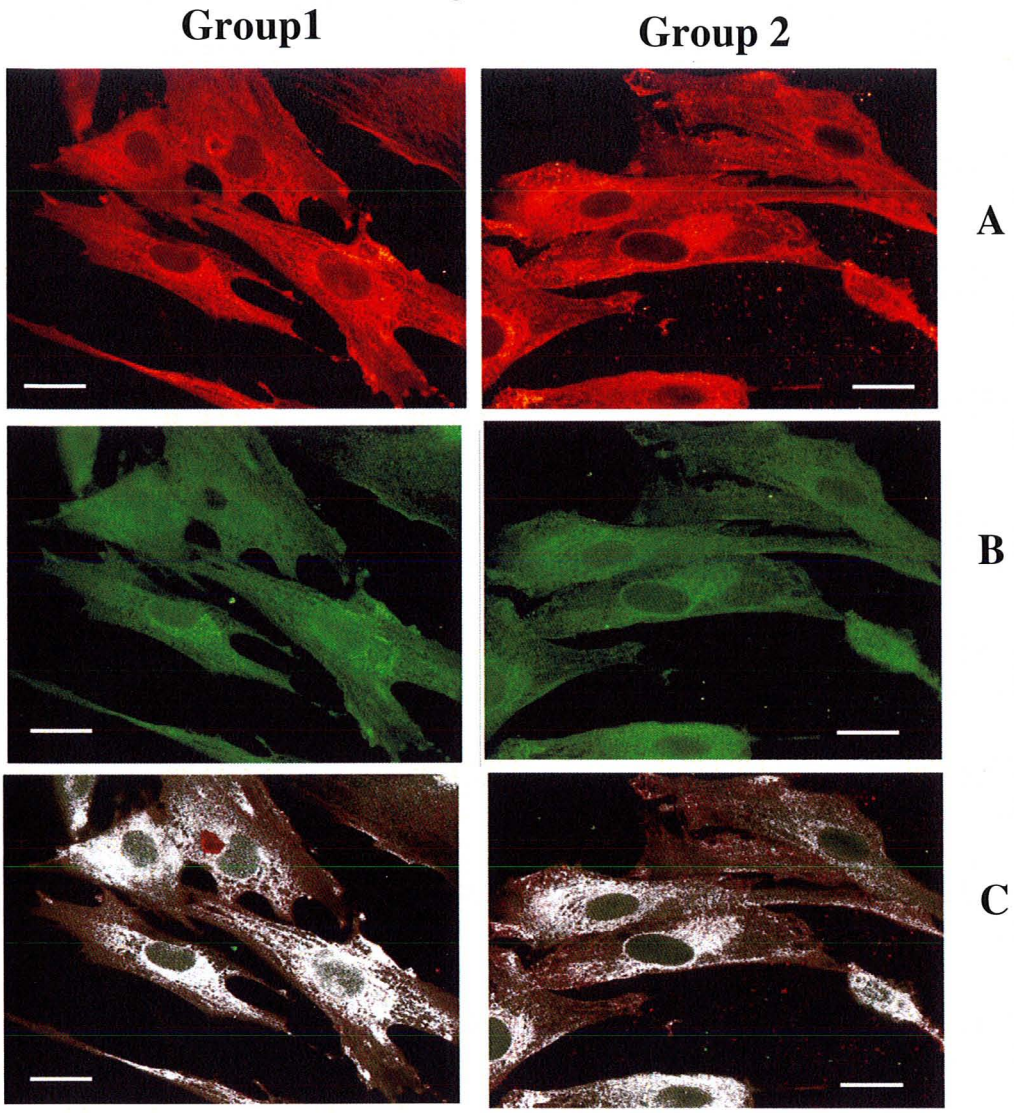

Figure 12: Images of Na^+ loaded SMC in NMG^+ buffer with or without thapsigargin labelled with anti NCX1 and SERCA2 antibodies. Images of middle stack of SMC **A**. SMC labelled with anti NCX1 (π11-13, 20x dilution) and secondary antibody goat anti rabbit alexa 568 (600 x dilution). **B**. Same cell labelled with anti SERCA2 (IID8, 20x dilution) with secondary antibody goat anti mouse alexa 488 (600x dilution). **C**. Overlap of NCX1 and SERCA1. White portion indicates overlap. **Group 1**represents images of SMC without thapsigargin. **Group 2** represents images of SMC with thapsigargin. Scale bar 20 μm

Figure 13 shows the steps involved in co-localization analysis of NCX1 and SERCA2. Four middle slices were selected from the stacks obtained for 561 nm (for NCX1) and 488 nm (for SERCA2) excitation channel. These stacks are interleaved to obtain a combined stack of 8 slices from both channels. Small areas near the plasma membrane are cropped from this combined stack to perform co-localization analysis for two proteins NCX1 and SERCA2. Prospective cropped areas are numbered as seen in figure 13 A. The cropped area is then rotated parallel to x-axis as seen in figure 13B. This cropped area contains a total of eight slices; four for NCX1 and four for SERCA2. This combined crop area is reduced into two substacks of four slices each for proteins NCX and SERCA. Figure 13 C and D shows substacks formed for NCX1 and SERCA2 respectively. These substacks are further analyzed for co-localization analysis using Just another co-localization plugin (JACoP). Mander's coefficients M1 (indicates fraction of NCX overlapping with SERCA) and M2 (indicates fraction of SERCA overlapping with NCX) and Pearson coefficient using Costes randomization are calculated using threshold. Table 4 indicates the values obtained for co-localization analysis for SMC in NMG buffer with or without thapsigargin.

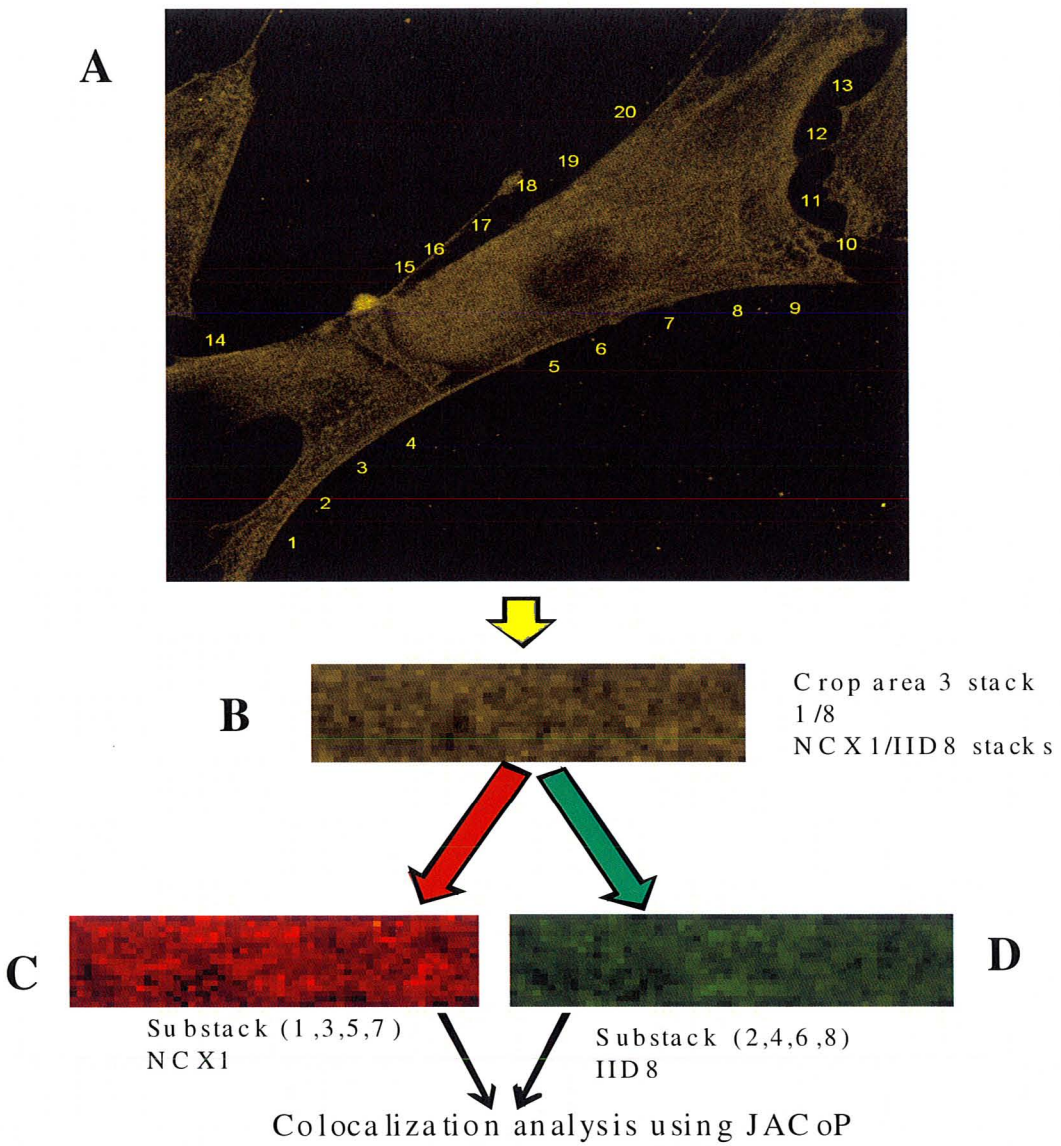

Figure 13: Steps involved in co-localization analysis of NCX1 and SERCA2 in SMC
A. Image of four middle stacks from each 561 (anti NCX1) and 488 channel (anti SERCA2 antibody) combined together. The image is numbered for the prospective areas that are cropped further. **B.** Represents combined stack of 8 slices (four of each channel) for a small area near plasma membrane. This area is then rotated parallel to X-axis. C and D are the image formed after separating four slices for each channel (antibody). Image C forms substack for channel 561 (NCX1) image D forms substack for channel 488 (SECRCA2). These substacks are further analyzed for colocalization using JACoP.

The images were analyzed to obtain Pearson's coefficient with Costes method (Roriginal). The stacks were also randomized using Costes approximation to obtain values for Rrandom. Table 3 shows the effect of thapsigargin on Roriginal values obtained from SMC (Na^+ loaded) in NMG buffer. The table indicates Roriginal obtained from individual experiments and pooled results for all experiments. P values were also calculated. A significant difference was obtained for the Roriginal value between NMG and NMG thapsigargin groups in four different experiments. The pooled value for Roriginal for NMG thapsigargin (0.415 ± 0.006; n=617) was significantly higher ($p<0.001$) compared to NMG group (0.356± 0.007 ; n= 514).

Mander's coefficient M1 and M2 was also calculated. M1 indicated the fraction of NCX overlapping with SERCA, and M2 indicated fraction of SERCA overlapping with NCX. To eliminate the possibility of higher values for M1 and M2 due to brighter staining of antibodies in a particular experiment or to eliminate the possibility of other artifacts, the difference between M1 and M2 (M1-M2) and the ratio of M1 and M2 (M1/M2) were also calculated. Table 4 summarizes the M1, M2, Roriginal, Rrandom, M1-M2 and M1/M2 for five individual experiments carried out on different days and the pooled values for all the experiments. M1 and M2 values were significantly higher ($p<0.001$) in NMG thapsigargin (M1=0.663± 0.004, M2 =0.616± 0.004) group compared to NMG group (M1= 0.617± 0.005, M2= 0.595± 0.005). There was no significant difference between the two groups in the Rrandom values. This indicates that the co-localization obtained is real and not due to random chance.

Table 3: The effect of thapsigargin treatment on the Pearson's coefficient (R original) value in Na^+-loaded cells in NMG^+ solution

	NMG (Mean ± SEM) (n)	NMG-Thapsi (Mean ± SEM) (n)	t-value	P value #
Experiment 1	0.373 ± 0.011 (161)	0.452 ± 0.013 (129)	4.654	**<0.001**
Experiment 2	0.460 ± 0.014 (112)	0.520± 0.011 (150)	3.365	**0.001**
Experiment 3	0.286± 0.014 (85)	0.321± 0.014 (142)	1.646	0.101
Experiment 4	0.329± 0.013 (90)	0.394± 0.011 (125)	3.946	**<0.001**
Experiment 5	0.262± 0.014 (63)	0.351± 0.014 (71)	4.513	**<0.001**
Pool Data	0.356± 0.007 (514)	0.415± 0.006 (617)	6.704	**<0.001**

P values in red indicates significant difference between NMG and NMG thapsigargin group

Table 4: The effect of thapsigargin treatment on various overlap parameters (Mander's and Pearson coefficient using Costes randomization) in Na^+-loaded cells in NMG^+ solution

	NMG (Mean ± SEM) (n)	NMG-Thapsi (Mean ± SEM) (n)	t-value	P value
Expt 1- M1	0.611± 0.008 (161)	0.671± 0.008 (129)	5.103	<0.001
M2	0.648 ± 0.007 (161)	0.657 ± 0.007 (129)	0.853	0.394
Roriginal	0.373 ± 0.011 (161)	0.452 ± 0.013 (129)	4.654	<0.001
Rrandom	0.095± 0.006 (161)	0.104± 0.008 (129)	0.953	0.341
M1-M2	-0.037 ± 0.004 (161)	0.015 ± 0.004 (129)	8.713	<0.001
M1/M2	0.943 ± 0.007 (161)	1.027 ± 0.005 (129)	9.235	<0.001
Expt 2- M1	0.659 ± 0.012 (112)	0.680 ± 0.009 (150)	1.480	0.140
M2	0.657 ± 0.011 (112)	0.676 ± 0.008 (150)	1.454	0.147
Roriginal	0.460 ± 0.014 (112)	0.520± 0.011 (150)	3.365	0.001
Rrandom	0.143± 0.011 (112)	0.094± 0.007 (150)	3.941	<0.001
M1-M2	0.002± 0.006 (112)	0.004± 0.005 (150)	0.310	0.757
M1/M2	1.006± 0.009 (112)	1.009± 0.008 (150)	0.283	0.777
Expt 3- M1	0.610± 0.010 (85)	0.646± 0.010 (142)	2.323	0.021
M2	0.562± 0.010 (85)	0.597± 0.009 (142)	2.450	0.015
Roriginal	0.286± 0.014 (85)	0.321± 0.014 (142)	1.646	0.101
Rrandom	0.065± 0.008 (85)	0.074± 0.009 (142)	0.664	0.507
M1-M2	0.048± 0.009 (85)	0.050± 0.006 (142)	0.125	0.901
M1/M2	1.102± 0.020 (85)	1.087± 0.010 (142)	0.764	0.446
Expt4 – M1	0.634± 0.008 (90)	0.720± 0.007 (125)	8.051	<0.001
M2	0.528± 0.006 (90)	0.600± 0.006 (125)	8.268	<0.001
Roriginal	0.329± 0.013 (90)	0.394± 0.011 (125)	3.946	<0.001
Rrandom	0.077± 0.007 (90)	0.111± 0.005 (125)	4.095	<0.001
M1-M2	0.106± 0.007 (90)	0.120± 0.006 (125)	1.492	0.137
M1/M2	1.208± 0.015 (90)	1.204± 0.013 (125)	0.155	0.877
Expt5 – M1	0.545± 0.011 (63)	0.548± 0.012 (71)	0.165	0.869
M2	0.488± 0.009 (63)	0.482± 0.008 (71)	0.537	0.592
Roriginal	0.262± 0.014 (63)	0.351± 0.014 (71)	4.513	<0.001
Rrandom	0.018± 0.005 (63)	0.025± 0.005 (71)	1.020	0.309
M1-M2	0.057± 0.008 (63)	0.066± 0.008 (71)	0.826	0.410
M1/M2	1.118± 0.016 (63)	1.138± 0.015 (71)	0.925	0.357
Pooled data				
M1	0.617± 0.005 (514)	0.663± 0.004 (617)	7.254	<0.001
M2	0.595± 0.005 (514)	0.616± 0.004 (617)	3.402	0.001
Roriginal	0.356± 0.007 (514)	0.415± 0.006 (617)	6.704	<0.001
Rrandom	0.088± 0.004 (514)	0.087± 0.004 (617)	0.150	0.881
M1-M2	0.022± 0.004 (514)	0.047± 0.003 (617)	5.159	<0.001
M1/M2	1.051± 0.007 (514)	1.085± 0.006 (617)	3.881	<0.001

- P values in red indicates significant difference between NMG and NMG Thapsi group

The effect of thapsigargin was also determined on SMC placed in Na^+ containing buffer after Na^+ loading. Colocalization analysis was performed and Roriginal, R-random, Mander's coefficient M1 and M2 were calculated. Five different experiments were carried out on different days with slightly different microscope parameters. Table 5 shows values for various colocalization parameters compared between two groups for five individual experiments. Three experiments performed showed higher values for Roriginal in the Na^+ thapsigargin group compared to the Na^+ group, while two experiments showed higher values for Roriginal in the Na^+ group compared to the Na^+ thapsigargin group. As these experiments were performed under different microscope conditions, it is difficult to compare the values between experiments. The results are inconclusive and need to be performed under the same conditions.

Table 5: The **effect of thapsigargin treatment on various overlap parameters (Mander's and Pearson coefficient using Costes randomization) in Na^+-loaded cells in Na^+ solution**

	Na (Mean ± SEM) (n)	Na-Thapsi (Mean ± SEM) (n)	t-value	P value #
Expt 1- M1	0.590±0.016 (47)	0.678±0.018 (40)	3.595	0.001
M2	0.655±0.015 (47)	0.621±0.014 (40)	1.620	0.109
Roriginal	*0.500±0.026 (47)*	*0.601±0.020 (40)*	*2.959	*0.004*
Rrandom	0.029±0.005 (47)	0.050±0.007 (40)	2.444	0.017
M1-M2	-0.065±0.013 (47)	0.056±0.010 (40)	7.171	<0.001
M1/M2	0.906±0.019 (47)	1.090±0.017 (40)	6.999	< 0.001
Expt 2- M1	0.658±0.020 (65)	0.667±0.014 (60)	0.378	0.706
M2	0.657±0.018 (65)	0.665±0.012 (60)	0.383	0.702
Roriginal	*0.735±0.013 (65)*	*0.655±0.018 (60)*	**3.601	*<0.001*
Rrandom	0.052±0.011 (65)	0.021±0.003 (60)	2.544	0.012
M1-M2	0.001±0.009 (65)	0.002±0.010 (60)	0.062	0.951
M1/M2	1.001±0.015 (65)	1.007±0.016 (60)	0.263	0.793
Expt 3- M1	0.489±0.016 (45)	0.650±0.015 (48)	7.300	<0.001
M2	0.484±0.013 (45)	0.557±0.019 (48)	3.126	0.002
Roriginal	*0.302±0.015 (45)*	*0.477±0.029 (48)*	*5.207	*<0.001*
Rrandom	0.028±0.005 (45)	-0.030±0.016 (48)	3.290	0.001
M1-M2	0.005±0.013 (45)	0.093±0.013 (48)	4.618	<0.001
M1/M2	1.022±0.028 (45)	1.531±.358 (48)	1.359	0.178
Expt4 – M1	0.596±0.025 (36)	0.740±0.020 (51)	4.493	<0.001
M2	0.583±0.016 (36)	0.676±0.018 (51)	3.667	<0.001
Roriginal	*0.598±0.024 (36)*	*0.685±0.023 (51)*	*2.537	*0.013*
Rrandom	0.034±0.004 (36)	0.048±0.006 (51)	1.848	0.068
M1-M2	0.013±0.015 (36)	0.064±0.009 (51)	2.986	0.004
M1/M2	1.017±0.028 (36)	1.095±0.016 (51)	2.555	0.012
Expt5 – M1	0.686± 0.026 (34)	0.589±0.013 (40)	3.400	0.001
M2	0.597±0.014 (34)	0.451±0.008 (40)	8.204	<0.001
Roriginal	*0.351±0.020 (34)*	*0.200±0.010 (40)*	**6.950	*<0.001*
Rrandom	0.036±0.004 (34)	0.095±0.008 (40)	3.900	<0.001
M1-M2	0.089±0.015 (34)	0.138±0.006 (40)	3.726	<0.001
M1/M2	1.140±0.024 (34)	1.340±0.019 (40)	5.236	<0.001

- P values in red indicates significant difference between Na^+ and Na^+ thapsigargin group. *R original values were larger with thapsigargin in 3 experiments and **smaller in the other two.

4.0 DISCUSSION

An increase in cytosolic Ca^{2+} was observed in SMC due to NCX mediated Ca^{2+} entry. This NCX mediated increase in cytosolic Ca^{2+} decreased upon inhibition of the SERCA pump with thapsigargin. SER depletion with thapsigargin also increased the co-localization of NCX and SERCA in the subplasma membrane domain. Validation of the protocols, interpretation of the results, observations from literature and significance of these findings are discussed further.

4.1 Cytosolic Ca^{2+} measurements

4.1.1 Rationale for monitoring Cytosolic Ca^{2+}

Ca^{2+} is an important signaling molecule and an increase in $[Ca^{2+}]i$ is involved in many cellular processes such as muscle contraction and signal transduction pathways. Increase in $[Ca^{2+}]i$ by NCX mediated Ca^{2+} entry can be measured using Ca^{2+} sensitive fluorescent dyes. This measurement technique indicates the actual change in $[Ca^{2+}]i$.

4.1.2. Validation of parameters

Fluorescence based measurement of $[Ca^{2+}]i$ is based on the effects of Ca^{2+} on changes in excitation or emission spectra or quantum yield of fluorescence of specific dyes. The use of visible spectrum excitable dyes offers several advantages such as reduced interference from samples, less cellular damage and reduced autofluorescence over the use of UV excitable dyes. $[Ca^{2+}]i$ was measured using the Ca^{2+} sensitive fluorescent dye Fluo-4. Fluo 4 can be excited using visible light (excitation maximum of 485 nm) and is an analog of Fluo 3 with greater fluorescence intensity and decreased loading time. A decreased loading time reduces the signal to noise ratio as well as it reduces the amount of dye being compartmentalized in subcellular compartments. Fluo 4 has a lower Kd than Fluo 3. It can be obtained commercially as

acetoxymethyl (Fluo 4-AM). Once inside the cell, the ester group is cleaved by the cellular esterases resulting in a Ca^{2+} sensitive membrane impermeant Fluo 4. This prevents the loss or leak of the signal. Initially, the fluorescnce was measured in individual single smooth muscles cell using a confocal microscope. However, the cells moved or contracted during the experiments and hence the experiments could not be completed. Therefore, measurements of $[Ca^{2+}]i$ were carried out using a fluorescence plate reader. Increase in $[Ca^{2+}]i$ lead to an increase in fluorescent intensity. Conversion of the fluorescence changes to $[Ca^{2+}]i$ changes requires the determination of Fmax values when the dye is saturated with Ca^{2+} and Fmin values when no dye is bound to Ca^{2+}. The extreme conditions needed for these determinations caused the cells to move and hence the calibrations were not reliable. Therefore increase in $[Ca^{2+}]i$ were expressed as a percent change in fluorescence intensity.

Reversal of the Na^+ gradient caused NCX to operate in Ca^{2+} entry mode. This was achieved by loading cells in a buffer containing high Na^+ and then transferring the cells to a minimal Na^+ buffer (NMG buffer). The Na^+ loaded cells which were transferred to a Na^+ containing buffer had no Na^+ gradient therefore NCX was not operating in those cells. Hence, the difference obtained in the fluorescence intensity between the two groups was considered as an NCX mediated increase in fluorescence intensity. The increase in fluorescence intensity due to NCX mediated Ca^{2+} entry was linear for the first five minutes and was then seen to plateau. Consequently, a five-minute time interval was chosen for subsequent experiments.

4.1.3. Validation using pharmacological inhibitors

Increase in $[Ca^{2+}]i$ due to NCX mediated Ca^{2+} entry activity was validated using the NCX inhibitors KB-R7943 and SEA 0400. KB-R7943 at higher concentrations may inhibit L-

type VOCC but this possibility was eliminated here since the VOCC inhibitor nitrendipine was also present in excess. KB-R7443 decreased the NCX mediated increase in $[Ca^{2+}]i$. Using another NCX1 specific inhibitor SEA 0400, similar results were obtained. Thus, the results with the two NCX inhibitors were consistent with the initial assumption that the difference in the fluorescence intensity in absence or presence of Na^+ was due to NCX mediated increase in cytosolic Ca^{2+}.

4.2 Fluorescence microscopy

In order to determine if there was a structural basis for the effects of the SERCA inhibitor on the NCX activity, immunofluorescence confocal microscopy was used to examine the spatial relationship between the NCX1 and SERCA2 proteins.

4.2.1. Limitations of method and alternatives

Confocal microscopy has been used extensively to determine cell structure with respect to specific proteins using immunofluorescence. However, the method has its limitations. The optical system of the microscope can result in chromatic and spherical aberrations leading to optical artifacts. Use of the confocal microscope eliminates the out-of-focus light by using a pinhole. Changing the size of the pinhole changes the amount of out-of-focus light and this can be a source of variability. In initial experiments large differences in co-localization were observed when the pinhole size was altered. Another drawback in the use of fluorescence light microscopy is the photobleaching of the sample. The molecular structure of the dye can be altered due to absorption of the emitted light. In addition, the fluorescent dye used can be phototoxic to the sample. This limitation can be overcome by using genetically modified fusion

proteins. A precaution was taken in these experiments; the samples were not exposed to room light during processing and they were subjected to a minimal amount of light during the micrscopy phase. Another drawback of fluorescence microscopy is that labelling of the proteins may not be 100%. Therefore, the co-localization observed can be concluded based on the immunolabeled proteins and hence can be underestimated. Cross-reactivity between antibodies of same species can be a major problem. This was avoided by using antibodies from two different species. Cross talk between two channels is another major problem associated with co-localization studies. Sequential scanning is carried out by exciting dye of a higher wavelength (561 nm) first, followed by dye of a lower wavelength (488 nm). In addition to this, an appropriate emission bandwidth was used to avoid overlap and cross talk of two channels being excited. In order to minimize variability, the experiments using the thapsigargin treated and untreated cells were conducted in parallel and under identical conditions. Typically stacks of 3-5 images of each wavelength were used for subsequent analysis. Sometimes only stacks of three were used since it is not always possible to obtain the same stack number. Therefore, whether or not the results for co-localization of the two proteins depended significantly on this source of variability was tested. Ten samples (from one cell), stacks of three or five images of each wavelength were analyzed but the same results were obtained. Hence, this parameter was not considered as a further source of variability.

Due to the wavelength of light, light microscopy permits only a resolution of approximately 200 nm. Use of electron microscopy can be an alternative for this as it has a much higher resolution but it is laborious and expensive and sample processing for electron microscopy is also cumbersome.

4.2.2. Justification of parameters used

Co-localization was analyzed using two methods: Pearson's coefficient (Roriginal) using Costes randomization (Rrandom) and determination of Mander's coefficients M1 and M2. Pearson's coefficient gives a value for co-localization between two proteins in a range of -1 to $^{+}$1. Negative values indicate reciprocal relation while positive values indicate direct relation. A Pearson's coefficient from 0.3 to 0.6 indicates medium co-localization, whereas, values from 0.6 to 1.0 indicate a strong positive co-localization. To determine if the observed co-localization is a random phenomenon, the pixel locations in the images can be randomized several times and then compared. The co-localization values are verified by randomization of pixels for 50 rounds. In most instances, randomizing the pixels gave a Rrandom value which was significantly lower and different than the Roriginal value. This validates the fact that the difference in Roriginal value obtained was real and not due to random chance. Pearson's coefficient does not indicate how much of NCX co-localizes with SERCA or vice versa. Hence co-localization was also analyzed using Mander's coefficients. Mander's coefficient gives split values of M1 (fraction of NCX1 overlapping with SERCA2) and M2 (fraction of SERCA2 overlapping with NCX1).

4.3. Novel findings:

Inhibition of SERCA with specific inhibitors like thapsigargin and CPA resulted in an increase in $[Ca^{2+}]i$ in SMC (figure 8). Inhibition of SERCA with thapsigargin had an influence on NCX activity in Na^{+} loaded SMC, resulting in a diminished NCX mediated increase in $[Ca^{2+}]i$ in Na^{+} loaded SMC (figure 9). This indicates that NCX mediated increase in $[Ca^{2+}]i$ is sequestered into the subsurface SER close to the plasma membrane. Inhibition of SERCA pump with thapsigargin affects the pump's function to sequester Ca^{2+} in SER leading in an increase in

localized Ca^{2+} near the plasma membrane and subsurface SER. Under this condition, diminished NCX mediated Ca^{2+} entry led to a smaller increase in $[Ca^{2+}]i$. These results indicate that inhibition of activity of one protein has influence on the activity of the other protein.

Immunofluorescence studies were carried to examine co-localization between NCX1 and SERCA2 in the presence and absence of the SERCA inhibitor thapsigargin. Co-localization is defined as finding of two fluorescently labeled proteins in the same compartment. Na^{+} loaded SMC in NMG buffer with thapsigargin showed a significantly higher value of co-localization (Roriginal) compared to the cells without SERCA inhibitor thapsigargin. SMC with the SERCA inhibitor thapsigargin had overall higher values for the fraction of the coefficient M1 (NCX overlapping with SERCA) and M2 (SERCA overlapping with NCX) compared to SMC without thapsigargin. The value of M1 was significantly higher than that of M2 for both the groups, with and without thapsigargin. This might be due to the distribution and abundance of SERCA in the cell; SERCA is widely distributed in cell. Overall these results indicate that inhibition of SER with thapsigargin leads to increased proximity of the plasma membrane protein NCX1 and SER protein SERCA2. Figure 14 gives a schematic model for these results. Such an increase may lead to a decrease in the subsarcolemmal space where Ca^{2+} entered via NCX1 could accumulate readily and prevent further entry by altering the Ca^{2+} gradient.

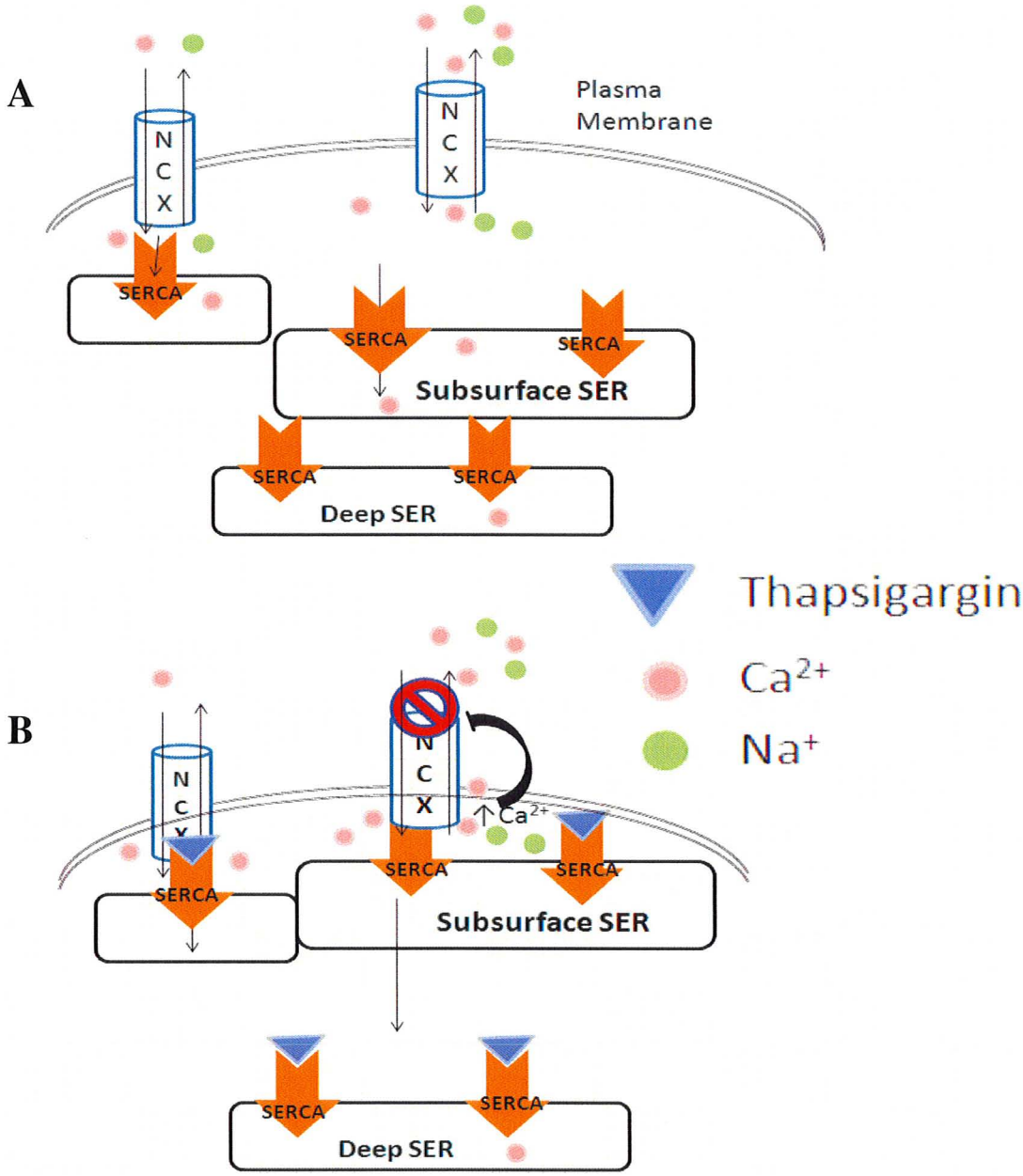

Figure 14: Schematic model for interactions of NCX and SERCA

A. Increase in cytosolic Ca^{2+} by NCX activity is sequestered in SER by SERCA.

B. On inhibition of SERCA by thapsigargin, no Ca^{2+} can be sequestered in SER increasing cytosolic Ca^{2+} near PM. SERCA is observed to move closer to NCX and diminished NCX activity is observed.

4.4. Consistency with literature

Literature supports the hypothesis of a functional linkage between NCX and SERCA. An increase in cytosolic Ca^{2+} through the reverse mode of NCX is sequestered into the SER (Ashida and Blaustein, 1987). Removal of Ca^{2+} from the extracellular space caused a rapid loss of SER Ca^{2+} and a decline of cytoplasmic Ca^{2+} concentration. These effects were not influenced by alterations in membrane potential. Removal of Ca^{2+} from the extracellular space induces Ca^{2+} release from SER. When Na^{+} Ca^{2+} exchange is blocked, SER maintains its Ca^{2+} levels by the process of recycling through SERCA (Nazer and Van Breeman, 1998). The bulk of Ca^{2+} reloading in SER is mediated by the reversal of NCX, which is linked to Ca^{2+} uptake into SER by SERCA. This process would require that the PM and SER membrame be closely apposed and separated by a narrow space for restricted diffusion of calcium ions (Lee *et al.*, 2005). In a previous study by our lab using radioactive Ca^{2+} to examine the NCX mediated entry, the inhibition of SER with thapsigargin decreased the magnitude of the NCX mediated Ca^{2+} entry in vascular smooth muscle cells (Davis *et al.*, 2009). The results obtained in the present study are consistent with the results obtained using radioactive Ca^{2+}.

The functional linkage between NCX and SERCA is also consistent with the previous studies for localization of NCX and SERCA. There is a 25-fold higher density of NCX in the areas of plasma membrane close to subsurface SER (junctional PM) compared to the non-junctional plasma membrane (Fameli *et al.*, 2007). In immunofluorescence and biochemical studies, there was a difference in the co-localization between the two proteins; the fraction of NCX co-localized with SERCA was higher than the fraction of SERCA co-localized with NCX (Kuszczak *et al.*, 2010, 2011).

The results obtained for co-localization of plasma membrane protein NCX and SER protein SERCA, on depletion of SER stores by thapsigargin is supported by other studies that indicated that the subsarcolemmal SER may move closer to the plasma membrane upon Ca^{2+} depletion from the SER. The depletion of SER with thapsigargin resulted in a redistribution of SER calcium sensor protein STIM1 (to micrometer scale) to SER- plasma membrane junctions containing the plasma membrane protein Orai (Calloway *et al.*, 2008). Another study by Sampieri *et al.*, (2009) confirms the finding that store depletion results in an association between SER and plasma membrane proteins. Upon SER depletion, STIM1 is dissociated from the microtubule tracking protein EB1 and associates with the plasma membrane protein Orai (Sampieri *et al.*, 2009). In addition to SOCE proteins STIM1 and Orai, store depletion with thapsigargin has shown to influence the association of plasma membrane protein TRPC1 and SER protein STIM1. STIM1 and TRPC1 are associated within the lipid raft domain. Store depletion caused increased partioning between these two proteins into the plasma membrane lipid raft domain (Pani *et al.*, 2008).

4.5. Summary

Figure 14 shows an overview of the interaction between NCX and SERCA in smooth muscle cells. The primary function of vascular smooth muscle cell is to regulate the vascular tone, which is achieved by the Ca^{2+} dependent contractile filaments. Ca^{2+} needs to be regulated precisely in order to regulate vascular tone. SER plays a large role in calcium regulation in smooth muscle cells. SMC contain high affinity SERCA pumps to sequester Ca^{2+} in SER. These pumps keep $[Ca^{2+}]_i$ levels low and under tight control. NCX is responsible for expelling large amounts of Ca^{2+}. NCX can also operate in Ca^{2+} entry mode under certain pathological conditions

and physiological condition of SER refilling. It was observed that depletion of SER caused increased co-localization of NCX and SERCA. NCX mediated increase in cytosolic Ca^{2+} was also inhibited under this condition.

The NCX activity may be diminished to decrease Ca^{2+} entry and thus prevent Ca^{2+} overload in SMC when it cannot be sequestered in SER due to SERCA inhibition. NCX and SERCA act in series to contribute to the maintenance of cytosolic Ca^{2+} concentrations in vascular SMC. The proximity between the two proteins is consistent with the co-ordinated action of these two proteins in Ca^{2+} refilling of the SER and is responsible for vectorial control of free Ca^{2+} in defined nanospaces. Higher colocalization of NCX and SERCA in the presence of thapsigargin in NMG containing Na^{+} loaded cells indicates that the two proteins move closer to each other for precise regulation of $[Ca^{2+}]i$. These interactions may be crucial to smooth muscle Ca^{2+} regulation and may act as potential therapeutic targets during oxidative stress.

4.6 Future experiments

This proposal raises many questions. Inhibition of SERCA pump with thapsigargin caused inhibition of NCX activity. This also caused an increase in the co-localization of SERCA and NCX. However the nature of this interaction is unknown. It remains to be explored if there is any physical interaction between NCX and SERCA which can lead to the inhibition of NCX activity on SER depletion.

NCX in its Ca^{2+} entry mode seems to play an important role in SER refilling. The localization of NCX and SERCA and their interaction might play an important role for regulation of $[Ca^{2+}]i$. During pathological conditions like ischemia-reperfusion and oxidative stress, varieties of ROS are generated. These ROS are known to damage SERCA. However, the effects

of ROS on NCX –SERCA interactions and co-localization are unknown. Further studies on $[Ca^{2+}]i$ and fluorescence microscopy need to be carried out in order to examine the effects of ROS on NCX-SERCA interactions and co-localization.

Cultured smooth muscle cells were used to access NCX-SERCA interactions for this project. However, culturing smooth muscle cells changes their phenotype and upregulates proteins involved in Ca^{2+} regulation. Therefore it is important to examine the NCX-SERCA interactions in freshly isolated cells or in intact tissues.

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