THE SODIUM CALCIUM EXCHANGER IN THE PORCINE CORONARY ARTERY

COMPARASION OF THE SODIUM CALCIUM EXCHANGER IN THE PORCINE CORONARY ARTERY ENDOTHELIAL AND SMOOTH MUSCLE CELLS

By

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ABSTRACT

Calcium (Ca^{2+}) is an important signaling molecule and hence its movement across cell membranes must be tightly regulated. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_{i)}$ in smooth muscle and endothelium controls the coronary tone. After stimulation, decreasing the $[Ca^{2+}]_i$ back to resting levels is achieved mainly by the sodium calcium exchanger (NCX), the plasma membrane calcium pump (PMCA) or the sarcoendoplasmic reticulum calcium pump (SERCA). The present study will focus on NCX and its interactions with SERCA in the smooth muscle and endothelium of pig coronary artery.

Aim 1 of my thesis is determination of activity levels of NCX in smooth muscle cells (SMC) and endothelial cells (EC). The NCX activity in cultured cells was approximately 5 times greater in EC than in SMC. The NCX inhibitors KB-R7943 and SEA 0400 blocked the NCX mediated Ca^{2+} entry, as did collapsing the Na^+ gradient with monensin. NCX1 is the isoform largely responsible for NCX activity in SMC and EC. NCX activity was also assayed as the Ca^{2+} efflux in cultured cells and as Ca^{2+} uptake in plasma membrane vesicles isolated from freshly isolated smooth muscle.

Aim 2 is to assess the existence of a functional NCX mediated Ca^{2+} entry linked to SERCA in SMC. In the absence of thapsigargin, BAPTA loading SMC increased the NCX mediated uptake. Thapsigargin did not affect the Ca^{2+} uptake in BAPTA loaded cells but it inhibited the Ca^{2+} uptake in cells that were not loaded with BAPTA. These data are consistent with a model in which SER acts as a sink for the NCX mediated Ca^{2+} entry. However, with BAPTA chelation and the resulting lower intracellular Ca^{2+}, the

need for SER to act as a sink is eliminated, and NCX is driven in full force. EC did not demonstrate a NCX-SERCA linkage.

Arterial SMC and EC differ in their structure and function. The function of SMC is the generation of tone which is achieved by the Ca^{2+} dependent contractile filaments. Since these filaments are distributed throughout the cell, Ca^{2+} must be transported to and removed from deep within the cell. As a result, the SER may play a large role in Ca^{2+} regulation in the SMC. Furthermore, SMC also contain higher levels of high affinity Ca^{2+} pumps (SERCA and PMCA) and thus Ca^{2+} is more tightly regulated. Endothelial cells release nitric oxide in response to an increase in $[Ca^{2+}]_i$, which relaxes the smooth muscle. The endothelial nitric oxide sythase produces nitric oxide and is located adjacent to the PM in EC. The SER that removes Ca^{2+} from deep within the cell cytosol may play a small role in Ca^{2+} dependent modulation of the endothelial nitric oxide synthase activity. Based on the Western blot data, EC contain a greater amount of the high capacity NCX, thus the larger quantities of Ca^{2+} can be removed from the cell and the vicinity of endothelial nitric oxide sythase.

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Abbreviations

AM - acetoxymethyl

ATP - Adenosine 5'-triphosphate

$[Ca^{2+}]_i$ - Intracellular calcium concentration

DMEM - Dulbecco's modified eagle's medium

EC - Endothelial cells

EDTA - Ethylenediaminetetraacetic acid

EGTA - Ethylene glycol tetraacetic acid

eNOS - Endothelial nitric oxide synthase

df – degrees of freedom

DTT - dithiothreitol

GSH - Glutathione

GSSG - Glutathione disulfide

HEPES - N-2-hydroxyethyl-piperazine-N8-2-ethanesulfonic acid

IP_3R - Inositol trisphosphate receptor

MOPS - 3-(N-Morpholino)-propanesulfonic acid

NCX - Na^+-Ca^{2+} exchanger

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

NHE - Na^+- Hydrogen Exchanger

NMG - N-methyl-glutamate

NO - Nitric oxide

NOS - Nitric oxide synthase

Abbreviations (cont'd)

PKA - Protein kinase A

PKC - Protein kinase C

PLEM - Phospholemman

PMCA - Plasma Membrane Calcium Pump

PMSF - Phenylmethylsulfonylfluoride

ROCC - Receptor operated calcium channel

RyR - Ryanodine receptor

SER - Sarco-endoplasmic reticulum

SERCA - Sarco-endoplasmic reticulum calcium pump

SMC - Smooth muscle cells

SOCC - Store operated calcium channel

VOCC - Voltage operated calcium channel

XIP - Exchange inhibitory peptide

1. Introduction

Calcium (Ca^{2+}) is an important signaling molecule and hence its movement across cell membranes must be tightly regulated. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the cytosol is maintained at a level 10000x lower than the extracellular $[Ca^{2+}]$. Organelles such as the sarcoendoplasmic reticulum (SER), mitochondria and Golgi may act as sources and sinks for Ca^{2+}. For example, SER stores have very high concentrations of Ca^{2+} that are buffered by proteins such as calsequestrin, calbindin and parvalbumin. $[Ca^{2+}]_i$ can increase by opening Ca^{2+} channels in the plasma membrane (PM) or the SER membrane. Decreasing the $[Ca^{2+}]_i$ back to resting levels is achieved mainly by the Na^+ calcium exchanger (NCX), the PM calcium pump (PMCA) or the SER calcium pump (SERCA).

$[Ca^{2+}]_i$ plays a critical role in the vascular tone of the coronary artery. Smooth muscle and endothelium have unique functional roles. Identifying the differences in the mechanism involved in calcium regulation between the two tissues will associate the mechanisms of calcium removal with the physiological function of the artery. Past studies in our lab have focused on the roles of PMCA and SERCA in $[Ca^{2+}]_i$ regulation in smooth muscle and endothelium. The present study will focus on NCX and its interactions with SERCA in the smooth muscle cells (SMC) and endothelial cells (EC) of pig coronary artery.

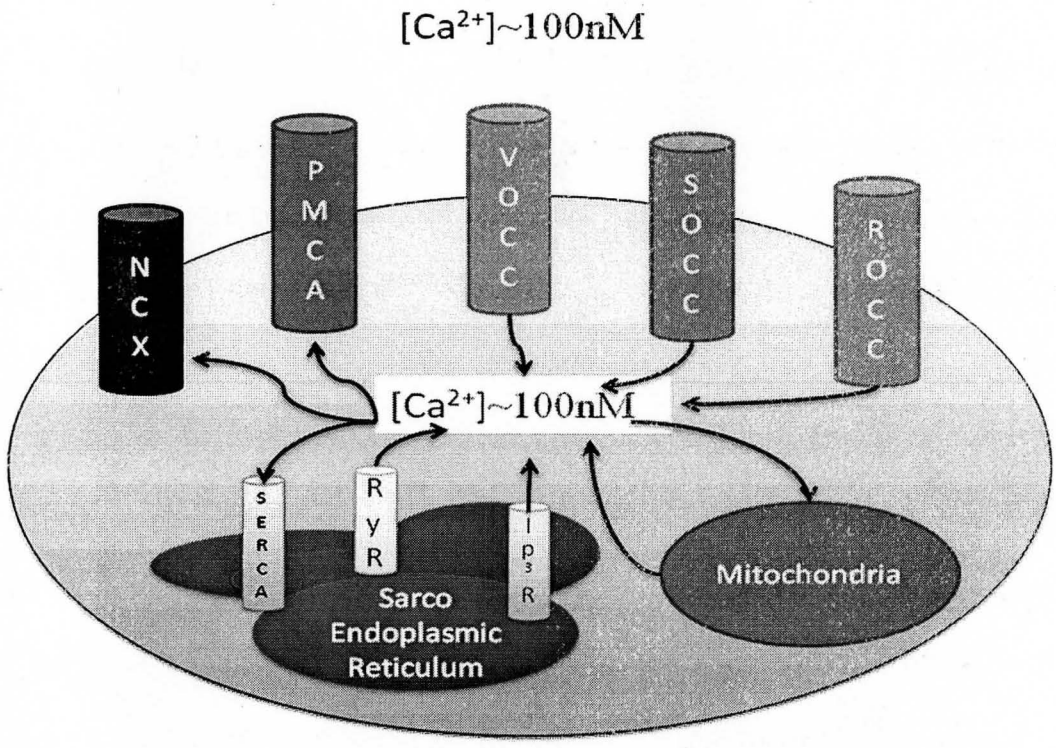


Figure 1. Overview of Calcium Homeostasis. $[Ca^{2+}]_i$ is maintained at around 100 nM meanwhile extracellular Ca^{2+} is around 1 mM, thus Ca^{2+} must be highly regulated inside the cell. Inside the cell two organelles are responsible for storage of calcium: the SER and the mitochondria (the mitochondria will not be elaborated). Mechanisms to raise $[Ca^{2+}]_i$ on 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 triphospate receptors (IP_3R). Mechanisms to reduce $[Ca^{2+}]_i$ are PMCA, SERCA and NCX.

1.1 Calcium homeostasis

Elevated $[Ca^{2+}]_i$ plays an important role in cell signaling. Ca^{2+} signaling regulates a variety of cellular processes such as the modulation of muscle and non-muscle motility, metabolic routes, synthesis and release of hormones, cell growth, cell cycle, and apoptosis (Carafoli, et al. 1994; Rosado, et al. 2006, Watanabe, et al. 2006). The resting $[Ca^{2+}]_i$ must be kept low in order for Ca^{2+} to function effectively as a signaling molecule (see Figure 1 for an overview of calcium regulating mechanism, Lodish, et al. 2000). The resting $[Ca^{2+}]_i$ is maintained at about 0.1 μM, while the extracellular $[Ca^{2+}]_i$ is 1-1.5 mM (Carafoli, 2002). From the cytosol, organelles such as SER actively sequester Ca^{2+} which is then bound to attain concentrations as high as 15 mM (van Breeman & Saida, 1989). Therefore, Ca^{2+} has a large electrochemical gradient across the PM and the SER membrane and this gradient is important for cell signaling since it allows release of Ca^{2+} into the cytosol. However, because of this strong electrochemical gradient, decreasing $[Ca^{2+}]_i$ requires energy (Meis et al., 2000).

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

$[Ca^{2+}]_i$ can be raised by several pathways. Firstly, Ca^{2+} channels located in the PM, such as the voltage- (VOCC), receptor- (ROCC) or store- (SOCC) operated channels, can open upon cell stimulation and allow an entry of extracellular Ca^{2+} (Coiffi et al., 2005; Feske et al., 2006; Parekh 2006; Poteser et al., 2006; Rosker, 2004; Van Breeman & Saida, 1989; Vig et al., 2006; Yao et al., 2005). VOCC have been associated with excitable cells, where they are activated by membrane depolarization typically

caused by agonists (Lipskaia & Lompre, 2004; Marin et al., 1999). ROCC are non-specific cation channels with a certain preference for divalent cations (Yamada et al., 1992). Agonist binding to receptors stimulates intracellular messengers, such G-proteins that may increase the concentrations of metabolites such as diacylglycerol thereby opening the ROCC (Marin et al., 1999). The SOCC are a major subfamily of the ROCC that open in response to a depletion of SER Ca^{2+} stores (Cioffi et al., 2005). The mechanisms by which the SER signals the SOCC are unclear; two hypotheses include (1) the involvement of a diffusible messenger and (2) a physical interactions between the plasma membrane and the SER (Ma et al., 2005; Kawanabe & Nauli, 2005). A direct linkage has also been proposed between the openings of such channels by receptors that bind IP_3, an agent that causes the release of Ca^{2+} from SER (Dietl et al., 1996).

Ca^{2+} that is sequestered in the SER can be released in response to different stimuli, thereby increasing $[Ca^{2+}]_i$ (Carafoli et al., 2001). Ca^{2+} may be released from the SER by two pathways both of which are voltage independent and not observed in the PM (Marin et al., 1999). These are the Ca^{2+} induced Ca^{2+} release (also known as activation of ryanodine receptors - RyRs) and Ca^{2+} release by activation of inositol triphospate receptors (IP_3R, Marin et al., 1999). The distribution and properties of these receptors vary between different tissues.

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

After cell excitation, $[Ca^{2+}]_i$ must be lowered to resting levels. This is accomplished by Ca^{2+} extrusion from the cell by either NCX or PMCA, or by

Ca^{2+}sequestration into SER by SERCA (Carafoli, 2002). Mitochondria also contain a low affinity electroneutral antiporter that can sequester Ca^{2+} (Lipskaia & Lompre, 2004). Under physiological conditions, the mitochondria are thought to be of minor importance (Carafoli, 2002; Lipskaia & Lompre, 2004).

Ca^{2+} pumps

There are three types of Ca^{2+} pumps all of which have high affinity for Ca^{2+}: PMCA, SERCA, and secretory pathway calcium ATPase also known as SPCA, the latter of which is present in the Golgi apparatus (Carafoli, 2002; Guerini, 1998; Pande & Grover, 2005; Penniston & Enyedi, 1998; Philipson & Nicoll, 2002; Schnetkamp, 2004; Stauffer, 1995; Strehler & Zacharias, 2004; Strehler & Treiman, 2001). These pumps utilize energy obtained from the hydrolysis of ATP (adenosine 5'-triphosphate) to move Ca^{2+} against its concentration gradient (Carafoli et al., 2001). Here, I will focus only on PMCA and SERCA which have been studied in many tissues, most notably in SMC and EC.

PMCA

PMCA belong to the P2 subtype of P-type ion ATPases. They have 10 transmembrane domains, 6 cytosolic domains, and 5 extracellular domains (Carafoli, 2002). PMCA are encoded by the genes PMCA1,2,3 and 4 with PMCA 1 and 4 being expressed widely (Carafoli, 2002). Our lab has found that pig coronary artery endothelial cells (EC) express mainly PMCA 1 while pig coronary artery smooth muscle cells (SMC)

express both PMCA1 and PMCA 4. PMCA transcripts may also be alternatively spliced. The various PMCA splice variants are regulated differently by calmodulin, protein kinases and acidic phospholipids (Carafoli, 2002; Guerini, 1998; Pande & Grover, 2005; Penniston & Enyedi, 1998; Stauffer et al., 1995; Strehler & Zacharias, 2004; Strehler & Treiman, 2001).

Lanthanum and vanadate are non-specific inhibitors of all PMCA isoforms that bind to the cytosolic domain of the pump (Pande & Grover, 2005). Recently, specific inhibitors of PMCA called caloxins have been developed, furthermore these caloxins have been shown to demonstrate isoform specficity (Pande et al., 2005).

SERCA

SERCA pumps sequester Ca^{2+} into the SER with a high affinity (Misquitta et al., 1999; Lytton et al., 1992; Grover & Samson, 1986). There are 3 genes that encode SERCA pump isoforms – SERCA1, SERCA 2 and SERCA3. SERCA 1 is expressed in fast twitch skeletal muscle. SERCA 2 gene is found in the cardiac and slow twitch skeletal muscles in greater abundance than in most other tissues including vascular smooth muscle and endothelium (Carafoli et al., 1994; Periasamy & Kalyanasundaram, 2007). SERCA2 gene is alternatively spliced to produce SERCA2a and SERCA2b (Periasamy & Kalyanasundaram, 2007). SERCA2a is most abundant in slow twitch skeletal and cardiac muscle while SERCA2b is expressed in most tissues (Periasamy & Kalyanasundaram, 2007). SERCA3 expression is most abundant in endothelial cells,

platelets, pancreas and kidney; it is also alternatively spliced (Periasamy & Kalyanasundaram, 2007).

All SERCA isoforms are inhibited by cyclopiazonic acid and thapsigargin (Inesi et al., 2005). SERCA2 pumps can be activated directly by protein kinases and inhibited by phospholamban while in the dephosphorlyated state (Colyer, 1998).

Oxidative stress by the production of hydroxyl radicals directly impairs SERCA activity (Flesch et al., 1999). Hydroxyl radicals and other reactive oxygen species (ROS) such as peroxynitrite have been found to impair smooth muscle contractility; evidence also suggests that this impairment is due to SERCA dysfunction (Walia et al., 2003). Furthermore, the hydroxyl radical's induced contractile dysfunction can be reduced by SERCA overexpression (Hiranandani et al., 2006).

SERCA susceptibility to ROS differs between different isoforms of SERCA. SERCA2a is more resistant to the harmful affects of peroxynitrite therefore the cells expressing SERCA2a such as EC may have an advantage during exposure to oxidative stress (Grover et al., 2003).

Exchangers

NCX

NCX is an ubiquitously expressed antiporter in the plasma membrane (Annunziato et al., 2004). NCX moves three Na^+ ions, in exchange for one Ca^{2+} ion and is thus electrogenic (Annunziato et al., 2004). Therefore, whether NCX extrudes Ca^{2+} (forward mode) or allows Ca^{2+} entry (reverse mode), depends on the electrochemical

gradients of Na^+ and Ca^{2+}. However, the rate at which this movement occurs depends on the number of NCX molecules, their binding affinities of Na^+ and Ca^{2+} to the exchanger, and concentrations of Na^+ and Ca^{2+} (Aiello et al., 2005; Iwamoto et al., 1996; Matsuoka, 2004). An exchanger that transports 4 Na^+ for 1 Ca^{2+} and 1 K^+ has also been reported; it is known as the Na^+-Ca^{2+}-K^+ exchanger (NCKX, Dong et al., 2006). However, as it is quite rare and there is no evidence for it in pig coronary artery, NCX will be the focus of this thesis.

NCX structure and splicing

NCX are encoded by three genes, NCX1, 2 and 3, of which NCX1 is best characterized. NCX1 is composed of 938 amino acids; it contains 9 transmembrane domains, two α-repeats that are crucial to ion translocation, and many regulatory sites in the large cytoplasmic domain (Figure 2, Annuziato et al., 2004; Goto et al., 1996; Iwamoto et al., 1996). Of these isoforms NCX1 is the most ubiquitously expressed (Annuziato et al., 2004).

The two α-repeats are located between TM2-TM3 and TM7 -TM8, they are named α-1 and α-2 respectively (figure 2). The α-repeats are crucial to the ion translocation pathway since they contain a highly conserved amphipathic amino acid sequence (Shigekawa & Iwamoto, 2001). The center of the α-2 repeat contains a GIG sequence similar to the GYG sequence present in the K^+ channel that is crucial for specificity of the K^+ ion translocation (Philipson & Nicoll, 2000). Therefore this GIG sequence may also be critical to the specificity of the NCX exchanger (Philipson &

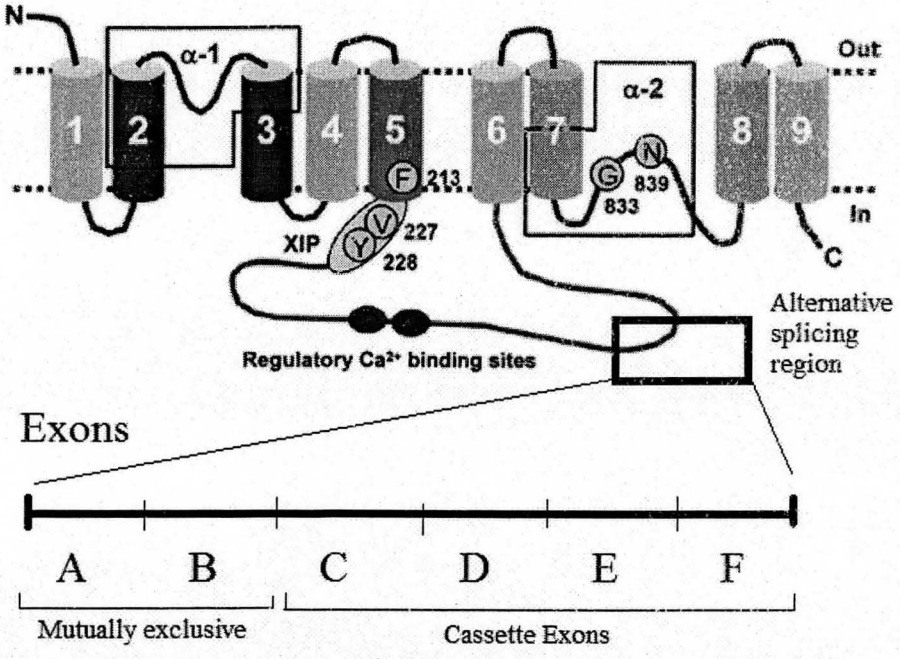


Figure 2. NCX structure. The nine transmembrane domain model of NCX 1 is taken from topology analysis (modified from Iwamoto, 1996). Key segments are highlighted in this model such as: α-1 and α-2, exchange inhibitory peptide region (XIP), regulatory Ca^{2+} binding site, key amino acids, alternative splicing region and alternative exons A-F (modified from Quednau et al., 1997)

Nicoll, 2000). Curiously, the α-1 and α-2 are found on opposite sides of the membrane to each other. It is hypothesized that the hydrophilic faces of the α-repeats form a portion of the ion translocation pathway (Philipson & Nicoll, 2000).

The large cytoplasmic domain located between transmembrane 5 and 6, is called the f-loop (Nicoll et al., 1999). The f-loop contains several key sequences that are involved in the regulation of NCX; it is not responsible for the actual translocation of Na^+ and Ca^{2+} across the membrane. A Ca^{2+} regulatory segment is located in the middle of the f-loop segment; it is 130 amino acids long and is characterized by the presences of a pair of three aspartyl residues and by a group of four cysteines (Nicoll et al., 1999; Qiu et al., 2001). Submicromolar concentrations (0.1– 0.3 μM) of $[Ca^{2+}]_i$ are needed to activate the NCX in both forward and reverse mode presumably via this Ca^{2+} regulatory site (DiPolo, 1979; Hilgemann et al., 1992). The regulatory function of low micromolar Ca^{2+} is evident in the reverse mode, although it is not completely clear in the forward (Matsuoka et al., 1995). Furthermore, the removal of $[Ca^{2+}]_i$ completely inhibits all NCX activity (Philipson & Nicoll, 2000).

A short 20 amino acid (219-238) auto inhibitory domain is also located in the f-loop at the N terminal close to the plasma membrane. This domain is called the XIP and it is rich in both basic and hydrophobic residues (Matsuoka et al., 1997). When intracellular Na^+ increases, an NCX dependent fast outward current is seen followed by an inactivation process (Hilgemann et al., 1992). The XIP region is involved in this intracellular Na^+ inactivation of NCX (Li & Kimura, 1991). The inactivation occurs by a

Exons	Terminology	Exons	Terminology	Exons	Terminology
NCX1		**NCX 2**		**NCX 3**	
ACDEF	NCX 1.1	AC	NCX 2.1	AC	NCX 3.1
ACDE	NCX 1.8			B	NCX 3.2
ACD	NCX 1.6			BC	NCX 3.3
ADF	NCX 1.5				
AD	NCX 1.4				
BCD	NCX 1.2				
BDF	NCX 1.7				
BD	NCX 1.3				
BDE	NCX 1.9				
BDEF	NCX 1.10				
BCDEF	NCX 1.11				
ADEF	NCX 1.12				

Table 1. Splice isoforms and exons of NCX 1, 2 and 3 (modified from Quednau, 1997)

conformational change in the C-terminal portion of the f-loop, thus resulting in the inhibition of the ion transport (Li & Kimura, 1991). Exogenous XIP peptides which have the same amino acid sequence as XIP act as inhibitors of NCX activity (Li, 1991; Pignataro, 2004; Annunziato, 2004).

The f-loop segment also contains alternative splicing sites. NCX splice variants are recognized based on the expression of one of two mutually exclusive exons, A or B and additional cassette exons, C-F (Annuziato, et al., 2004; Hilgemann, 1990; Hurtado et al., 2006; Khananshvili, 1990; Quednau et al., 1997). NCX1 has the largest number of splice variants; there are twelve known arrangements. NCX2 only has one known expressed form NCX2.1, which is present at detectable levels inside cells. NCX3 has three splice variants (Table 1, Quedau et al., 1997).

NCX1 is expressed ubiquitously in all somatic cells. Isoforms NCX1.1 and 1.8 are expressed in heart tissue, NCX1.4, 1.5 and 1.6 are expressed in brain tissue and NCX1.3 and 1.7 are expressed in lung, kidney, skeletal muscle, intestine, aorta, eye, liver, stomach, pancreas, smooth muscle and endothelium (Qiu et al., 2001; Quedau et al., 1997). NCX2 has a single splice variant present in glial cells and skeletal muscle (Quedau et al., 1997). NCX3 exon A splice variants are found in skeletal muscle; exon B splice variants are present in specific subpopulations of neuronal cells and skeletal muscle (Quedau et al., 1997). Both NCX2 and NCX3 show low levels of mRNA transcripts present in other cell types (Quedau et al., 1997; Quedau et al., 2004).

Variations of splice isoforms can also regulate the activities of NCX. For example, cardiac tissue (NCX1.1) and kidney (NCX1.3) NCX isoforms differ in their

voltage dependence and in their Ca^{2+} regulation (Schluze et al., 2002). The alternatively spliced isoforms of NCX1 show tissue-specific expression patterns, suggesting functional adaptation to tissues (Schluze et al., 2002).

Regulation of NCX

NCX may be activated by a variety of mechanisms. These mechanisms include intracellular ATP, protein kinases, phospholemman (PLEM), phosphoarginine, intracellular pH, nitric oxide (NO), and redox agents (Annuziato et al., 1997). A brief summary of NCX regulation by each mechanism is given below.

ATP increases NCX activity by increasing the affinity of both $[Ca^{2+}]_i$ and extracellular Na^+ for NCX. ATP does not affect the maximum transporter rate at saturating concentrations therefore ATP acts like a catalyst for binding and not as an energy source (Blaustein, 1977). ATP either directly participates in the NCX molecule phosphorylation process by protein kinase A (PKA) and protein kinase C (PKC) (Caroni & Carafoli, 1983), increases PIP2 production (Hilgeman et al., 1996) or activates G-protein-coupled receptors, via endogenous and exogenous ligands (DiPolo & Beauge, 1998).

Phosphorylation of NCX and NCX-mediating proteins plays a significant role in the regulation of NCX. NCX may be activated by protein kinases. Each of the NCX isoforms has distinctive putative phosphorylation sites, although their roles have not yet been elucidated (Linck et al., 1998). PKC increases the affinity of NCX for $[Ca^{2+}]_i$ and extracellular Na^+ (Iwamoto et al., 1995; Iwamoto et al., 1996). PKA has also shown an

increase in NCX activity in exon A-carrying splice isoforms of NCX (Aiello et al., 2005, Asano et al., 1998, Iwamoto & Shigekawa, 1998, Schulze et al., 2002).

Another compound involved in the regulation of NCX is PLEM, a 15 kDa protein which can inhibit NCX activity when phosphorylated by PKA (Zhang et al., 2006). It is structurally similar to the SERCA pump regulatory protein phospholamban (Zhang et al., 2006). However, phosphorylation of phospholamban removes the SERCA inhibition (Zhang et al., 2006). Thus, the PKA phosphorylation has opposite effects on NCX and SERCA. The presence and activity of PLEM in cells can also be regulated. Studies have demonstrated that PLEM expression in the left ventricle is decreased in heart failure (Bossuyt et al., 2005; Palmer et al., 1991). This decrease in PLEM may lead to the deregulation of NCX which in turn may contribute to heart dysfunction (Cheung et al., 2007).

Phosphoarginine, an energy storing compound similar to phosphocreatine, increases NCX activity in the forward mode at millimolar concentrations (DiPolo & Beauge, 1998; DiPolo et al., 2004). This activation is unique as it binds with a new area of NCX named the phosphoarginine region, which is related to intracellular transport sites for Na^+ and Ca^{2+} (DiPolo et al., 2004).

Intracellular H^+ may inactivate NCX in the forward mode. Intracellular pH changes as little as 0.4 can induce a 90% inhibition of NCX activity (Doering & Lederer, 1993). This H^+ ion modulatory action is attributed to the antiporter's hydrophilic intracellular loop (Espinosa-Tanguma et al., 1993). Intriguingly, such inhibitory action depends on the presence of intracellular Na^+ ions (Doering & Lederer, 1994). This

method of NCX inactivation has not been further characterized. A large body of work implicates the regulation of NCX by sodium hydrogen exchanger (NHE) via changes in the Na^+ gradients (Aiello et al., 2005; Doering & Lederer, 1994, Espinosa-Tanguma et al., 1993; Matsumoto et al., 2002).

NO can directly stimulate NCX activity in forward and reverse mode (Matsuda et al., 2006). Functionally, NCX is involved in NO-induced depletion of Ca^{2+} in the endoplasmic reticulum, leading to ER stress (Matsuda et al., 1995). In addition, a direct relationship between the constitutive form of nitric oxide synthase (NOS), the enzyme involved in NO synthesis, and NCX has recently been demonstrated. This complex was formed under conditions of heat stress which induced NOS phosphorylation causing NOS to form a complex with NCX, thus decreasing its activity (Kiang et al., 2003).

Oxidizing and reducing agents are also known to affect the activity of NCX. ROS, such as peroxide and GSH/GSSG have been proven to enhance NCX mediated Ca^{2+} fluxes. On the other hand, the strong oxidant HOCL inhibited NCX activity (Amoroso et al., 2000; Reeves et al., 1986; Santacruz-Toloza et al., 2000). Other findings suggest the stimulation of the exchange activity requires the combination of a reducing agent, DTT, GSH, or Fe^{2+}, with an oxidizing agent, H_2O_2 and GSSG (Santacruz-Toloza et al., 2000). The antiporter's sensitivity to changes in the redox status can assume particular relevance during oxidative stress (Huschenbatt et al., 1998). In fact, in this condition, the modulation of ROS could affect the transport of Na^+ and Ca^{2+} ions through the plasma membrane (Huschenbatt et al., 1998).

NCX inhibitors

NCX inhibitors are crucial to understanding the role of NCX. They also have potential therapeutic uses. There are a variety of different types of inhibitors, although most NCX inhibitors are not very selective or are difficult to administer.

The divalent cations; Ni^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, and the trivalent cation; La^{3+}, have been reported to block NCX (Iwamoto et al., 1998). This inhibitory effect can be either due to a direct action on the exchanger molecule or to the replacement of Ca^{2+} ions as a substrate for the antiporter. However, this inhibition is non-specific since the affinity of these cations for other Ca^{2+} transporters is greater (Colvin et al., 2000; Hilgemann, 1989; Hobai et al., 1997; Kimura et al., 1987).

There are a variety of peptides that inhibit NCX: endogenous constrained cyclic peptide: FACACF, the molluscan tetrapeptide: FMAF, callipeltin A and XIP. Of these, XIP is the only peptide that is relatively specific for NCX inhibition. XIP, as mentioned previously, is an auto inhibitory segment located on the f-loop segment of NCX (Li & Kimura, 1991; Pignataro et al., 2004). Administration of synthetic XIP peptide with the same amino acid sequence into the cell will inhibit NCX (DiPolo et al., 1994; Li & Kimura, 1991). However, XIP is non-specific since it also inhibits the calmodulin stimulated PMCA activity in erythrocyte ghosts with high affinity (Li & Kimura, 1991). Furthermore, XIP does not readily pass through the cell membrane. Therefore XIP was modified to bear a molecule of glucose attached to the Tyr-6 residue (Namane et al., 1992). The modified peptide penetrates into the cell by active transport of the glucose molecule by glucose transporters 1 and 3 (Namane et al., 1992).

Isothiourea derivatives such as KB-R7943 have been used extensively to examine NCX although only at low μM concentrations does it exclusively inhibit NCX in the reverse mode (Iwamoto et al., 1998). KB-R7943 inhibits the reverse mode NCX with an EC_{50} value of 1-3 μM (Iwamoto et al., 1996). It also has a 3 fold higher affinity for NCX3 than NCX1 or NCX2 (Iwamoto et al., 1998). At very high concentrations (EC_{50}= 30 μM), it can also inhibit NCX in the forward mode but it will also start to inhibit the L-type Ca^{2+}-channels (EC_{50}= 60 μM, Iwamoto et al., 1996). Thus, only at concentrations below 10 μM, is KB-R7943 is a selective inhibitor of NCX in reverse mode (Shigekawa & Iwamoto, 2002).

The ethoxyaniline derivative SEA 0400 is one of the most potent (EC_{50} = 5–92 nM) NCX inhibitors available although it may also interfere with Ca^{2+} movement across the cell membrane by other pathways (Matsuda et al., 2001; Reuter et al., 2002). This compound, similarly to KB-R7943, inhibits NCX in the reverse mode. SEA 0400 demonstrates stronger isoform preference than KB-R7943, it is most selective for NCX1. It blocks NCX1 with a high affinity (EC_{50} = 56nM), it has a lower affinity for NCX2 (EC_{50} value = 980 nM), and does not inhibit NCX3 (Iwamoto et al., 2004).

Quinazolinone derivatives are being developed as new and potent NCX inhibitors. A new compound belonging to quinazolinone family named SM-15811 has been found to inhibit Na^{+}-induced Fura-2-monitored Ca^{2+} increase (Hasegawa et al., 2003). This drug demonstrated a high affinity for the exchanger with an EC_{50} value of 17 nM, although its selectivity to inhibit only NCX is still under investigation (Hasegawa et al., 2003).

There are a variety of other non specific compounds that inhibit NCX such as amiloride derivatives, diarylaminopropylamine derivatives, benzofuran derivatives such as amiodarone, imidazoline derivatives such as cibenzoline, phenylalkylamines such as verapamil and methoxyverapamil, oxime derivatives, acridines-quinacrine, opiate derivatives and various anaesthetics (Annuziato et al., 1997). These compounds are useful in single channel patch clamp electrophysiology experiments. Otherwise due to their non specific nature, it is difficult to use these NCX inhibitors to elucidate the function of NCX.

NCX is involved in a variety of pathological states such as ischemia-reperfusion, hypertension, and oxidative stress (will be discussed in more detail in the NCX and pathology section, Annuziato et al., 1997). As a result, NCX inhibitors are being investigated as pharmaceutical tools to help combat hypertension and ischemia-reperfusion (Bell et al., 2000; Coleman & Khalil, 2002; Iwamoto et al., 2004; Matsuda et al., 2001).

Functional properties of NCX

Since NCX works in a bidirectional manner – forward or reverse mode, each mode has different physiological effects (Aiello et al., 2005; Iwamoto et al., 1996). The direction of NCX is governed by electrochemical potentials of Na^+ and Ca^{2+} but the rate of transport is determined by concentrations of Na^+ and Ca^{2+} and the effects of various modulating agents (Annuziato et al. 1997; Dipolo et Beauge, 1987; Grover, 1984). Several hypothetical models suggest the NCX mechanism of ion translocation. One

model implies that the transporter can only bind and transport one species of ion at a time, whereas another model suggests that both species of ions may be transported simultaneously (Blaustein & Lederer, 1999). A model for the transport cycle of cardiac NCX1 assumes conformations of the ion transport sites as inward-facing E_1 and outward-facing E_2. The exchange activity is regulated in the forward mode largely by intracellular Na^+ (K_i=20mM) and in the reverse mode largely by $[Ca^{2+}]_i$ (K_i= 6μM, Shigekawa & Iwamoto, 2001).

Forward mode NCX may be crucial in cells that need to extrude a large amount of Ca^{2+} rapidly; for example: cardiac muscle cells (Blaustein & Lederer, 1999). During quiescent periods, the electrochemical driving force of the exchanger can be modulated by the transmembrane potential or intracellular Na^+ (Fujioka et al., 2000; Matsuoka et al., 1992; Matsuoka et al., 1995; Shigekawa & Iwamoto, 2001; Szewczyk et al., 2007). Consequently, only a small fraction of the NCX exchangers are active at the normal resting $[Ca^{2+}]_i$ in most cells (Dipolo, 1979). In contrast, the exchanger is fully activated in the forward mode when $[Ca^{2+}]_i$ is in the low micromolar range, about the concentration expected during peak activity in many types of excitable and secretory cells (Blaustein & Lederer, 1999).

The reverse mode is largely used as a research tool to track the entry of Ca^{2+} although it also has a variety of physiological roles. Reverse mode NCX may bring Ca^{2+} in the cell during contractile cycle of the cardiac muscle (Grantham & Cannell, 1996). Furthermore, reverse mode NCX has been reported to be involved in re-filling the SER with Ca^{2+} after asynchronous Ca^{2+} release (Fameli et al., 2007; Lee et al. 2001). The

reverse mode of NCX is implicated in many diseases as it can cause Ca^{2+} overloading in the cell leading to many harmful effects (elaborated in the NCX and pathology section, Annuziato et al., 2007; Grantham & Cannell, 1996).

There are several interesting functional aspects of NCX that are predominant in the literature. PMCA and NCX operate in parallel to remove $[Ca^{2+}]_i$, although they each contribute differently to Ca^{2+} removal (Blaustein & Lederer, 1999). NCX is a low affinity high capacity transporter useful for transporting large amounts of Ca^{2+} when the $[Ca^{2+}]_i$ levels are high (Grantham & Cannell, 1996). PMCA is a high affinity low capacity transporter which is important for regulation of Ca^{2+} at lower resting levels (Zylinska & Soszynski, 2000). Selective inhibitors will elucidate the functional role of these Ca^{2+} extrusion mechanisms. Unfortunately, NCX does not have a selective inhibitor in the forward mode (see section on NCX inhibitors). PMCA selective inhibitors, caloxins, have just been recently designed (Pande & Grover, 2005).

Another interesting feature of NCX is its localization in restricted portions of the plasma membrane of some types of cells. NCX has been known to localize in specialized lipid based structures in the plasma membrane called caveolae (Bossuyt et al., 2002; Cha et al., 2004; Scriven et al., 2005; Teubl et al., 1999). The localization of NCX in conjunction with other Ca^{2+} transporters may lead to specialized Ca^{2+} regulation pockets (Blaustein et al., 1999).

NCX and pathology

NCX activity has been implicated in pathologies such as white matter degeneration after spinal cord injury, brain trauma, and optical nerve injury (Annuziato et al., 1997). More specifically, relating to the coronary artery, NCX may also play a role in ischemia reperfusion, hypoxia/anoxia and hypertension (Annuziato et al., 1997; Bell et al., 2000; Coleman & Carafoli, 2002; Iwamoto et al., 2004; Matsuda et al., 2001).

NCX mediates Ca^{2+} induced white matter damage caused by anoxia or trauma (Stys, 1998). Elevated Na^{+} and depolarization during anoxia or trauma promotes Ca^{2+} overload mediated primarily by reverse mode NCX (Stys, 1998). Drugs capable of inhibiting NCX activity, such as bepridil and amiloride derivatives, reduce the white matter damage in different experimental models of white matter injury (Li et al., 2000, Stys et al., 1990; Stys, 1998; Wolf et al., 2001).

The use of various ischaemic models has produced conflicting results on the role of NCX during ischemia (Graham & Trafford, 2007). Some models show that NCX activity is neuroprotective while others indicate that it is neurodamaging. Recent *in vitro* studies suggest that proteolytic cleavage of NCX1 and NCX3 may render increased susceptibility to ischaemic and excitotoxic death (Annuziato et al., 2007).

NCX activity has been linked to apoptosis, potentially by causing NCX dependent Na^{+} efflux and thus cell shrinkage (Lang et al., 2004). Deregulation of NCX activity has been linked to a shift from apoptosis to necrosis due to NCX dependent Ca^{2+} overloading in the cell (Shigeki et al., 2005). The impairment of Ca^{2+} homeostasis in neuronal cells is considered to be the major triggering event that leads to the development of brain aging

(Annunziato et al., 2002). In aged rats, the activity of NCX is markedly reduced in the forward and reverse mode (Annuziato et al., 1992; Michaelis et al., 1984). Nevertheless, during the aging process, NCX is not the only Ca^{2+} sensitive membrane extrusion system that is impaired (Michaelis, 1989).

Selective NCX inhibitors and genetically engineered mice show that salt-sensitive hypertension may be triggered by Ca^{2+} entry through NCX1 in arterial smooth muscle (Iwamoto et al., 2005). The NCX inhibitor SEA 0400 selectively suppresses salt-dependent hypertension (Iwamoto et al., 2005). This antihypertensive profile is unique and differs from that of Ca^{2+} -channel blockers, which lower blood pressure in most hypertensive models (Iwamoto et al., 2005).

Measuring NCX activity

NCX activity may be monitored using: electrophysiology techniques to measure ion currents; pharmacological techniques to measure $^{45}Ca^{2+}$ fluxes; or fluorescent imaging techniques to measure direct changes in $[Ca^{2+}]_i$ with Ca^{2+} sensitive fluorescent probes.

Since NCX is electrogenic, a large body of work on it has been carried out by monitoring ion currents in squid axons using whole cell patches or membrane patches. In measuring NCX ion current, the non specific channels must be excluded with the use of ouabain (inhibits the Na^+ pump) and tetrodotoxin (blocks a variety of non-specific Ca^{2+} channels, Blaustein, 1977). Therefore forward mode NCX ion currents are defined as external Na^+-dependent Ca^{2+} efflux and an internal Ca^{2+} -dependent ouabain- and tetrodotoxin -insensitive Na^+ influx (Blaustein, 1977). Reversal of the Na^+ gradient can

drive NCX into the reverse mode, this mode of exchange is usually identified as the internal Na^+-dependent Ca^{2+} influx and an external Ca^{2+} -dependent, ouabain-insensitive Na^+ efflux (Blaustein, 1977).

Measuring $[Ca^{2+}]_i$ can be performed by loading the cells with acetoxymethyl (AM) esters of fluorescence dyes such as Fura-2 or Fluo-3 (Roe et al., 1990). The dyes are non-polar therefore they pass through the cell membrane easily where they are de-esterifed and then trapped inside the cell (Roe et al., 1990). The fluorescence intensity of the dye changes with different $[Ca^{2+}]_i$ (Roe et al., 1990).

In measuring NCX, by electrophysiology techniques or fluorescent imaging techniques, NCX activity in cells can be stimulated by voltage changes or changes in extracellular Na^+ (Blaustein & Lederer, 1999). Inhibitors are used to block the ion current or changes in $[Ca^{2+}]_i$ to establish the NCX activity. These experiments have yielded a large amount of information concerning the regulation of NCX in various cell types and tissue types.

Reversal of the Na^+ gradient can drive NCX into the reverse mode, which can be followed by monitoring $^{45}Ca^{2+}$ uptake (Aiello et al., 2005, Iwamoto et al., 1995). This is accomplished by loading the cells with Na^+ in the presence of 1mM ouabain, 25μM nystatin and 10 μM nitrendipine. The cells are then placed in buffers containing Na^+ and/or Na^+-free buffer in which Na^+ is replaced with N-methyl-glucamine (NMG). In the Na^+ free buffer the Na^+ gradient is reversed so that Na^+ is expelled from the cell and $^{45}Ca^{2+}$ is taken up into the cells. The Na^+ containing buffer acts as the negative control.

Another measure of NCX activity can be accomplished by removing extracellular K^+ and Ca^{2+} (Elmoselhi et al., 1994). This will inhibit the Na^+/K^+ ATPase, thus preventing the removal of Na^+, and causing the cell to depolarize. The combination of high intracellular Na^+ and cell depolarization is the ideal condition for Ca^{2+} entry via NCX. Thus, the addition of extracellular Ca^{2+} into the buffer will activate NCX in the reverse mode, and Ca^{2+} will enter into the cell. This Ca^{2+} can be measured by using $^{45}Ca^{2+}$ or loading the cells with Ca^{2+} dyes such as Fura-2 or Fluo-3 and measuring $[Ca^{2+}]_i$ transients. Since the exchanger is in reverse mode, KB-R7943 or SEA 0400 can also be used to inhibit the NCX activity.

1.2 Coronary artery

The coronary artery allows transport of oxygen and necessary nutrients throughout the heart. The artery is constructed of three different layers: intima, media and adventia (Malhorta et al., 2003). The innermost layer, the intima, is comprised of a monolayer of endothelial cells (Malhorta et al., 2003). The endothelium functions as a barricade between the blood and the underlying tissues. The endothelium also has the ability to regulate the tone of the artery. The next layer, the media, is composed of extracellular matrix proteins and most importantly smooth muscle. The outermost layer is named the adventia and functions as mechanical support (Malhorta et al., 2003). Varicosities are primarily found in the adventitia and the outer layer of arterial muscle (Daniel et al., 1995; Lee et al, 1988; Wu et al., 1988).

Damage to the coronary artery can lead to drastic physiological effects. The most frequent damage is the development of atherosclerotic plaques. These plaques narrow or even block the arteries lowering the blood supply to the heart leading to many complications such as coronary spasms and ischemia reperfusion.

Endothelium

Endothelial cells are involved in many processes: vasoconstriction, vasodilation, blood clotting, atherosclerosis, formation of new blood vessels, inflammation and swelling (Kinlay et al., 2001; Tousoulis et al. 2006). They also control the passage of white blood cells and other material from the blood stream into the rest of the body (Brooks, 1988). Some types of endothelium are highly specialized at filtration such as in the blood brain barrier and the renal glomerulus (Takakura et al., 1991).

Endothelium located in the lumen of most arteries is composed of a monolayer of cells (Ghitesco & Robert, 2002). It has three discrete cell surfaces; luminal, abluminal and lateral. Each surface plays an important role in arterial functioning. The luminal surface is in direct contact with the blood. It contains many receptors and adhesion molecules. Furthermore, it can secrete anti-thrombotic molecules which travel into the blood plasma or remain on the cell surface (Malhorta et al., 2003). The abluminal surface functions as a regulator of the vascular tone; it secretes many specialized substances towards the smooth muscle such as NO and endothelin (Ghitescu & Robert, 2002). The lateral surface joins the neighboring cells together through intercellular junctions (Dejana et al., 1995). These junctions allow the endothelial cells to communicate with each other

by passage of ions and other substances. More importantly, the tight junctions create an endothelial barrier, thus protecting the smooth muscles cells. The endothelial cells are distributed throughout the entire body and depending on their location, demonstrate different physiological variations (Luscher et al., 1997).

Endothelial dysfunctions are found in a large variety of disease such as atherosclerosis, hypertension and diabetes (De Meyer et al., 1997; Hill & Whitten, 1997; John et al., 2000; King et al., 1994; Quyyumi, 1998; Stehouwer et al., 1997; Toborek & Kaiser, 1999). Other diseases, for instance the inhibition of blood vessel growth (an endothelial cell dependent process) in solid tumors, still represent the site of a concentrated and promising research effort (Drevs et al., 2000; Folkman, 1995; Martelli et al., 2006; Skobe et al., 1997,).

Endothelial cells are easy to identify in culture. They contain unique proteins called von-Willebrand factor and endothelial NOS (Hristov & Weber, 2004). Through the use of Western blots or by immunocytochemistry, these unique proteins can be used to differentiate endothelium from smooth muscle cells.

Various agents such as ATP, bradykinin, thapsigargin and A23187 increase $[Ca^{2+}]_i$ in endothelial cells by different mechanisms (Li & van Breeman, 1995; Schilling et al., 1988; Uchida et al., 1999). Therefore, it is likely that the role of NCX would differ with the type of stimuli. Some of these pathways may alter NCX mediated $^{45}Ca^{2+}$ extrusion and others may affect $^{45}Ca^{2+}$ entry.

Smooth muscle

Smooth muscle is a type of non-striated muscle found within the walls of hollow organs and places such as the bladder and abdominal cavity, the uterus, gastrointenstinal tract, respiratory tract and vasculature, the skin and the ciliary muscle and the iris of the eye (Gollash et al., 2000). Smooth muscle is different from skeletal muscle and cardiac muscle in both structure and function (Gollash et al., 2000). Different types of smooth muscles which are located in various organs perform diverse functions: control of blood pressure in blood vessels, childbirth in myometrium, and aid in digestive and excretory functions in the gastrointestinal canal, and respiratory functions in the trachea (Gollash et al., 2000).

Smooth muscle cells are elongated and spindle shaped, each containing loose bundles of thick and thin filaments within the cytoplasm (Craig et al., 2006). Myosin makes up the primary structural component of thick filaments (Craig et al., 2006). Upon an increase in $[Ca^{2+}]_i$, contraction of smooth muscle occurs via cross-bridge formation between myosin and actin (Lodish et al., 2000). Smooth muscle cells differ from endothelium in the expression of myosin and actin (Craig et al., 2006).

There are several ways in which $[Ca^{2+}]_i$ is increased in smooth muscle cells. Membrane depolarization causes Ca^{2+} entry by opening L-type VOCC. $[Ca^{2+}]_i$ in the cytosol can trigger Ca^{2+} sensitive mechanisms such the induction of the store operated Ca^{2+} entry or Ca^{2+} induced Ca^{2+} release from the SER (Dong et al., 2006; Zhang et al., 2005). The hormone angiotensin II binding to AT1 receptors may trigger either $G_{\alpha q}$ (linked to IP_3 stimulated Ca^{2+} release from SER) or $G_{\alpha i}$ (inhibit adenyl cyclase) and lead

to different variations of $[Ca^{2+}]_i$ transients (Grover et al., 1994; Tekunkara et al., 2003). Another example is endothelin which may mobilize Ca^{2+} through multiple pathways (Aiello et al., 2005; Bell et al., 2000; Elmoselhi et al., 1994).

NCX in smooth muscle and endothelium

NCX in smooth muscle and endothelium has been well examined by a large variety of mechanisms. Depending on the type of smooth muscle and the type of endothelium, NCX demonstrates different activity levels, isoforms, regulation and functional properties; therefore in each model it is important to reassess NCX. In the coronary artery, NCX controls the level of $[Ca^{2+}]_i$ which in turn regulates the coronary tone. In the coronary artery SMC and EC, activity of the isoform NCX1 is predominant (Quedau et al., 1997; Qiu et al., 2001).

NCX has been studied in the pig coronary artery smooth muscle cells, in which NCX has been implicated in the isopreterenol induced reduction of $[Ca^{2+}]_i$ and tension (Yamanaka et al., 2003) . In porcine coronary smooth muscle, NCX has been implicated in SER Ca^{2+} unloading (Heaps et al., 2000; Jones et al., 2000). NCX activity in porcine endothelium has been established in cardiac micro vascular endothelial cells and $[Ca^{2+}]_i$ has been identified as a potential physiological regulator of NCX expression (David & Bos, 1999).

There is also evidence for a linkage between NCX and SERCA. The actions of NCX and SERCA have been proposed to be linked through a limited junctional cytoplasmic space model. Evidence for this model is based on several key theories.

The first theory linking NCX-SERCA assumes that the PM containing NCX and the SER membrane containing SERCA are in close proximity. Liang et al. (2004) demonstrates that in rabbit aortic endothelial cells, ryanodine receptors (which are located on the SER) and NCX are in close functional proximity near the plasma membrane.

Secondly, if NCX and SERCA are linked, inhibition of one Ca^{2+} transporter should affect the other, whereas other Ca^{2+} transporters such as PMCA should not be affected. Thus, inhibition studies are useful to provide evidence linking NCX and SERCA. In a study based on $[Ca^{2+}]_i$ measurements, when NCX was arrested by removing both external Na^+ and Ca^{2+}, Ca^{2+} released from the SER was resequestered. However, when both NCX and SERCA were blocked, the Ca^{2+} released from the reticulum was then extruded from the cells by PMCA (Nazar & van Breeman, 1998). In pulmonary artery endothelial cells, inhibition of PMCA or NCX demonstrated that the two pathways contributed to the Ca^{2+} extrusion in a non-additive fashion (Klishin et al., 1998; Sedova & Blatter, 1999).

Thirdly, the NCX-SERCA linkage may serve some physiological purpose. During agonist induced asynchronous Ca^{2+} oscillations in vascular smooth muscle the SER must be refilled with Ca^{2+} (Fameli et al., 2007). Evidence suggests that it is plausible and possible for sufficient Ca^{2+} to pass through the NCX and SER junctions to replete the SER during the regenerative phase of the Ca^{2+} release (Fameli et al., 2007).

Lastly, if NCX and SERCA are linked, the cytoskeleton may aid in maintaining the NCX-SERCA linkage. Disruption of the cytoskeleton with calyculin inhibits the

phenylephrine-induced generation of asynchronous Ca^{2+} waves in smooth muscle cells maintained by NCX and SERCA (Lee et al., 2005).

There is sufficient evidence for a linkage between NCX and SERCA; however, other processes may also participate in this linkage. For instance, store operated Ca^{2+} channels may also be associated with NCX activity, especially in the Ca^{2+} entry mode (Arnon et al., 2000, Zhang et al., 2006).

The literature has not yet been able to prove a functional model for the NCX-SERCA linkage. Our lab proposes that the linkage between NCX and SERCA is crucial in specific cell types for Ca^{2+} regulation. Using our past experience working with pig coronary artery SMC and EC in addition to having studied other Ca^{2+} handling mechanisms such as PMCA and SERCA, we plan to associate a functional relationship to this linkage that is unique to SMC and not EC. We also plan to investigate this linkage in disease states. The hypothesis is that oxidative stress can destroy this linkage leading to disruption of Ca^{2+} handling and increasing the severity of various pathological states.

1.3 Experimental model

Pig is used as our model for several reasons. Firstly, it is similar in its physiology and anatomy to humans. Secondly, the abattoir located close to our facility can supply large numbers of hearts needed for this work, and thirdly, our lab has completed extensive research and has a great expertise using the pig model, specifically in Ca^{2+} related studies.

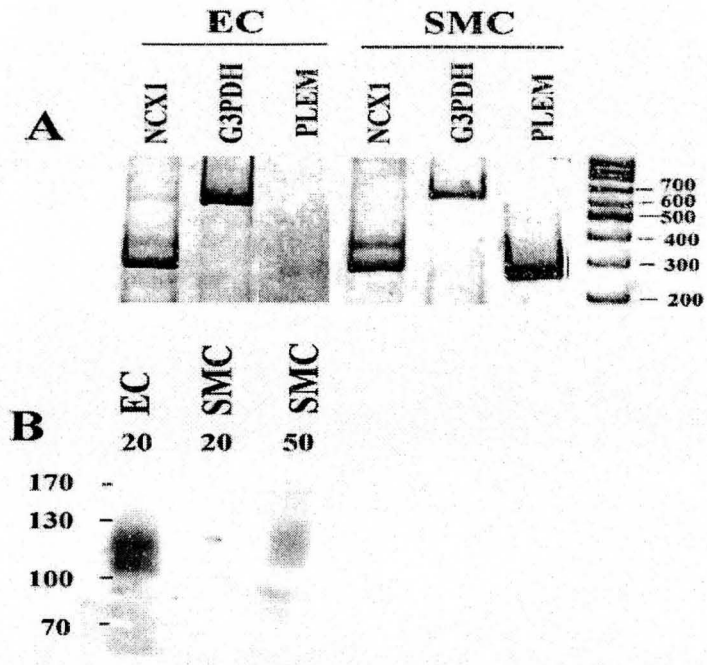
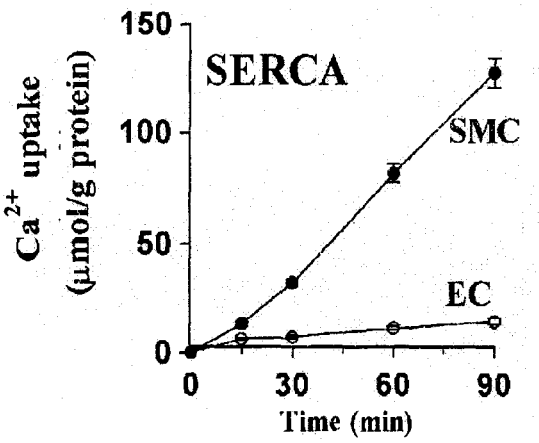


Figure 3. Expression of NCX in EC and SMC. a) RT-PCR analysis in EC and SMC of NCX1 and PLEM, G_3PDH is used to control expression levels. Both EC and SMC express a strong band at 309-bp corresponding to NCX 1.3 and a larger but weaker band corresponding to NCX1.7. SMC express phospholemman mRNA, but EC does not. b) Western Blot analysis of PM-enriched fractions of SMC and EC examining NCX1 protein abundance. EC exhibits a doublet at 116 kD and 112 kD, SMC exhibits a very faint band (modified from Szewczyk et al., 2007).

A



B

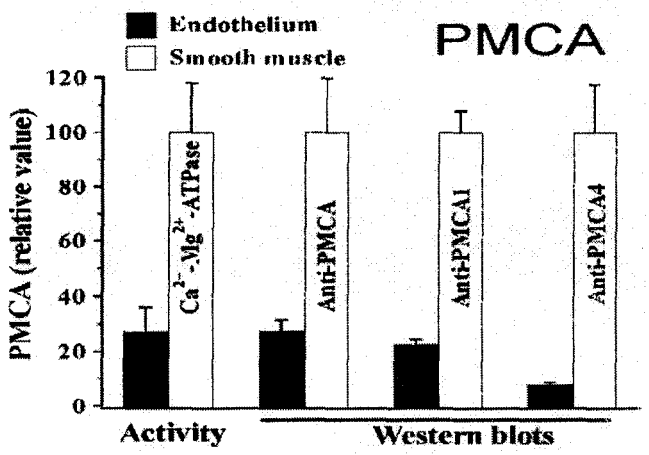


Figure 4. Activity of SERCA and PMCA in the coronary artery. A) SERCA activity measured by Ca^{2+} uptake in permeabilized SMC and EC. B) PMCA activity, PMCA1, PMCA 4 & total PMCA expression in EC and SMC, relative to SMC as 100% control value (modified from Szewczyk et al., 2007).

1.4 Data from our lab

RT-PCR experiments showed that NCX1 mRNA is present in cells cultured from both EC and SMC (figure 3A, Szewczyk et al., 2007). Two different splices can be identified in figure 3A, NCX1.3 and NCX 1.7, in both endothelium and smooth muscle Furthermore, mRNA for the protein PLEM has been identified in SMC but not EC. In addition, the presence of NCX1 has also been confirmed with Western blot analysis (Figure 3b) of plasma membrane enriched fractions made from cultured cells. EC shows a significantly higher expression of NCX than SMC. Membranes isolated from freshly isolated smooth muscle tissue also contain the NCX1 protein (unpublished).

Our lab has also examined PMCA and SERCA in these two cell types. Both SERCA and PMCA demonstrated higher activity levels in SMC (Szewczyk et al., 2007). PMCA expression was also found to be significantly higher while NCX has demonstrated a much lower expression in SMC (Figure 4, Szewczyk et al., 2007). The amounts of Ca^{2+} buffering proteins like calmodulin, calreticulin, calbindin, and calsequestrin may also be different in SMC and EC. Thus regulation of Ca^{2+} between EC and SMC is very different.

1.5 Overall Objective

Arterial SMC and EC differ in their structure and function. The function of SMC is the generation of tone which is achieved by the Ca^{2+} dependent contractile filaments. Since these filaments are distributed throughout the cell, Ca^{2+} must be transported to and removed from deep within the cell. As a result, the SER may play a large role in Ca^{2+}

regulation in the SMC. Furthermore, SMC also contain higher levels of high affinity Ca^{2+} pumps (SERCA and PMCA) and thus Ca^{2+} is more tightly regulated. Endothelial cells release NO in response to an increase in $[Ca^{2+}]_i$, which relaxes the smooth muscle. The endothelial nitric oxide synthase (eNOS) and is located adjacent to the PM in EC. The SER that removes Ca^{2+} from deep within the cell cytosol may play a small role in Ca^{2+} dependent modulation of the eNOS activity. Based on the Western blot data, EC contain a greater amount of the high capacity transporter NCX, thus larger quantities of Ca^{2+} can be removed from the cell and from the vicinity of eNOS.

Aim 1 is determination of ACTIVITY levels of NCX in SMC and EC. This is examined mainly as the NCX mediated Na^+ dependent Ca^{2+} entry. Physiological and pathophysiological implications of this entry were discussed on p19. It includes characterization of NCX in the cultured cells. There have been suggestions in the literature that there may be a functional linkage between NCX and SERCA. Aim 2 is to assess the existence of this linkage in the two cell types. NCX activity is determined by monitoring in the Ca^{2+} extrusion mode and whether the cells contain any NCKX activity.

2. Methods

2.1 Materials

The following chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada): Bovine serum albumin, 3-(N-Morpholino)-propanesulfonic acid (MOPS), N-methyl glucamine (NMG), ethylene glycol tetraacetic acid (EGTA), N-2-hydroxyethyl-piperazine-N8-2-ethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), ouabain, dithiothretol, $LaCl_3$, nystatin, phenylmethylsulfonylfluoride (PMSF), thapsigargin, monensin, calyculin A and cytochalasin. KB-R7943 was purchased from Tocris Biosciences (Ellisville, Missouri, USA). Nitrendipine was purchased from Miles Pharmaceuticals (West Haven, Connecticut, USA). Fura-2 Am was purchased from Teflabs (Austin, Texas, USA). BAPTA and Dulbecco's modified Eagle's medium (DMEM) and Ca^{2+} and Mg^{2+} free Hanks balanced solution were purchased from Invitrogen (Burlington, Ontario, Canada). SEA 0400 was synthesized by Dr. Lorand Kiss at the University of Szeged, Hungary. Cariporide was a gift from Aventis Pharma, Frankfurt, Germany. The radioisotope $^{45}Ca^{2+}$ were purchased from Perkin Elmer (Waltham, Massachusetts, USA). Tissue culture supplies were purchased from Fisher (Nepean, Onatrio, Canada), 96 well plates were from Sarstedt (Montreal, Quebec, Canada), scintillation cocktail from Beckman Coulter (Mississauga, Ontario, Canada) and Bradford reagent from BioRad (Mississauga, Ontario, Canada).

2.2 Tissue dissection, membrane isolation and $^{45}Ca^{2+}$ uptake experiments for fresh smooth muscle tissue

Thirty to sixty hearts obtained from Maple Leaf Meats (Burlington, Ontario, Canada) were placed in ice cold buffered saline solution containing 10 mM HEPES (pH 6.4), 140 mM NaCl, 5mM KCl, 1mM $MgCl_2$, and 1.5 mM $CaCl_2$. Tissue slices containing the left coronary artery were dissected and placed in ice cold HEPES-Krebs' solution containing the following: 116 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 11 mM glucose and 11 mM HEPES (pH 6.4). Coronary artery smooth muscle from these arteries were dissected and used to isolate plasma membrane vesicles as described previously (Grover et al., 1985). The plasma membrane was centrifuged at 500,000 g for 15 min, the pellet was washed and re-suspended in 8 % sucrose, 50 mM imidazole-HCl, and 0.1 M NaCl (pH 7.4). This sample was placed on ice and used for $^{45}Ca^{2+}$ uptake experiments.

Ten µl of the plasma membrane suspension was placed in 500 µl of the $^{45}Ca^{2+}$ uptake solution containing: 20 mM MOPS-tris (pH 7.4 at 37°C), 50 µM CaCl2, 10 µM nitrendipine, including a trace amount of $^{45}Ca^{2+}$, and either 140 mM Na^+ or NMG at 37°C for 5 min (pH 7.4 at 37°C). The $^{45}Ca^{2+}$ uptake was stopped by filtering the membranes through 0.45 µM nitrocellulose filters under suction, and washing the filters with an ice cold wash buffer containing 20 mM MOPS, 100 mM $MgCl_2$, and 1 mM $LaCl_3$ (pH 7.4). The filters were placed into vials and dissolved by 5 ml of scintillation cocktail. Then the solution was counted for radioactivity.

2.3 Cell cultures

Smooth muscle

Smooth muscle cells from the left coronary artery were isolated and cultured as previously described (Grover et al., 1985). The cells were grown to confluence and then replated into a larger flask. The culture medium, 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 split into 60 millimeter petri dishes and used on day 7 of growth for $^{45}Ca^{2+}$ experiments. 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 that these cells are SMC. Further characterization using immunoblots established that the SMC cells contain high levels of PMCA, SERCA 2b and a low level of NCX1.

Endothelium

Endothelial cells from the left coronary artery were isolated and cultured as previously described (Grover et al., 1985) using the same medium as used for the smooth muscle cells. The coronary artery is dissected in a similar fashion to the smooth muscle cell dissection described above. Once the cells grew to confluence, they were removed by trypsinization in 0.25% trypsin, 1 mM EDTA in Ca^{2+} and Mg^{2+} free Hanks balanced solution for 4 min at 37°C and replated. At the third passage the cells were frozen into

aliquots of 2 million cells/ml. Passage 3 cells were thawed and replated as needed. Confluent cells from passage 4 were split into 60 mm petri dishes and used on day 7 of growth for the $^{45}Ca^{2+}$ uptake experiments. EC were characterized to ensure cell purity of each batch. The EC reacted positively to anti-eNOS and anti-von Willebrand factor. They tested negative to anti-smooth muscle α-actin, confirming that these cells are not smooth muscle cells. Further characterization establishes that EC contain low levels of SERCA (isoforms SERCA 2b and SERCA 3) and PMCA but higher levels of NCX1 relative to smooth muscle cells.

2.4 Experimental Procedure for the Na^+ dependent $^{45}Ca^{2+}$ influx experiments

EC (passage 5) and SMC (passage 4) were grown to confluence. Typically, the cells were loaded with 20 μM BAPTA-AM for 2 hours in 37°C at DMEM media. Where specified in the Results, cells were also used without BAPTA loading. The cells were washed twice in a MOPS–tris buffer (pH 7.4) containing 140 mM Na^+, 2 mM $MgCl_2$, 20 mM MOPS, and then Na^+ loaded in MOPS-tris buffer with 1 mM ouabain, 25 μM nystatin and 10 μM nitrendipine. The cells were then quickly washed twice in 2 ml of the above mentioned MOPS buffer with only nitrendipine. The cells were placed in $^{45}Ca^{2+}$ solutions with 20 mM MOPS-tris, 50 μM $CaCl_2$ with trace amounts of $^{45}Ca^{2+}$, 10 μM nitrendipine, and either 140 mM Na^+ or NMG, with additives as specified in the Results. To terminate the $^{45}Ca^{2+}$ uptake, the cells were then washed 6 times with ice cold 20 mM MOPS (pH 7.4) containing 100 mM $MgCl_2$ and 1 mM $LaCl_3$ to remove extracellular radioactivity. The cells were dislodged by scraping in 1 milliliter of cold 0.1 % triton-100

with 1 mM EGTA. Typically, 10 µl of the radioactive solution without cells was diluted with MOPS-tris buffer to 1 ml final volume was used for determining total radioactivity with the scintillation cocktail. Protein concentration was estimated in each sample.

2.5 Experimental Procedure for the Na^+ dependent $^{45}Ca^{2+}$ efflux experiments

EC (passage 5) were grown to confluence. The cells were loaded overnight with $^{45}Ca^{2+}$ at 37° degrees in DMEM. Cells were washed twice in Balanced Saline Solution containing: 10 mM HEPES, 146 mM NaCl, 146 mM NMG, 2 mM $MgCl_2$, 10 mM glucose, 4 mM KCl, and BSA 0.1% (pH 7.4). They were then placed in 1 ml of the saline solution containing either 146 mM Na^+ or 146 mM NMG for various times. Nine hundred µl of the saline efflux solution was removed from the plate and used for radioactivity determination. In initial experiments, it was determined that the efflux solution obtained did not contain detectable amounts of protein. The cells were then scraped and used for protein estimation and radioactivity determination. For each sample, $^{45}Ca^{2+}$ efflux was calculated as a percentage of the total cell $^{45}Ca^{2+}$ content.

2.6 Protein Estimation

The protein estimation was carried out with Bradford reagent. In a 96 well plate, 10 µl of cell solution or 20 µl of efflux solution was placed. In each well 250 µl of Bradford solution (made from stock solution: diluted with water by 5X) was added. The solution was allowed to incubate for 5 minutes and then the absorbance at a wavelength of 595 nm was read with a 96 well plate reader. The concentration of the protein was

estimated by interpolation of a standard curve using bovine serum albumin as the standard (the range used in μg: 0.5, 1.0, 2.0, 3.0, and 4.0).

2.7 Data analysis

NCX dependent Ca^{2+} uptake was calculated as the difference in $^{45}Ca^{2+}$ uptake between the cells in NMG and the cells in Na^+ buffer. The values were calculated as Ca^{2+} uptake per μmol per gram protein. NCX dependent Ca^{2+} efflux was calculated as the difference of the $^{45}Ca^{2+}$ efflux between the cells in Na^+ buffer and the cells in NMG buffer. Ca^{2+} efflux was calculated as percent of total $^{45}Ca^{2+}$ uptake.

Each experiment was repeated 2-4 times, analyzed separately and one representative experiment was selected and presented. Values are presented as a mean ± SEM of the specific number of replicates. Statistical analysis was performed by the computer program INSTAT. Either an unpaired t-test (2 groups) or an ANOVA (3+ groups) followed by a Tukey-Kramer multiple comparison test was used to test the null hypothesis.

Statistical values are given in the figure legends. When an unpaired t test is used (2 groups): t values and the degrees of freedom (df) are given. When an ANOVA is used (3 or more groups): the F value and the df are given, in addition q values for the Tukey Kramer analysis are also displayed. P values <0.05 were considered to negate the null hypothesis.

3. Results

The NCX mediated Ca^{2+} entry and the NCKX mediated Ca^{2+} entry in cultured SMC and EC was examined. In addition the NCX mediated Ca^{2+} efflux in cultured EC was examined. Furthermore the NCX mediated Ca^{2+} uptake experiments were conducted using plasma membrane vesicles isolated from fresh smooth muscle tissue. This thesis focuses on the NCX mediated Ca^{2+} entry in SMC and EC.

3.1. NCX mediated Ca^{2+} uptake in cultured smooth muscle and endothelial cells

NCX mediated Ca^{2+} entry represents NCX activity in the reverse mode. These experiments were performed by Na^+ loading the cells; washing them, and then placing them in a buffer containing Ca^{2+} and Na^+ or NMG (see Methods section for details). The replacement of Na^+ with NMG creates a strong Na^+ gradient which allows the NCX mediated expulsion of Na^+ and uptake of Ca^{2+} to occur. Ca^{2+} entry via voltage sensitive Ca^{2+} channels was blocked with nitrendipine. The NCX mediated Ca^{2+} entry was determined as the difference between Ca^{2+} uptake in the NMG buffer and the Na^+ buffer.

3.1.1 Time course of NCX mediated Ca^{2+} Uptake in cultured SMC and EC

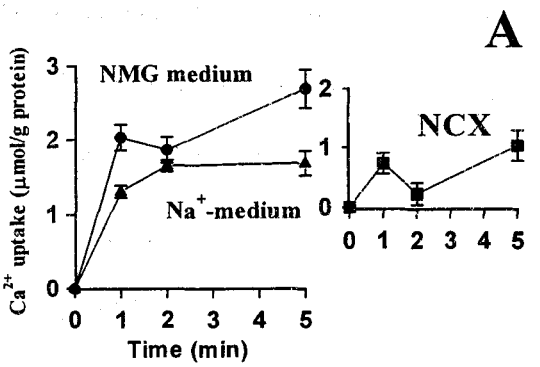
The NCX mediated Ca^{2+} entry was examined in cultured SMC and EC. The cells were Na^+ loaded and the Ca^{2+} uptake was examined in Na^+ or NMG containing solutions as described in the Methods.

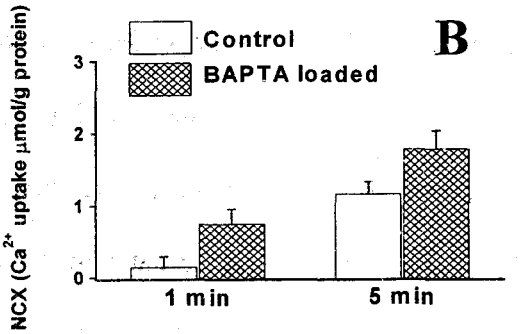
Characterization of the NCX mediated Ca^{2+} uptake in cultured SMC commenced with a time course analysis as shown in Figure 5A. The difference between Ca^{2+} uptake

in Na^+ containing buffer and NMG containing buffer was significant at 1 and 5 min ($p<0.05$), but not at 2 min ($p>0.05$). The difference in the Ca^{2+} uptake between Na^+ and NMG buffer was established as the NCX mediated Ca^{2+} uptake. This uptake was not consistent amongst all the time points due to a large variability as demonstrated in figure 5A inset. In order to attempt to reduce variability the cells were BAPTA loaded.

The effects of BAPTA loading on the NCX mediated Ca^{2+} uptake in cultured SMC was investigated. BAPTA is a Ca^{2+} chelator that can be trapped inside the cells and used for decreasing free $[Ca^{2+}]_i$ to maintain a strong inward Ca^{2+} gradient. BAPTA-AM is a hydrophobic molecule that is able to pass freely through the plasma membrane. Inside the cell, the ester groups on BAPTA-AM are cleaved by esterases to form a charged BAPTA which thus remains trapped inside the cell. The cells were loaded with BAPTA-AM for 2 hours prior to the experiment. BAPTA loading significantly increased the amount of NCX mediated Ca^{2+} in SMC at 1 and 5 min ($p<0.05$, Figure 5B). Thus, BAPTA loading cells was effective at increasing the NCX mediated Ca^{2+} uptake and decreasing the signal to noise ratio. Furthermore, the difference between 1 and 5 min BAPTA loaded cells was significant ($p<0.05$). This indicates that the NCX mediated pathway is not yet saturated and it may be useful to investigate the effects of NCX at longer time periods.

Figure 5C is the time course of the NCX mediated Ca^{2+} uptake in BAPTA loaded SMC for the time periods; 2, 5 and 10 min. The Ca^{2+} uptake in the NMG buffer was significantly higher than in the Na^+ buffer at 2, 5 and 10 min ($p<0.05$). This indicates that BAPTA loading caused the Ca^{2+} uptake in NMG buffer to be significantly increased


A
NMG medium
Na+-medium
NCX
Ca2+ uptake (µmol/g protein)
Time (min)


B
Control
BAPTA loaded
NCX (Ca2+ uptake µmol/g protein)
1 min
5 min

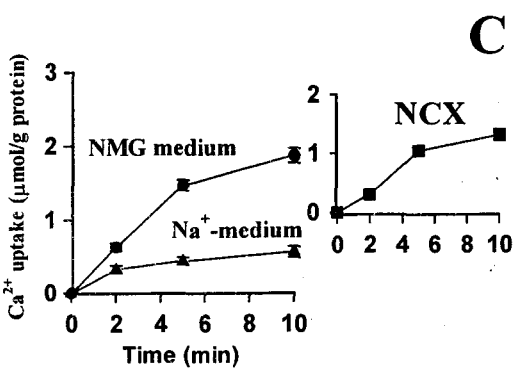
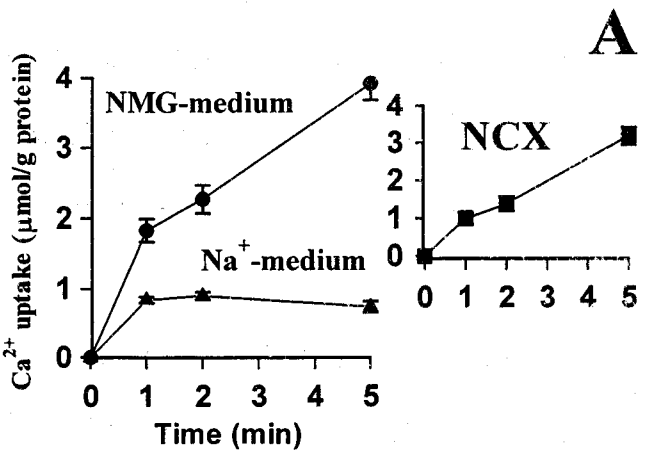

C
NMG medium
Na+-medium
NCX
Ca2+ uptake (µmol/g protein)
Time (min)

Figure 5. Time course of the NCX mediated Ca^{2+} uptake in cultured SMC. A. Ca^{2+} uptake in the presence of extracellular Na^+ or NMG. The Ca^{2+} uptake is significantly different in Na^+ and NMG at 1 min ($p<0.05$, $t=4.81$, degrees of freedom $df= 6$) and 5 ($p<0.05$, $t=2.996$ $df= 8$) but not at 2 min ($p > 0.05$, $t=1.002$, $df = 7$). Inset: The NCX dependent Ca^{2+} uptake is defined as the difference between the Ca^{2+} uptakes in the two media. The NCX component is not linear with time, B. The effect of loading SMC with BAPTA on the NCX dependent Ca^{2+} uptake. Analysis by ANOVA followed by Tukey-Kramer multiple comparisons test established that the NCX dependent Ca^{2+} uptake was greater in the BAPTA loaded cells at 1 min ($p<0.05$, $q=4.681$) and 5 ($p<0.05$, $q=4.371$) than in the controls cells ($p<0.05$, $F=22.982$ $df=17$). The NCX dependent uptake was greater in 5 min than in 1 for the control ($p<0.05$, $q=6.863$) and the BAPTA ($p<0.05$, $q=7.057$) loaded cells, C. Time course of NCX mediated Ca^{2+} uptake in BAPTA loaded SMC. At each time point, the values for the uptake in NMG were greater than those in Na^+ medium (2 min: $p<0.05$, $t=3.792$, $df=10$, 5min: $p<0.05$, $t=3.792$, $df=10$, 10min: $p<0.05$, $t=3.792$, $df=10$). Inset: The NCX component increased linearly with time up to 5 min.

and with decreased variability compared to non BAPTA loaded cells. The NCX mediated Ca^{2+} uptake was linear with time up to 5 min but not for longer periods (Figure 5C inset). Therefore, BAPTA loaded SMC and EC were used in all remaining experiments unless otherwise stated.

The NCX mediated Ca^{2+} entry was next examined in cultured EC. Preliminary time course analysis of NCX mediated Ca^{2+} uptake in EC which were not BAPTA loaded (Figure 6A). The NCX mediated Ca^{2+} uptake was linear with time up to 5 min ($p<0.05$, figure 6A inset). Figure 6B demonstrates the time course of the NCX mediated Ca^{2+} uptake in BAPTA loaded EC. BAPTA loading further increased the NCX dependent Ca^{2+} uptake.

The NCX mediated Ca^{2+} uptake was then compared in SMC and EC across all experiments that were performed (Figure 7). EC had significantly higher NCX mediated Ca^{2+} uptake than the SMC ($p<0.05$). The value in EC was 4-6 fold higher. To further elucidate the role of NCX in SMC and EC, NCX inhibitors were used to test the specificity of the NCX mediated Ca^{2+} uptake.


A
NMG-medium
Na+-medium
NCX
Ca2+ uptake (µmol/g protein)
Time (min)
0 1 2 3 4 5

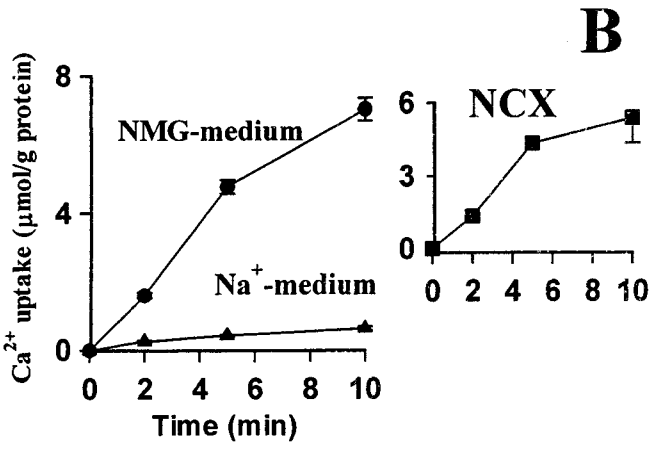

B
NMG-medium
Na+-medium
NCX
Ca2+ uptake (µmol/g protein)
Time (min)
0 2 4 6 8 10

Figure 6. **Time course of NCX mediated Ca^{2+} uptake in cultured EC.** A. Ca^{2+} uptake in the presence of extracellular Na^+ or NMG in cells not loaded with BAPTA. The difference between the Ca^{2+} uptake in the two media was significantly different at 1 ($p<0.05$, $t=5.4$ $df= 10$), 2 ($p<0.05$, $t=6.091$, $df=10$) and 5 min ($p<0.05$, $t=11.566$, $df= 10$). Inset: The NCX dependent Ca^{2+} uptake was linear with time up to 5 min. B. NCX mediated Ca^{2+} uptake in BAPTA loaded EC. At each time point, the values for the uptake in NMG were significantly greater than those in Na^+ medium (2 min: $p < 0.05$, $t=13.167$, $df=9$, 5 min: $p < 0.05$, $t=20.957$, $df=11$, 10 min: $p < 0.05$, $t=18.634$, $df=10$). Inset: NCX mediated Ca^{2+} uptake increased linearly with time for up to 5 min. This experiment was repeated twice with similar results.

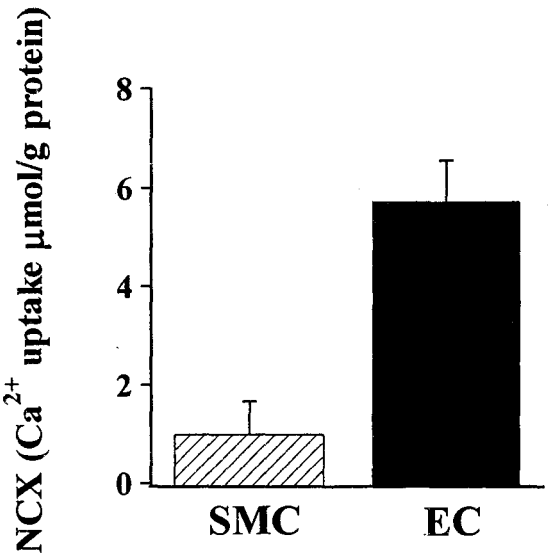


Figure 7. BAPTA loaded EC had greater NCX mediated Ca^{2+} uptake than EC. From 22 experiments for SMC and 14 for EC, the uptake was significantly greater in EC ($p<0.05$, $t=7.059$, $df=34$).

3.1.2. Effects of NCX specific inhibitors on the NCX mediated Ca^{2+} Uptake

The NCX inhibitors, KB-R7943 and SEA 0400, were used to validate that the difference in the Ca^{2+} uptake between the Na^+ and NMG containing buffers was due to NCX and not other Ca^{2+} entry pathways.

The reported value of K_i of KB-R7943 for NCX1 is 4.3 μM. However, it has been reported that it has a higher affinity for NCX3 than for NCX2 and NCX1 (Iwamoto et al., 1996). Furthermore, KB-R7943 at high concentrations (above 10 μM) can block L-type Ca^{2+} channels and voltage gated Na^+ channels (Iwamoto et al., 1996). However, in our experimental protocol these channels are largely blocked with nitrendipine (10 μM) to ensure minimal background noise, so we eliminate the possibility that KB-R7943 is blocking other Ca^{2+} entry pathways and affecting the Ca^{2+} entry.

Figure 8 demonstrates the effects of KB-R7943 on the NCX mediated Ca^{2+} uptake in SMC and EC. KB-R7943 did not significantly affect the basal Ca^{2+} uptake in the Na^+ containing buffer up to a concentration of 30 μM ($p>0.05$, $F=2.141$, $df=17$, data not shown). In contrast, KB-R7943 inhibited the NCX mediated Ca^{2+} uptake (Figure 8A) with a K_i of 2.9 ± 0.6 μM ($p<0.05$) in SMC. In EC (Figure 8B), it inhibited the NCX mediated Ca^{2+} uptake with a K_i of 3.0 ± 0.05 μM ($p<0.05$). Thus, SMC and EC had similar K_i values for the NCX mediated inhibition of Ca^{2+} uptake suggesting that similar isoforms are active in both EC and SMC.

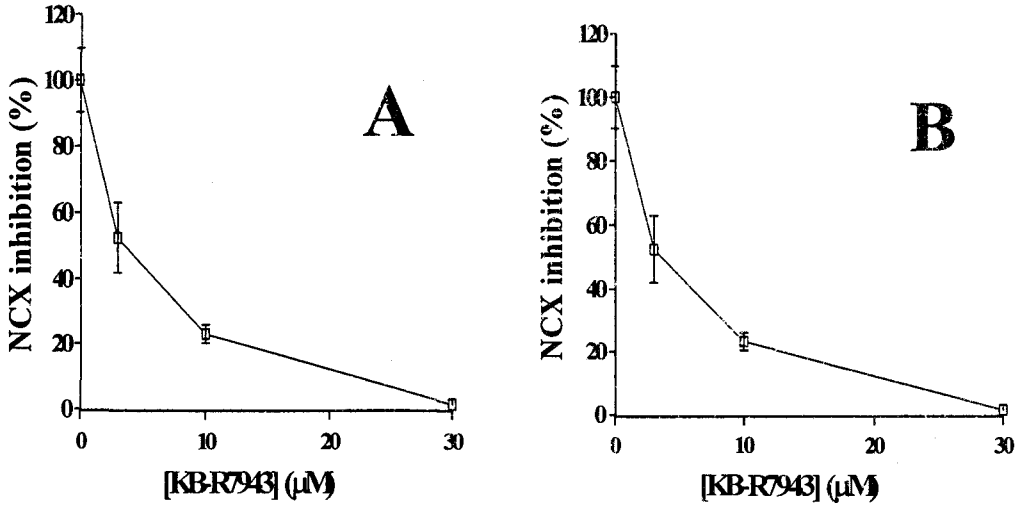


Figure 8. The NCX inhibitor KB-R7943 inhibited the NCX mediated Ca^{2+} uptake of cultured SMC (A) and EC (B), A. In SMC, KB-R7943 significantly lowered the NCX mediated Ca^{2+} uptake at all the concentrations of KB-R7943 ($p<0.05$, $F=10.603$, $df=25$); 3 μM ($p<0.05$, $q= 5.650$), 10 μM ($p<0.05$, $q=4.372$), and 30μM KB-R7943 ($p<0.05$, $q= 7.298$). The K_i value of KB-R7943 was computed to be 2.9 ± 0.6 μM. This experiment was repeated 4 times with similar results. B. EC. Compared to 0 μM KB-R7943 (control): 3 ($p<0.05$, $q=5.709$), 10 ($p<0.05$, $q=9.247$), and 30μM KB-R7943 ($p<0.05$, $q=11.835$) significantly inhibited the Ca^{2+} uptake ($p<0.05$, $F=27.406$, $df=26$). The K_i of Ca^{2+} uptake inhibition for KB-R7943 was 3.0 + 0.5 μM. This experiment was repeated twice with similar results.

SEA 0400 is another NCX inhibitor that preferentially inhibits NCX in the reverse mode. It is more potent than KB-R7943 for NCX1 (K_i = 0.056 μM). SEA 0400 also demonstrates isoform specificity as it exerts a strong effect on NCX1, mildly blocks NCX2 and has very little effect on NCX3 (Iwamoto et al., 1996). SEA 0400 at concentrations up to 3 μM had no significant effect on the basal Ca^{2+} uptake in the Na^+ containing buffer in either SMC or EC ($p>0.05$, $F=0.2369$, $df=17$, replicated in 3 experiments, data not shown). Figure 9 demonstrates inhibition of NCX mediated Ca^{2+} uptake with SEA 0400 in SMC and EC. In SMC (figure 9A), the inhibition is significant at 0.3 and 3 μM SEA 0400 ($p<0.05$). Since SEA 0400 is an NCX1 specific inhibitor and it completely blocks the NCX mediated Ca^{2+} uptake in SMC and EC, it was concluded that in both cell types, the NCX activity is largely due to NCX1.

The protocol used to investigate the NCX mediated Ca^{2+} uptake requires a drastic change in extracellular Na^+ concentration. The following section investigates the possible interference of other Na^+ sensitive pathways in the NCX dependent Ca^{2+} uptake.

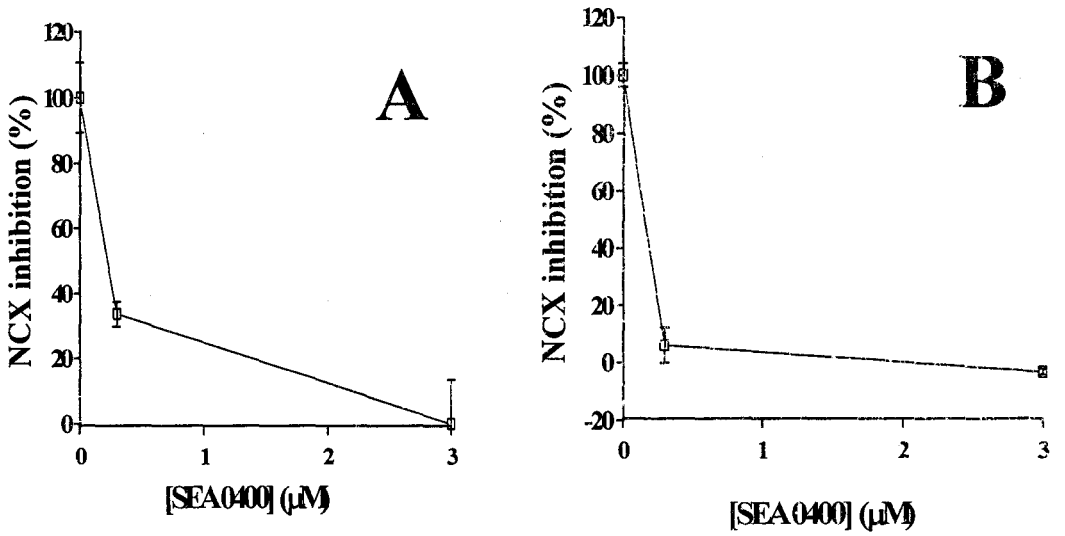


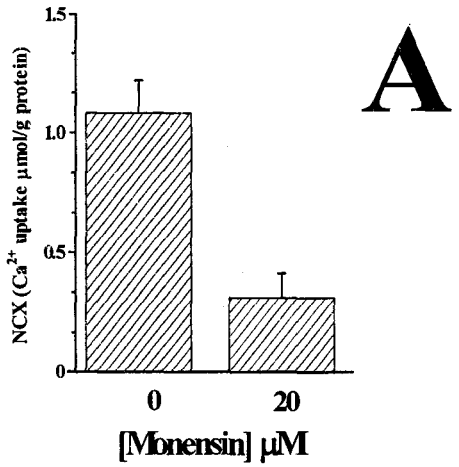
Figure 9. The NCX1 inhibitor SEA 0400 inhibited the NCX mediated Ca^{2+} uptake of cultured SMC (A) and EC (B). A. SMC. Compared to control (no SEA 0400), 0.3 ($p<0.05$, $q=8.199$), and 3 μM SEA 0400 ($p<0.05$, $q=12.349$) significantly inhibited the Ca^{2+} uptake ($p<0.05$, $F=39.491$, $df=14$). This experiment was repeated twice with similar results. B. EC. SEA 0400 significantly inhibited the NCX mediated Ca^{2+} uptake ($p<0.05$, $F=441.73$, $df=14$). As compared to control (no SEA 0400), 0.3μM ($p<0.05$, $q=36.452$), and 3 μM SEA 0400 ($p<0.05$, $q=36.167$) differed significantly. This experiment was repeated twice with similar results.

3.1.3. Effects of Na^+ sensitive pathways on the NCX mediated Ca^{2+} Uptake

The following section investigates the possible interference of other Na^+ sensitive pathways in the NCX dependent Ca^{2+} uptake NCX inhibitors. Monensin (Na^+ ionophore) and cariporide (NHE exchanger) were used to examine the NCX mediated Ca^{2+} uptake.

Since the Na^+ gradient is a major driving force of NCX, abolishing it would inhibit the NCX mediated Ca^{2+} uptake. Monensin functions as a Na^+ ionophore and collapses the Na^+ gradient driving NCX. In the literature, 20 μM monensin was sufficient to reduce the Na^+ gradient (Hattori & Wang, 2006; Malinen et al., 2007). Monensin did not affect basal Ca^{2+} uptake in Na^+ containing medium ($p<0.05$, $t=0.2599$, $df=10$, data not shown, replicated in 3 different experiments). The effect of collapsing the Na^+ gradient with monensin in both SMC and EC is shown in Figure 10. At 20 μM in both SMC and EC, NCX mediated Ca^{2+} uptake was significantly inhibited ($p<0.05$). In conclusion, in SMC and EC the NCX dependent Ca^{2+} uptake is largely driven by a strong Na^+ gradient.

The exclusion of Na^+ in the buffer results in NHE inhibition. NHE is responsible for removing intracellular protons and may thus cause intracellular acidosis if inhibited. Intracellular acidosis can affect cell permeability, Ca^{2+} regulation and Ca^{2+} sensitive processes (Madden, 2000; Xiong, 2006). Therefore, the effects of inhibiting NHE with cariporide were tested on the NCX mediated Ca^{2+} uptake to determine whether acidosis is playing a role (Figure 11). Cariporide did not affect the basal Ca^{2+} uptake in Na^+ containing buffer ($p>0.05$) or the NCX mediated uptake at 2 or 10 μM ($p<0.05$) in both SMC and EC. Therefore, the observed NCX mediated Ca^{2+} uptake cannot be attributed to intracellular acidosis.



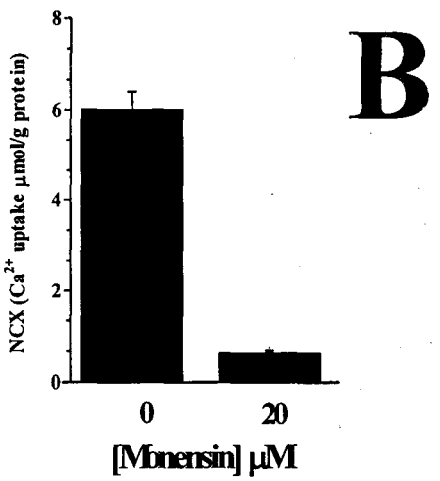


Figure 10. The effect of monensin on the Na^+ mediated Ca^{2+} uptake in SMC (A) and EC (B). Monensin (20 µM) significantly inhibited the Ca^{2+} uptake compared to the absence of monensin (control) in SMC (A; $p<0.05$, $t=3.926$, $df=9$) and EC (B; $p<0.05$, $t=3.144$, $df=9$). This experiment was repeated twice with similar results.

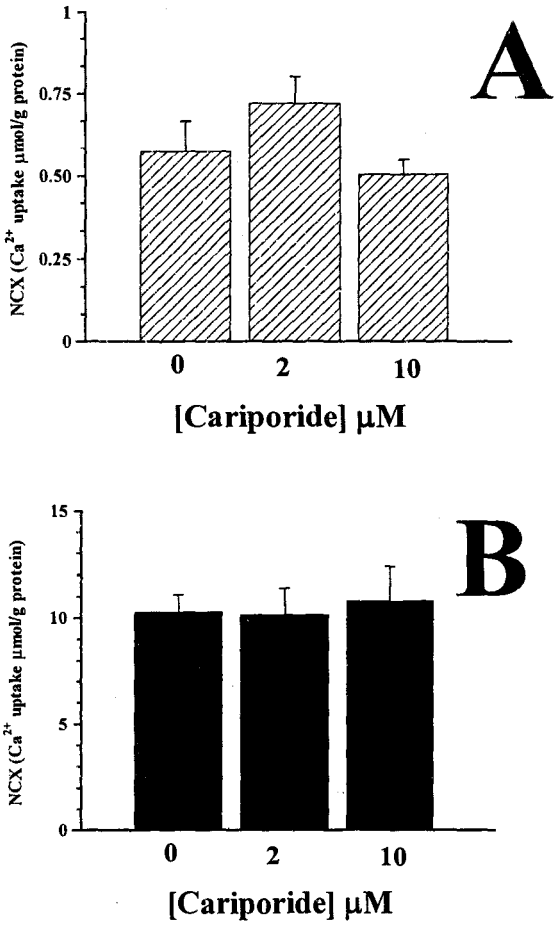


Figure 11. Cariporide did not affect the Na^+ mediated Ca^{2+} uptake in SMC (A) and EC (B). Using an ANOVA test, it was determined that compared to 0 µM cariporide, NCX dependent Ca^{2+} uptake with 2 and 10 µM cariporide did not significantly differ in SMC ($p>0.05$, $t=1.438$, $df=17$) or EC ($p>0.05$, $t=0.7224$, $df=14$). These experiments were repeated twice with similar results.

3.1.4. Linkage between NCX and SERCA using the NCX dependent Ca^{2+} uptake assay

The hypothesis was tested that Ca^{2+} which enters the cells via an NCX mediated pathway is sequestered into the SER by the SERCA pump, hence inhibiting the SERCA pump would also inhibit NCX mediated Ca^{2+} uptake.

Thapsigargin is a SERCA pump inhibitor, and it was used to block SERCA sequestration. In SMC, in the presence of thapsigargin, the NCX mediated uptake was inhibited ($p< 0.05$, Figure 12A). Thapsigargin did not affect the Ca^{2+} uptake in BAPTA loaded cells ($p< 0.05$, Figure 12A). These data are consistent with a model in which SER acts as a sink for the NCX mediated Ca^{2+} entry. However, with BAPTA chelation and the resulting lower $[Ca^{2+}]_i$, the need for SER to act as a sink is eliminated, and NCX is driven in full force.

A similar experiment was also conducted with EC (Figure 12B). BAPTA loading EC increased the NCX mediated Ca^{2+} uptake ($p<0.05$). However, thapsigargin did not inhibit the Ca^{2+} uptake in control cells nor in those loaded with BAPTA ($p>0.05$). Therefore, it is hypothesized that in EC, NCX is not linked to SERCA or not linked as strongly as in SMC.

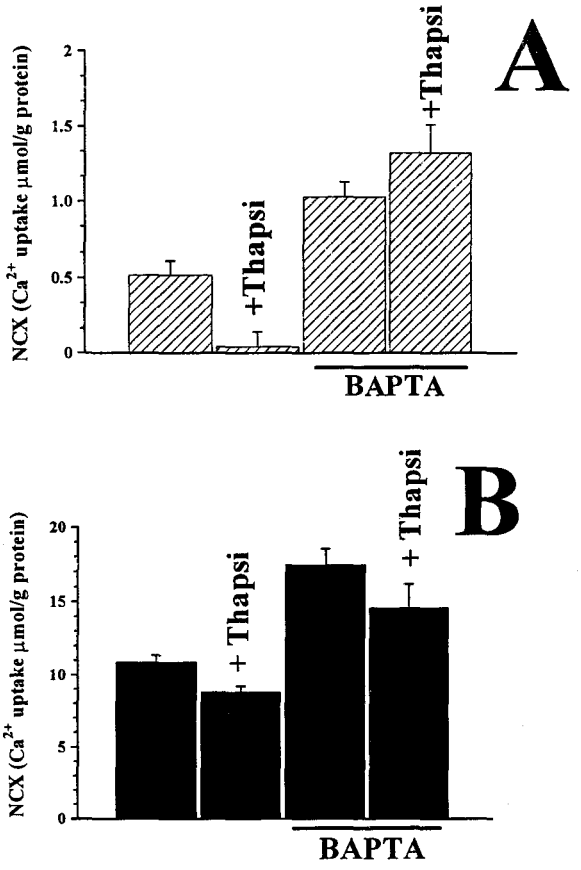


Figure. 12. Thapsigargin on the NCX mediated Ca^{2+} uptake in control and BAPTA loaded cells. A. SMC. BAPTA loaded cells showed greater NCX mediated Ca^{2+} uptake ($p<0.05$, $t=3.747$, $df=8$). Thapsigargin significantly inhibited NCX mediated Ca^{2+} uptake in the control cells ($p<0.05$, $t=3.493$, $df=8$) but not in the cells loaded with BAPTA ($p>0.05$, $t=1.367$, $df=8$). This experiment was repeated twice with similar results. B. EC. BAPTA loaded cells showed greater NCX mediated Ca^{2+} uptake. ($p<0.05$, $t=4.810$, $df=7$). Thapsigargin did not significantly affect the NCX mediated Ca^{2+} entry in control cells ($p<0.05$, $t=1.579$, $df=6$) or in the BAPTA loaded cells ($p>0.05$, $t=1.518$, $df=7$). This experiment was repeated twice with similar results.

3.2 NCX mediated Ca^{2+} efflux in cultured EC

Under normal physiological conditions NCX largely functions in the forward mode to lower $[Ca^{2+}]_i$. NCX in the forward mode expels Ca^{2+} and takes up Na^+. In order to quantify the amount of Ca^{2+} expelled, first the cells were loaded with $^{45}Ca^{2+}$ for 16-18 h, washed, and then placed in an efflux buffer that contained either Na^+ or NMG (see Methods for details). $^{45}Ca^{2+}$ released was determined as a percent of the total $^{45}Ca^{2+}$ initially present in the cells. The difference between the two was designated NCX mediated Ca^{2+} efflux.

Figure 13 is a time course of Ca^{2+} efflux in Na^+ and NMG buffers. At 30 seconds, 1 min ($p<0.05$) but not at 2 min ($p>0.05$) the uptake between the Ca^{2+} efflux in the Na^+ and NMG efflux buffer was significantly different. Thus this assay could be used only for very short time intervals and even then the signal to noise ratio was very high. As a result it was not an ideal assay for characterization of the NCX activity in SMC and EC.

3.3. NCKX dependent Ca^{2+} uptake in cultured SMC and EC

NCKX is a similar transporter as NCX, it transports 4 Na^+ for 1 Ca^{2+} and 1 K^+ (Dong et al., 2006). It is possible that NCKX is partially responsible for the NCX mediated Ca^{2+} uptake. Extracellular KCl was added to the Ca^{2+} uptake buffer in attempt to stimulate NCKX Ca^{2+} uptake. The difference in Ca^{2+} uptake between NMG and NMG+KCl is considered the NCKX mediated Ca^{2+} uptake. From figure 14A it was concluded that SMC did not show a detectable level of NCKX mediated Ca^{2+} uptake. In

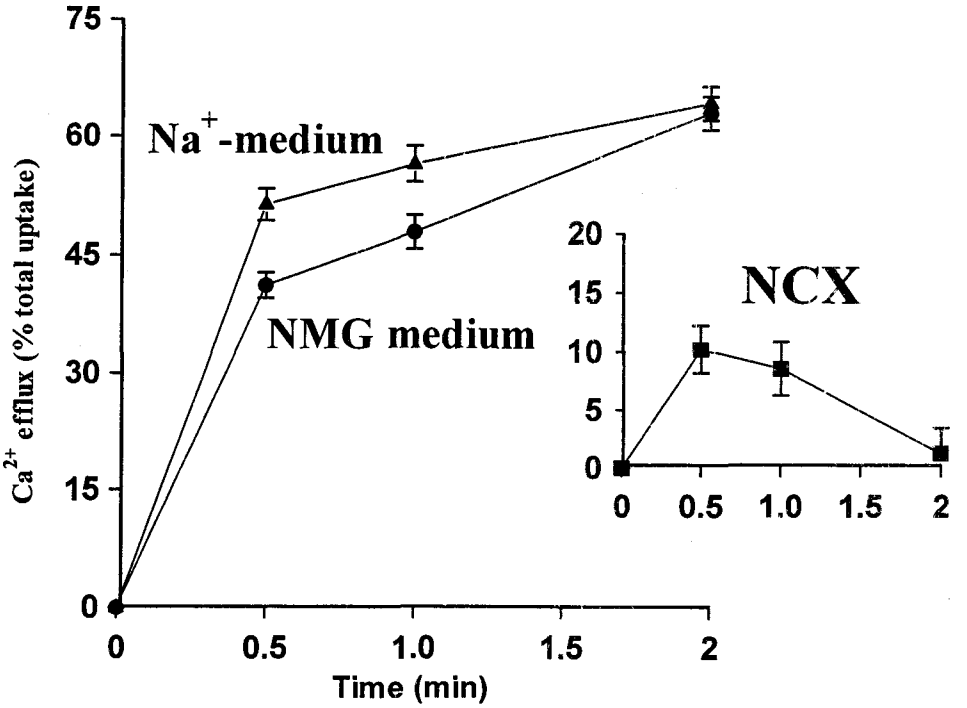


Figure 13. Time course of NCX mediated Ca^{2+} efflux in cultured EC. Ca^{2+} efflux in the presence extracellular Na^+ or NMG is shown as a percent of the total cellular $^{45}Ca^{2+}$ content. The difference between the Ca^{2+} efflux in the two media (NCX mediated Ca^{2+} efflux) is significant at 30 sec ($p<0.05$, $t=3.675$ $df= 12$), 1 ($p<0.05$, $t=2.713$, $df=12$) but not at 2 min ($p<0.05$, $t=0.3894$, $df= 10$). The NCX mediated Ca^{2+} efflux (inset) was not linear with time.

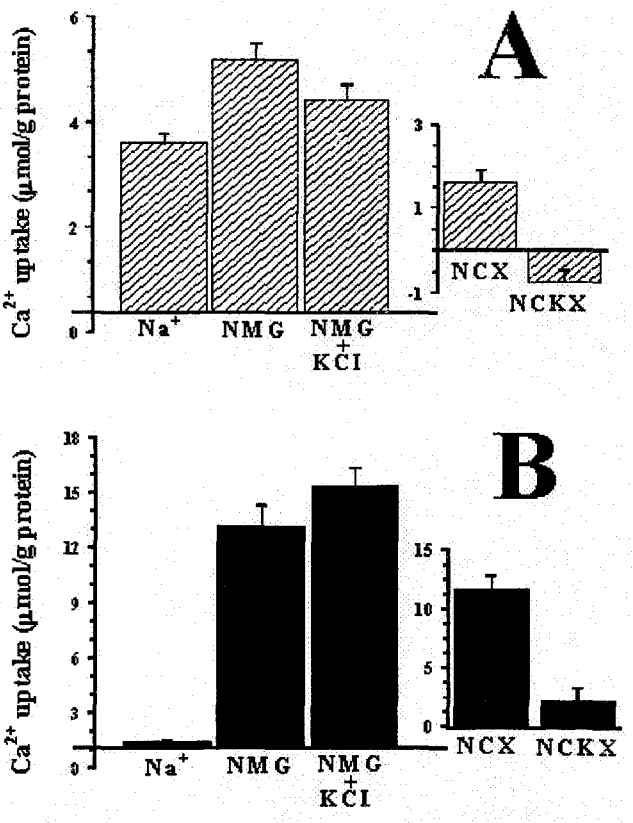


Figure 14. NCKX mediated Ca^{2+} uptake was not significant in SMC (A) and EC (B). A. SMC. The uptake was significantly higher in NMG ($p<0.05$, $q=5.407$) and NMG+KCl buffer ($q>3.482$ than $p<0.05$, $F=7.312$, $df=33$) than in Na^+ buffer. The uptake in the NMG and NMG+KCl buffers did not differ significantly ($p>0.05$, $q=2.656$). Inset: The NCKX Ca^{2+} uptake was not significantly different than NCX Ca^{2+} uptake. The experiment was repeated twice with similar results. B. EC. Ca^{2+} uptake in Na^+ and NMG ($p<0.05$, $q=17.905$) and the NMG and NMG+ KCl ($p<0.05$, $q=4.213$) buffers significantly differed from each other ($p>0.05$, $F=149.70$, $df=16$). However, this difference between NMG and NMG+KCl was not obtained upon repeating the experiment. Inset: NCX and NCKX mediated Ca^{2+} uptake.

figure 14B, EC demonstrate that NMG and NMG+KCl containing buffers did significantly differ ($p<0.05$) in one experiment but not observed in subsequent experiments suggesting that NCKX does not play a significant role on EC.

3.4. NCX and NCKX dependent Ca^{2+} uptake in membranes isolated from fresh smooth muscle tissue

NCX and NCKX mediated Ca^{2+} uptake experiments were performed on plasma membrane vesicles isolated from fresh coronary artery smooth muscle (figure 15). Often the process of culturing cells alters the phenotypic characteristics of cell, thus NCX measurements were also performed in fresh smooth muscle. Fresh endothelium is very difficult to obtain in large amounts therefore this experiment was not performed.

The figure 15 inset compares the activity of NCX and NCKX in 2 min. The plasma membrane vesicles showed an NCX mediated but not NCKX mediated Ca^{2+} uptake. However, the results were not reproducible between experiments. This may be due to the high basal uptake in the Na^{+} buffer most likely due to an increase in cell permeability during the process of isolating the membranes.

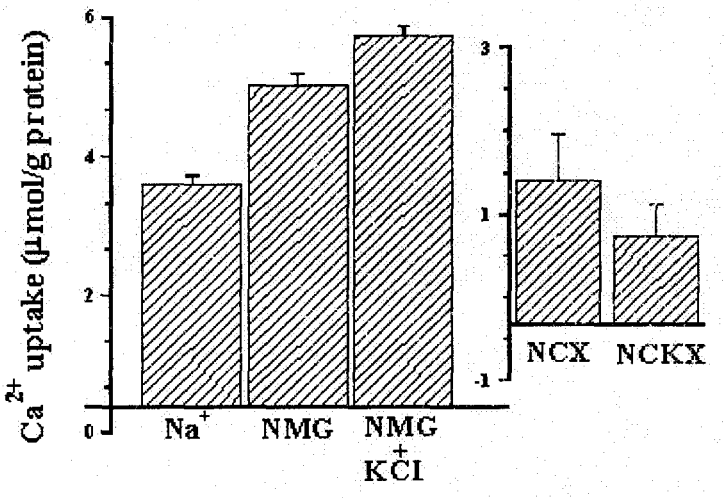


Figure 15. NCX and NCKX mediated Ca^{2+} uptake in plasma membrane vesicles isolated from fresh smooth muscle. Ca^{2+} uptake compared between Na^{+} and NMG medium ($p<0.05$, $q=8.749$) and between NMG and NMG+KCl medium ($p>0.05$, $q=4.580$) significantly differ ($p<0.05$, $F=45.864$, $df=23$), Inset: the NCX and the NCKX mediated Ca^{2+} uptake do significantly differ from each other ($p>0.05$, $t=2.815$, $df=14$). This experiment was repeated 4 times with similar results.

4. Discussion

4.1 Overview

NCX is important in regulation of Ca^{2+} handling and many Ca^{2+} sensitive processes. The results showed reverse mode NCX activity in cultured EC and SMC. The NCX inhibitors KB-R7943 and SEA 0400 blocked the NCX mediated Ca^{2+} uptake, as did collapsing the Na^+ gradient with monensin. The NCX activity in cultured cells was approximately 5 times greater in EC than in SMC. NCX1 is the isoform largely responsible for NCX activity in SMC and EC. NCX activity was also assayed as the Ca^{2+} efflux in cultured EC and as Ca^{2+} uptake in plasma membrane vesicles isolated from freshly isolated smooth muscle. NCX mediated Ca^{2+} entry was functionally linked to SERCA in SMC. Validation of the NCX assay, interpretation of the observations, comparison with the literature and significance of these findings are discussed further.

4.2 Validation of the Na^+ dependent Ca^{2+} uptake as NCX activity in EC and SMC

Ca^{2+} uptake using Na^+ loaded cells was designed to measure NCX activity. The results rule out the possibility of other Ca^{2+} channels or Na^+ sensitive pathways affecting the NCX mediated Ca^{2+} uptake.

In both SMC and EC, the possibility of non-NCX mediated Ca^{2+} uptake in the Na^+ loaded cells was eliminated with the use of NCX specific inhibitors KB-R7943 and SEA 0400. KB-R7943 has been reported to inhibit NCX and the Results demonstrated complete inhibition of Na^+ sensitive Ca^{2+} uptake. KB-R7943 may also inhibit L-type VOCC therefore the possibility needs to be considered that Na^+ loading depolarizes the

cell and may open non specific Ca^{2+} channels such as VOCC (Iwamoto et al., 1996). This possibility is eliminated since the uptake solutions contained 10 μM nitrendipine which is known to block the L-type VOCC in nanomolar concentrations.

The inhibition of Ca^{2+} uptake with SEA 0400, which is more selective for NCX than KB-R7943, further validates the role of NCX. It is also pointed out that SEA 0400 is more selective for NCX1, which from the Results and from the literature is expressed in SMC and EC (Szewczyk et al., 2007).

Alteration of the Na^+ gradient has the possibility of affecting many other Na^+ sensitive processes such as the NHE exchanger, Na^+ transporters, and the Na^+-K^+ ATPase (Doering & Lederer, 1993). These processes may have downstream effects on the $[Ca^{2+}]_i$ levels. The role of the Na^+ gradient as the driving force for NCX was demonstrated by inhibition of NCX mediated Ca^{2+} uptake by the Na^+ ionophore monensin. Cariporide was used to induce intracellular acidosis and thereupon the Ca^{2+} uptake was measured. Intracellular acidosis did not be attribute to the NCX mediated Ca^{2+} uptake via thus the extracellular Na^+ removal although it might cause intracellular acidosis will not affect the Ca^{2+} uptake. Thus, these experiments demonstrated that the Na^+ dependent Ca^{2+} uptake corresponded entirely to NCX mediated Ca^{2+} entry and not the involvement of other non specific mechanisms.

4.3 Levels of NCX activity in EC and SMC

NCX activity in EC was 5 times that in SMC (see Results). The levels of NCX activity in SMC and EC have not been compared in other labs. The disparity may arise

from a difference in the levels of NCX expression, NCX regulation in the two tissues or differences in morphology of SMC and EC. The higher levels of NCX expression in EC compared to SMC has been shown in our lab using Western blot analysis (Szewczyk et al., 2007). The level of expression in these studies was analyzed as anti-NCX per mg plasma membrane protein. However, in this thesis the NCX activity was expressed as per mg total cell protein. Therefore, one also needs to consider that the PM surface area to total protein ratio is higher in EC which are considerably smaller than SMC.

The comparatively high levels of EC activity can also be explained by regulation of NCX. Figure 3 (see Introduction) shows that phospholemman mRNA has a much higher expression in SMC than in EC (Szewczyk et al., 2007). Since phospholemman can act as an NCX inhibitor, it may contribute to the lower NCX activity in SMC. However, this is only a theory since the action of phospholemman itself requires activation by protein kinases (Zhang et al., 2006).

The difference in NCX activity between SMC and EC may be related to the unique Ca^{2+} handling requirements of the two cell types. In EC, the expression of the SERCA pump is very low and the SER is small and less intrusive than SMC. As a result, Ca^{2+} that enters cells though NCX has access to the large cytoplasm volume, and can diffuse quickly throughout the cell. This maintains the Ca^{2+} gradient for longer periods and hence results in a larger amount of NCX mediated Ca^{2+} uptake. In SMC, the tight Ca^{2+} regulation is performed by low capacity high affinity transporters such as PMCA. Furthermore, SMC contain a large amount of SER and SERCA, the SER (a) restricts the mobility of Ca^{2+} throughout the cell, and (b) sequesters large amounts of Ca^{2+}. Therefore

the presence of large PMCA and SERCA activity replaces the need for high capacity Ca^{2+} removal systems such as NCX. This concept is discussed further as linkage between NCX and SERCA.

4.4 NCX-SERCA Linkage

Evidence for functional linkage between NCX and SERCA

The functional linkage between NCX-SERCA is defined by the activity of one transporter affecting the other. Use of the SERCA inhibitor thapsigargin resulted in a decrease in NCX activity in SMC (Figure 12). This result indicates that SERCA may sequester Ca^{2+} from the cytoplasmic domain near NCX into SER and thus acts as a sink for the Ca^{2+} that enters the cell via NCX. In cells loaded with the Ca^{2+} chelator BAPTA , NCX mediated Ca^{2+} uptake is markedly increased and inhibition by thapsigargin is not evident (See Results). This is consistent with the sink hypothesis since chelation by BAPTA may act as an alternative to the SER as a sink. This hypothesis is supported by the observation that the effects of thapsigargin and BAPTA are minimal in EC which have much larger NCX activity and significantly less SERCA activity. Alternatively, one could consider that the NCX-SERCA functional linkage is present in SMC and not in EC because the linkage is triggered by a specific NCX or SERCA splice variant. Both SMC and EC have been reported to contain the same NCX splice variants but different SERCA isoforms and splice variants. EC contain both SERCA2b and SERCA3 while SMC contains only SERCA2b. This would mean that the abundance of SERCA2b may be associated with a NCX-SERCA linkage or that SERCA3 may interfere with the linkage.

The literature supports the proposal that the NCX-SERCA linkage in SMC is to regulate the SER Ca^{2+} levels (Blaustein, 1999). It was also demonstrated that NCX regulates SER Ca^{2+} content by showing that the removal of extracellular Na^{+} loaded an $[Ca^{2+}]_i$ pool, which was releasable by caffeine, thapsigargin, and physiological agonists. Fameli et al., (2007) suggest that it is plausible and possible for sufficient Ca^{2+} to pass through the NCX and SER junctions to replete the SER during the regenerative Ca^{2+} release, which underlies agonist, induced asynchronous calcium oscillations in vascular smooth muscle. Evidence implicating the role of NCX-SERCA functional linkage in endothelium is rare although it was found that NCX activity is involved in NO-induced depletion of Ca^{2+} in the ER (Matsuda et al., 2006). Thus, some types of ER may have a functional NCX-SERCA linkage.

A physical linkage between NCX and SERCA

The functional linkage is based on several assumptions; the first assumption is that NCX and SERCA are located close in proximity. Secondly, a structure must be in place to maintain the close contact between NCX and SERCA. Preliminary experiments were conducted to determine if the NCX-SERCA linkage in SMC is maintained by the cytoskeleton. Two compounds were used to damage the cytoskeleton: calyculin A and cytochalasin. Cytochalasin had no significant effect on NCX activity; unfortunately positive controls were not designed to ensure the cytoskeleton was damaged. Calyculin A removed the cells from the culture dishes and increased the background signal, thereby making the results difficult to interpret. However, there is evidence in the literature that

there is a close contact between NCX and SERCA in a variety of cells suggesting that this NCX and SERCA linkage may be wide spread in a variety of tissue but has not yet been fully investigated. Liang et al. (2004) presents evidence that in rabbit aortic endothelial cells ryanodine receptors (which are located on the ER) and NCX are in close functional proximity near PM. Brading et al. (2002) reached the conclusion that NCX activity between the SER and extracellular space took place at the locations where the PM and SER membranes made contact. Finally, recent advances in imaging techniques have allowed for a direct visualization of co-localization of the NCX, and the superficial SER (Lyashkov et al., 2006). Therefore, there is strong evidence of a physical linkage between NCX and SERCA. There may be some unknown cytoskeleton or scaffolding protein maintaining the close contact between the NCX and SERCA.

4.5 Physiology and Pathophysiology

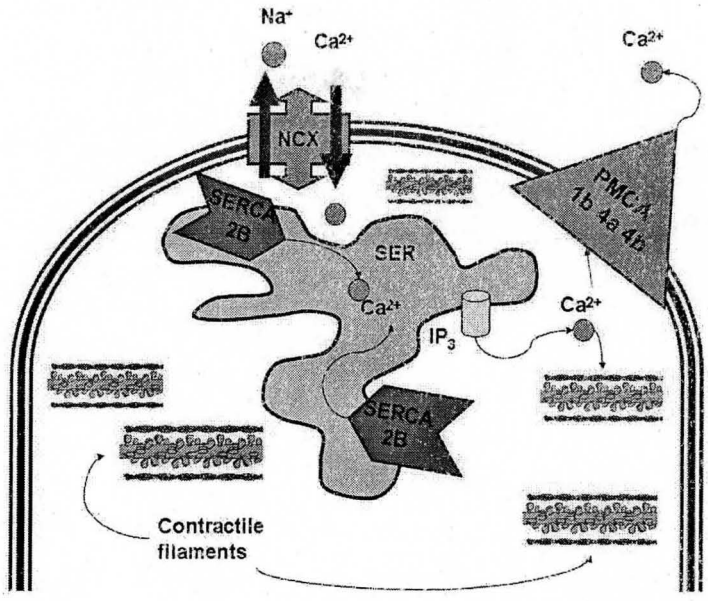
NCX plays a diverse functional role in the coronary artery, especially since it has the ability to both expel Ca^{2+} and also allow reverse mode NCX. In the literature, it is not clear whether secondary regulating proteins such as PKC, ATP, phosphoarginine, and H^{+} affect NCX mediated reverse mode as in the same way as the forward mode. Therefore it is important to distinguish the role of NCX as it may behave differently depending on the mode- forward or reverse - of activation.

NCX activity in the forward mode was tested in cultured EC as this mode plays an important role in Ca^{2+} removal. In the coronary artery, between SMC and EC, NCX in the forward mode can have opposing physiological effects. In vascular endothelium, high

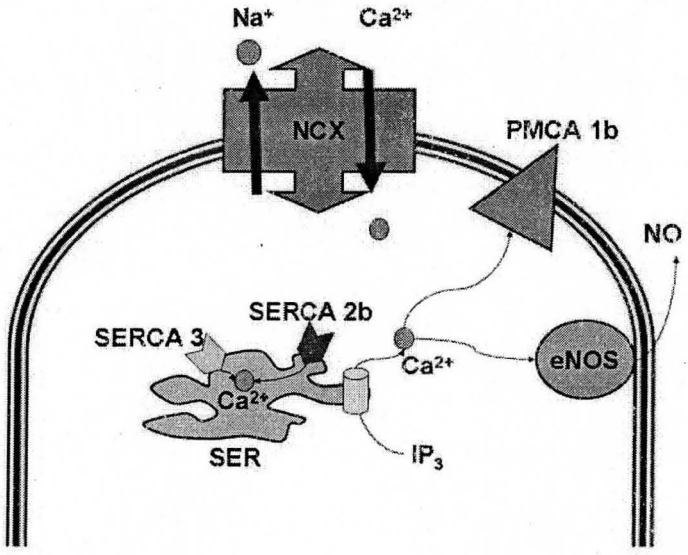
$[Ca^{2+}]_i$ stimulates NO synthesis, which is released to cause vasodilation (Amoroso et al., 2000). The high $[Ca^{2+}]_i$ can be removed by NCX, which then can stop or reduce NO synthesis and aid in sustaining coronary tension. The presence of NO can also stimulate NCX activity in forward mode, either through direct or indirect mechanisms, thus expelling more Ca^{2+} or the same amount of Ca^{2+} faster (Amoroso et al., 2000; Schulz & Triggle, 1994). This may be a feedback mechanism to decrease high levels of NO, especially under conditions of stress (Amoroso et al., 2000; Schulz & Triggle, 1994). Furthermore, there may be a physical relationship between eNOS and NCX that leads to a regulatory effect of eNOS (Schulz, 1994).

In SMC, NCX in the forward mode aids relaxation by removal of $[Ca^{2+}]_i$. Inhibition of NCX in this mode may lead to sustained contraction of the artery which can lead to coronary artery disease (Annuziato et al., 1997; Bell et al., 2000; Coleman & Khalil, 2002; Iwamoto et al., 2004; Matsuda et al., 2001).

Reverse mode was used in as an experimental tool but it is found in many physiological situations. Firstly reverse mode NCX is important in refilling the SER after asynchronous Ca^{2+} oscillations in smooth muscle (Fameli et al., 2007). Reverse mode NCX may also contribute to maintain a longer stronger contraction during cardiac contraction (Graham & Trafford, 2007). Reverse mode NCX has also been found in pathological situations when ion distributions are deregulated. The most harmful effect of NCX in reverse mode is the subsequent Ca^{2+} overloading in the cell (Annuziato et al., 1997). Inhibition of this entry by agents such as SEA 0400 and KB-R7943 may be therapeutically useful. In the coronary artery, Ca^{2+} deregulation can cause a dysfunction



SMC



EC

Figure 16. Summary of Ca^{2+} removal pathways in SMC (top) and EC (bottom).

in coronary tone, and may lead to pathological states such as hypertension, ischemia, and oxidative stress (Iwamoto et al., 2004).

NCX-SERCA linkage may also be damaged during pathophysiological states which way in turn, amplify the disturbance. SERCA2b is more susceptible to oxidative stress than SERCA3 (Grover et al., 2003). It remains to be demonstrated how this damage to SERCA2b affects NCX activity.

4.6 Summary and conclusions

Figure 16 shows an overview of the functional role of NCX and other Ca^{2+} transporting processes in SMC and EC in pig coronary artery. The primary function of SMC is the regulation of tone which is achieved by the Ca^{2+} dependent contractile filaments. These filaments are distributed throughout the cell, so Ca^{2+} must be transported and removed from deep within the cell. As a result, in SMC the SER plays a large role in calcium regulation. Furthermore, SMC contain higher activity levels of the high affinity pumps SERCA and PMCA, than EC. These pumps keep $[Ca^{2+}]_i$ levels low and under tight control. NCX is responsible for expelling large amounts of Ca^{2+}. In SMC, the NCX activity is lower than in EC, and the NCX inhibiting protein, phospholemman is present in higher quantities. Thus, even the small amount of NCX present in SMC has the potential to be highly regulated by phospholemman. NCX is also linked by close proximity or by physical linkage to SERCA which may be important in refilling the Ca^{2+} in the SER. This linkage may be triggered by (1) expression of specific SERCA isoforms, (2) required amount of SERCA expression and (3) different splice variants of

NCX. In conclusion, the mechanisms to regulate Ca^{2+} in smooth muscle work together to transport Ca^{2+} throughout the cell to the contractile filaments, meanwhile they also maintain very controlled Ca^{2+} levels.

EC release NO in response to an increase in $[Ca^{2+}]_i$, which relaxes the smooth muscle. The enzyme eNOS produces NO and is located adjacent to the PM in EC. The SERCA pump is responsible for removing Ca^{2+} from deep within the cell cytosol, since eNOS is located right on the interior of the PM, SERCA most likely does not play a large role in Ca^{2+} regulation and modulation of eNOS. Since SERCA does not play as large a role in EC, this may explain the absence of the NCX-SERCA linkage. EC contain a greater amount of the high capacity Ca^{2+} transporter NCX. In these cells, forward mode NCX has access to larger parts of the cytoplasm since SER is not present in large quantities in EC. Therefore larger quantities of Ca^{2+} can be removed from the cell and the vicinity of eNOS.

In conclusion, the functional role of NCX in SMC and EC has been studied with great interest. Understanding the role of NCX in the coronary artery may lead to development of specific inhibitors that can be used as strong pharmaceutical tools. In endothelium there is a great amount of NCX activity, therefore NCX must play a very important role in Ca^{2+} homeostasis. In smooth muscle, we see a functional linkage between NCX-SERCA. This linkage may be crucial to smooth muscle Ca^{2+} regulation and has the ability to be a potential therapeutic target during oxidative stress.

4.7 Future experiments

This proposal raises many unanswered questions some of which are presented here. Cultured cells were used in this study. Even though their phenotypes have been examined previously, their relationship to NCX in fresh tissue is not known. PM vesicles isolated from freshly isolated smooth muscle did exhibit an NCX mediated Ca^{2+} uptake although non specific Ca^{2+} uptake was also very large. It would be beneficial to examine if freshly isolated smooth muscle tissue strips can be used to examine the NCX-mediated Ca^{2+} entry and its linkage to SERCA. This technique will conserve the proper physiological localization of membrane proteins. This will hopefully allow a better understanding of NCX activity within SMC especially in relation to the physical and functionally regulation with SERCA.

The NCX mediated Ca^{2+} experiments unfortunately do not indicate the actual concentration of Ca^{2+} change in the cell, nor does it indicate where in the cell these changes occur, close to the plasma membrane or is the Ca^{2+} being sequestered into the SER. For instance, the $[Ca^{2+}]_i$ level changes could be influenced by Ca^{2+} buffering and other Ca^{2+} handling in the cell in addition to the NCX activity. Cell imaging using Ca^{2+} sensitive fluorescence dyes would be useful to elaborate our knowledge of NCX in the pig coronary artery.

Understanding the regulation of NCX by phosphorylation of phospholemman in SMC and EC would be of interest. Interest lies in the delineating the conditions of NCX phosphorlyation and the differences between isoforms. Also investigation is needed into the role of phosphorylation during the contraction of SMC and during oxidative stress.

The NCX-SERCA linkage in SMC remains unknown. This mystery may unfold with biochemical experiments such as immunoprecipitation or confocal microscopy to examine protein localization. It would also be interesting to determine if NCX is localized in caveolae, which may form a center for Ca^{2+} cell signaling.

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