LOCALIZATION OF NCX & SERCA

IN PORCINE CORONARY ARTERY SMOOTH MUSCLE

LOCALIZATION OF SODIUM CALCIUM EXCHANGER & SARCO/ENDOPLASMIC RETICULUM CALCIUM PUMP IN PORCINE CORONARY ARTERY SMOOTH MUSCLE

By

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ABSTRACT

Calcium (Ca^{2+}) is an important signaling molecule hence its movement across cell membranes must be tightly regulated. Ca^{2+} transporters play a key role in this regulation. The sarco/endoplasmic reticulum (SER) Ca^{2+} pump (SERCA) and plasma membrane Ca^{2+} pump (PMCA) keep $[Ca^{2+}]_i$ levels low. The sodium calcium exchanger (NCX) may work to expel Ca^{2+} or bring it into the cell. NCX has been proposed to play a role in refilling the SER Ca^{2+} pool. We recently reported a functional linkage between NCX1 and SERCA2 in smooth muscle cells cultured from pig coronary artery. The membrane invaginations termed caveolae (lipid rafts containing caveolin) may also play a role in directing Ca^{2+} movements during cell signalling. The overall objective of this thesis was to determine whether NCX, SERCA and caveolin proteins were spatially linked (co-localized) in pig coronary artery smooth muscle. This was investigated by examining their co-migration in detergent treated microsomal membranes upon sucrose density gradient fractionation. The fractions were analyzed for the abundance of NCX1, SERCA2, caveolin-1, lipid raft markers, and cytoskeletal proteins. Then, the Pearson's and Spearman's correlation coefficient between each proteins distribution was determined. The results indicate there was a significant correlation in the distribution of NCX1 and SERCA2, NCX1 and caveolin-1, and SERCA2 and caveolin-1 in the flotation; however the migration was not perfect. In conclusion, NCX1 and SERCA2 co-migrate to similar types of membrane domains in pig coronary artery smooth muscle. These domains are rich in lipid rafts and include caveolae. The results also indicate that although a spatial interaction exists, co-localization may not always be present. Thus, NCX1 may contribute

to the SER refilling but it may not be solely responsible for this process. The partial association of NCX1 and caveolin-1 suggests the role of caveolae in some Ca^{2+} signalling pathways but not others.

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Abbreviations

ATP - Adenosine 5'-triphosphate

BSA - Bovine Serum Albumin

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

CBD - Ca^{2+} binding domain

CICR - Ca^{2+}-induced Ca^{2+}-release

CIF - Ca^{2+} influx factor

CRAC - Ca^{2+} release activated Ca^{2+} channel

DTT – Dithiothreitol

EDTA – Ethylenediamine tetra-acetic acid

EGTA – Ethylene glycol-bis (2-aminoethyl ether)-N, N, N', N'-tetra-acetic acid

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

H_2O_2 – Hydrogen peroxide

IgG - Immunoglobulin G

IP_3 - Inositol 1, 4, 5-trisphosphate

MES - 2-(N-morpholino) ethanesulfonic acid

MIC - Microsomes

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

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

NO – Nitric oxide

PIP_2 – Phosphatidylinositol 4, 5-bisphosphate

PKA – Protein kinase A

PKC – Protein kinase C

PM - Plasma membrane

PMCA – PM Ca^{2+} pump

PMSF – Phenylmethylsulfonylfluoride

PSS - Physiological salt solution

PVDF - Polyvinylidene fluoride

ROCC – Receptor operated Ca^{2+} channel

ROS – Reactive oxygen species

SER - Sarco/endoplasmic reticulum

SERCA – SER Ca^{2+} pump

SOCC – Store operated Ca^{2+} channel

SOCE – Store operated Ca^{2+} entry

SPCA – Secretory pathway Ca^{2+} pump (in Golgi)

STIM1 – Stromal interacting molecule

TBS – TRIS Buffered Saline

TEMED – N, N, N', N'-Tetramethyl-1-,2-diaminomethane

TMS – Transmembrane segments

TRPC – Transient receptor potential channel

VOCC – voltage operated Ca^{2+} channel

XIP – Exchange inhibitory peptide

1.0 INTRODUCTION

Ca^{2+} is a universal signal transduction molecule. The extracellular Ca^{2+} concentration is relatively constant near 1 mM. To function effectively as a signaling molecule, the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) must be kept at nanomolar levels in resting cells and increased only during brief periods of cell excitation (Berridge, Lipp, and Bootman, 2000; Lipskaia and Lompre, 2004). After signal transmission, three major systems are responsible for returning $[Ca^{2+}]_i$ back to its resting levels. The sodium calcium exchanger (NCX) and the plasma membrane (PM) Ca^{2+} pump (PMCA) extrude Ca^{2+} from the cell. The sarco/endoplasmic reticulum (SER) Ca^{2+} pump (SERCA) sequesters cytosolic Ca^{2+} into the SER. An alteration in the function or regulation of any of these players has serious consequences, often associated with the pathogenesis of disease (Berridge, Lipp, and Bootman, 2000; Berridge, Bootman, and Roderick, 2003). Our laboratory has previously focused on the role of SERCA and PMCA in $[Ca^{2+}]_i$ regulation in smooth muscle. We have recently started to explore the role of NCX and its interactions with SERCA. Our initial results were consistent with a functional linkage existing between NCX and SERCA; however, the nature of this relationship has not yet been explored. The present study aims to characterize the nature of the NCX and SERCA linkage in pig coronary artery smooth muscle.

1.1 Overview of Ca^{2+} Homeostasis

Ca^{2+} is utilized by cells as a ubiquitous signalling molecule. At rest, mammalian cells maintain a $[Ca^{2+}]_i$ of approximately 0.1 μM, which is 10,000x lower than the

extracellular [Ca^{2+}] (1-1.5 mM) (Carafoli, 1987; Marin *et al.,* 1999). A rise in [Ca^{2+}]$_i$ regulates a broad range of cellular processes such as cell differentiation and proliferation, muscle and non-muscle motility, and apoptosis (Berridge, Bootman, Roderick, 2003; Carafoli *et al.,* 2001; Bootman *et al.,* 2001; Carafoli, 2002). [Ca^{2+}]$_i$ must thus be tightly regulated in time, space and amplitude for cells to extract specific information from these parameters (Berridge, Lipp and Bootman, 2000).

The cell has access to both extracellular and intracellular Ca^{2+}. The SER can store up to 5-10 mM Ca^{2+}, most of which is buffered by the high capacity, low affinity Ca^{2+} binding proteins such as calsequestrin and calreticulin (Raeymaekers *et al.,* 1993; Milner, Famulski, and Michalak, 1992). These proteins maintain the free lumen [Ca^{2+}] at around 1 mM (Meldolesi and Pozzan, 1998). An increase in [Ca^{2+}]$_i$ required for signaling functions occurs upon the release of sequestered Ca^{2+} from the SER or entry from the external environment (Marin et al., 1999). Following the rise in [Ca^{2+}]$_i$, Ca^{2+} is restored to lower levels and maintained at its resting concentration. Outlined below are the major mechanisms involved in removing Ca^{2+} from the cytosol, followed by the systems regulating Ca^{2+} release into the cytosol, as well as mechanisms involved in refilling the SER.

1.2 Mechanisms of lowering cytosolic Ca^{2+}

After cell excitation, Ca^{2+} is rapidly and efficiently removed from the cytoplasm to reduce excessive [Ca^{2+}]$_i$ and maintain homeostasis (Carafoli *et al.,* 2001; Brini and Carafoli, 2000). Ca^{2+} can also be buffered with Ca^{2+} binding proteins within the cell

(Carafoli *et al.,* 2001; Carafoli, 2002; Brini and Carafoli, 2000). The Ca^{2+} transporters that remove $[Ca^{2+}]_i$ following cell stimulation include the ion exchangers and the Ca^{2+} pumps. The Ca^{2+} transporting ion exchangers include NCX or the K^+ dependent Na^+ Ca^{2+} exchanger (NCKX) in the PM. The two key families of Ca^{2+} pumps include SERCA, which pump Ca^{2+} into the lumen of their internal store, and PMCA, which extrude Ca^{2+} out of the cell (Missiaen *et al.,* 1991). In general, the Ca^{2+} pumps have a high affinity for Ca^{2+}, whereas the ion exchangers are low Ca^{2+} affinity systems. The relative roles of the Ca^{2+} transporters in removing Ca^{2+} from the cytosol varies on the cell type due to the differences in their abundance and kinetic properties (Penniston *et al.,* 1997). Since the focus of this thesis is on NCX and SERCA, these Ca^{2+} transporters are first described in detail below, followed subsequently by a brief description of the other transport mechanisms.

1.2.1 Ion Exchangers

Sodium Calcium Exchanger (NCX)

General Properties

NCX is an ubiquitously expressed transmembrane protein which catalyzes the bidirectional exchange of Na^+ and Ca^{2+} (Blaustein and Lederer, 1999; Philipson and Nicoll, 1993). The movement of one Ca^{2+} is coupled directly to reciprocal movements of three Na^+, thus making NCX electrogenic (Pitts, 1979; Reeves and Sutko, 1979; Blaustein and Lederer, 1999). This process has been demonstrated in a variety of tissues (Reeves

and Sutko, 1979; Pitts, 1979; Blaustein *et al.,* 1991; Philipson and Nicoll, 1993; Rasgado-Flores, Santiago and Blaustein, 1989; DiPolo and Beauge, 1990). Whether NCX extrudes Ca^{2+} from cells or allow its entry depends on the electrochemical gradients of Na^{+} and Ca^{2+} (Blaustein and Santiago, 1977; Blaustein and Lederer, 1999; Annunziato, Pignataro and Di Renzo, 2004). The rate of ion movement depends upon the number of NCX molecules in the tissue, the ion concentrations of Na^{+} and Ca^{2+}, as well as on regulation of its activity, which is described in greater detail below. The binding affinity (K_m) of NCX for Na^{+} is approximately 25-45 mM, while the K_m for Ca^{2+} is approximately 2.6-7 μM (Blaustein and Lederer, 1999; Szewczyk *et al.,* 2007; Matsuoka and Hilgemann, 1992; Matsuoka *et al.,* 1995).

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NCX are encoded by a family of 4 genes: NCX1, NCX2, NCX3, and NCX4 (Nicoll, Longoni, and Philipson, 1990; Nicoll *et al.,* 1996; Li *et al.,* 1994; Marshall *et al.,* 2005). These gene products have similar hydropathy patterns, suggesting similar overall molecular structure (Blaustein and Lederer, 1999; Shigekawa and Iwamoto, 2001). There do not appear to be large functional differences amongst the gene products (Linck *et al.,* 1998). The NCX1 protein is the best characterized and is widely distributed including cardiac, skeletal, and smooth muscles, neurons, astrocytes, kidney, lung, and spleen (Quednau, Nicoll and Philipson, 1997; Kofuji *et al.,* 1992). In contrast, NCX2 and NCX3 are found in brain and skeletal muscle (Li *et al.,* 1994; Nicoll *et al.,* 1996). The *NCX4* gene has only recently been identified in fish species genomes (Marshall *et al.,* 2005).

Since coronary artery smooth muscle contains predominantly NCX1 and this isoform is the best characterized structurally and functionally, it will be the focus for the remainder of this section.

The NCX1 protein has an open reading frame of 970 amino acids, including a 32-residue signal peptide which is cleaved during protein maturation (Nicoll, Longoni, and Philipson, 1990). It contains nine transmembrane segments (TMS), two loops formed by alpha-repeats, a large cytosolic loop, and an extracellular amino terminus and intracellular carboxyl terminus (Nicoll *et al.,* 1999; Hryshko *et al.,* 1993; Doering *et al.,* 1998).

The alpha repeats, alpha 1 and alpha2, are conserved amongst all exchangers and span portions of TMS 2-3 and TMS 7-8, respectively (Nicoll *et al.,* 1999; Philipson and Nicoll, 1993). They are localized in close proximity to each other, on opposite sides of the membrane (Levitsky, Nicoll and Philipson, 1994; Iwamoto *et al.,* 2000). The repeats play an important role in the ion binding and translocation process (Levitsky, Nicoll and Philipson, 1994; Nicoll *et al.,* 1996; Qiu, Nicoll and Philipson, 2001; Iwamoto *et al.,* 2000).

The cytosolic loop (f loop) is approximately 550 amino acid residues long. It is a highly hydrophilic domain that connects TMS 5 and 6, and it can undergo dramatic conformational changes (Qiu, Nicoll and Philipson, 2001; Levitsky, Nicoll and Philipson, 1994). It is not essential for ion transport, however, and contains two Ca^{2+} binding regulatory domains (CBD_1 and CBD_2) thought to be responsible for the secondary Ca^{2+} regulation of NCX (Levitsky, Nicoll and Philipson, 1994; Matsuoka *et al.,* 1995). Although both CBD_1 and CBD_2 bind Ca^{2+}, CBD_1 has a seven fold higher affinity ($K_D \sim$

120 nM) compared to CBD_2 (K_D ~ 820 nM) (Levitsky, Nicoll and Philipson, 1994; Hilge, Aelen and Vuister, 2006). CBD_1 is considered to be the primary Ca^{2+} sensor and CBD_2 binds Ca^{2+} only at elevated concentrations. The two different sensitivity thresholds enable NCX to function dynamically over a wide range of Ca^{2+} concentrations (Hilge, Aelen and Vuister, 2006).

The f loop also contains a 20-residue long domain important in regulating exchange activity of NCX (Nicoll, Longoni, and Philipson, 1990; Li *et al.,* 1991; Matsuoka *et al.,* 1997). It is known as the autoinhibitory domain and the sequence, which is rich in both basic and hydrophobic residues, is called the exchange inhibitory peptide (XIP) site. This region is responsible for Na^+_i-dependent inactivation (see NCX regulation), as well as in NCX regulation by non-transported Ca^{2+} (Matsuoka *et al.,* 1997; Matsuoka *et al.,* 1995). The XIP site also has an autoregulatory function – exogenously added peptide, with the XIP sequence, potently inhibits NCX activity (see NCX inhibitors).

In addition to the abovementioned regulatory sites, the f loop also contains sites for alternative splicing. Besides the four isoforms mentioned above (NCX1-4), splice variants are generated from the NCX gene in a region corresponding to the COOH terminus of its intracellular loop, in a tissue-specific manner (Fig. 1) (Hurtado *et al.,* 2006; Lee, Yu, and Lytton, 1994; Quednau, Nicoll, and Philipson, 1997). Six cassette exons, A, B, C, D, E, and F, code for this alternatively spliced site (Kofuji *et al.,* 1992). Exons A and B are mutually exclusive and are followed by various combinations of the remaining four exons. Table 1 summarizes all the possible splice variants of NCX1-3.

The heart contains NCX1.1 and vascular tissue (smooth muscle) contains NCX1.3 and 1.7 (Szewczyk *et al.,* 2007; Quednau, Nicoll, and Philipson, 1997).

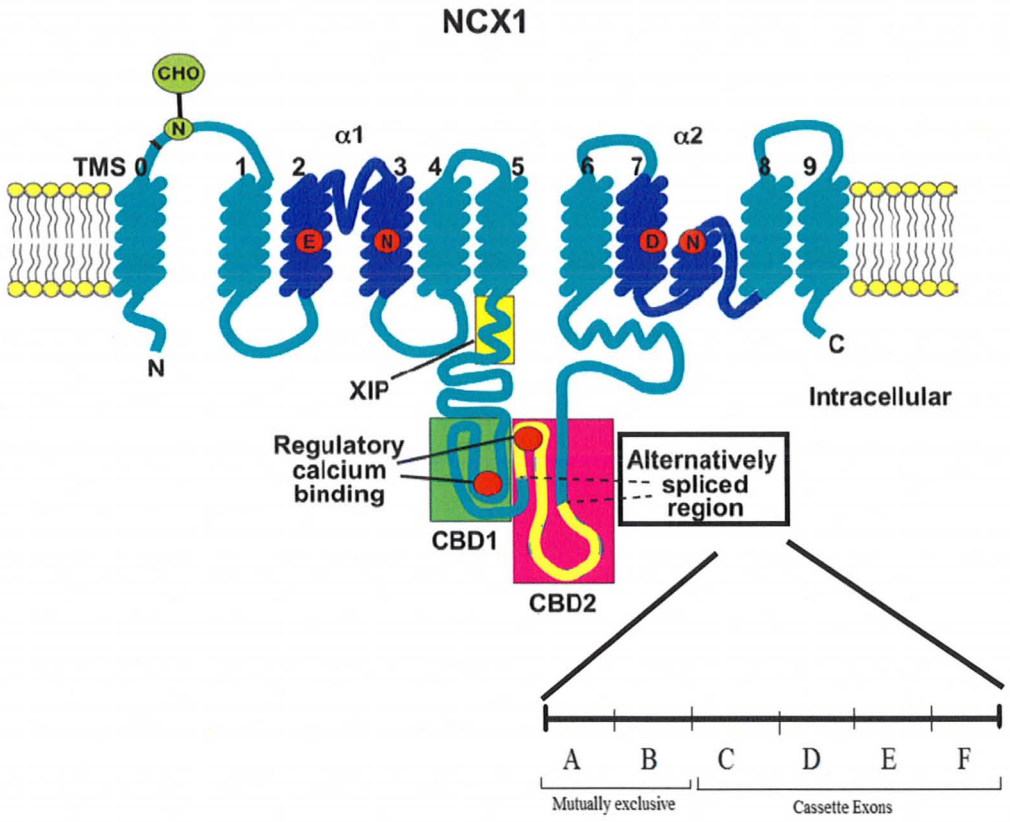

Figure 1. Topological model of NCX1.
NCX1 contains nine transmembrane spanning domains, a cleaved signal peptide (TMSO), and a pair of α repeats in two re-entrant loops (critical amino acids are highlighted in red). A single N-linked glycosylation site is shown in green (N-CHO). The regulatory regions of the cytosolic loop are also highlighted and include the XIP region, the two Ca^{2+} binding domains (CBD_1 and CBD_2), and the region where extensive alternative splicing occurs (modified from Lytton *et al.,* 2007).

Table 1. Splice isoforms and exons of NCX 1, 2 and 3

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				

(modified from Quednau, Nicoll, and Philipson, 1997)

Regulation

Several factors are involved in regulating NCX activity – intracellular concentrations of Na^+ and Ca^{2+}, intracellular pH, metabolic related compounds (i.e. ATP, phosphoarginine, PIP_2, PKA and PKC), phospholemman, redox agents, and nitric oxide.

NCX is subject to two forms of intrinsic regulation by either of the transport substrates, Na^+ and Ca^{2+} (DiPolo and Beauge, 1987; Hilgemann *et al.,* 1992). When $[Na^+]_i$ rises, Na^+ binds to the transport site of NCX (the XIP site), activates exchange activity, and then ultimately leads to Na^+-dependent inactivation. This inactivation is enhanced by acidic environments and attenuated by $[Ca^{2+}]_i$, ATP and PIP_2 (Hilgemann *et al.,* 1992; Hilgemann, Collins, and Matsuoka, 1992; Doering and Lederer, 1993; Hilgemann and Ball, 1996). The second regulatory state, known as Ca^{2+}-dependent regulation, occurs in the absence of $[Ca^{2+}]_i$ and is independent of Na^+ (Hilgemann, Collins, and Matsuoka, 1992; Philipson and Nicoll, 1993). Unlike Na^+, the site at which $Ca^{2+}{}_i$ regulates NCX (CBD_1 and CBD_2) is different from the one required for Ca^{2+} transport (Matsuoka *et al.,* 1995; Levitsky, Nicoll and Philipson, 1994). Submicromolar concentrations (0.1– 0.4 μM) of Ca^{2+} promote the recovery of the exchanger from this inactivation state (Hilgemann, Collins, and Matsuoka, 1992; Levitsky, Nicoll and Philipson, 1994; Matsuoka *et al.,* 1995; Matsuoka *et al.,* 1997). Recently, it has been suggested that the state of the Ca^{2+} regulatory site (active or inactive) influences the affinity of the extracellular Ca^{2+} and Na^+ transport sites (DiPolo and Beauge, 2008).

Differential regulation of NCX splice variants by Na^+ and Ca^{2+} has also been observed (Hurtado *et al.,* 2006). Specifically, inactivation of the Ca^{2+} entry mode is

significantly more pronounced in the NCX1.3 splice variant. Furthermore, an increase in $[Ca^{2+}]_i$ reduces Na^+-dependent inactivation in NCX1.1, but not NCX1.3 (Dyck *et al.*, 1999; Hryshko, 2002). Hence, ionic regulation is important in controlling NCX1 activity under conditions that promote Ca^{2+} overload (Hurtado *et al.*, 2006).

In addition to $[Na^+]_i$ and $[Ca^{2+}]_i$, NCX1 exchange activity is also regulated by intracellular pH. An acidic environment lowers the activity of NCX and a basic environment increases it (Doering and Lederer, 1993; Philipson, Bersohn and Nishimoto, 1982). The action site of protons is attributed to the NCX hydrophilic f loop and has no effect at the extracellular surface (Espinosa-Tanguma, DeSantiago, and Rasgado-Flores, 1993; DiPolo and Beauge, 2008; Doering and Lederer, 1993). This inhibitory action depends on the presence of Na^+_i. A large body of work implicates the regulation of NCX by the Na^+/H^+ exchanger via changes in the Na^+ gradient (Doering and Lederer, 1993; Espinosa-Tanguma, DeSantiago, and Rasgado-Flores, 1993; Matsumoto *et al.*, 2002).

Adenosine 5' triphosphate (ATP) increases NCX activity by acting as a phosphoryl donor molecule (Blaustein and Santiago, 1977). First, it may directly participate in the NCX phosphorylation process by protein kinase A (PKA) or C (PKC) (Caroni and Carafoli, 1983). Moreover, it can also activate G-protein-coupled receptors, via endogenous and exogenous ligands, and stimulate activity indirectly through the pathway involving PKC or PKA activation (DiPolo and Beauge, 1998; Iwamoto, Wakabayashi, and Shigekawa, 1995). The mechanism underlying the phosphorylating effect is related to an increase in NCX affinity for both internal Ca^{2+} and external Na^+ and to a decrease in its inhibition by $[Na^+]_i$. ATP does not affect the maximal transport rate at

saturating substrate concentrations, suggesting it is acting as a catalyst for binding and not as an energy source (Blaustein and Santiago, 1977). The second way ATP may influence NCX activity is by increasing PIP_2 production (Hilgemann and Ball, 1996). PIP_2 interacts with the XIP region of NCX, eliminates its Na^+-dependent inactivation and thus stimulates NCX function (Annunziato, Pignataro and Di Renzo, 2004; Posada, Beauge and Berberian, 2007). ATP cellular depletion inhibits NCX1 and NCX2 but does not affect NCX3 activity (Annunziato, Pignataro and Di Renzo, 2004).

Another metabolic regulator of NCX is phosphoarginine (DiPolo and Beauge, 1998). Unlike ATP, phosphoarginine does not affect $Ca^{2+}{}_i$ or $Na^+{}_i$ interactions with the f loop, but increases the affinity of the intracellular transport sites, particularly of Ca^{2+}. Millimolar concentrations of phosphoarginine in the cytosol activate NCX in a way that preferentially promotes the Ca^{2+} removal mode. Although phosphoarginine action is independent of ATP, the effects of the two regulators are additive (DiPolo and Beauge, 2008).

Another agent involved in regulating NCX is phospholemman (Cheung *et al.*, 2007; Lencesova *et al.*, 2004). This 15 kDa protein is phosphorylated by PKA at Ser68, and by PKC at both Ser63 and Ser68. Phosphorylation of phospholemman allows it to associate with and inhibit NCX1 (Song *et al.*, 2005; Wang *et al.*, 2006; Zhang et al., 2006). Overexpression of PLM inhibits NCX activity, whereas down-regulation enhances NCX1 current (Tucker *et al.*, 2006). The effects of PKA or PKC on NCX1 activity regulation may also indirectly occur by their phosphorylation of phospholemman. Phospholemman shares sequence similarity with the SERCA activity regulator,

phospholamban.

Nitric oxide (NO) stimulates the Ca^{2+} entry mode of NCX in neuronal preparations and astrocytes in a cyclic GMP dependent manner (Asano *et al.,* 1995). In contrast, stimulation in C6 glioma cells requires the presence of iron in the NO donor sodium nitroprusside and not NO release (Amoroso *et al.,* 2000). A direct relationship between the constitutive form of NO synthase, the enzyme involved in NO synthesis, and NCX has also been demonstrated. Heat stress causes NOS phosphorylation which leads to complex formation with the exchanger and a subsequent decrease in its activity. In addition, NCX activity is also involved in NO-induced depletion of Ca^{2+} in microglial ER, suggesting a role for the exchanger in regulating ER Ca^{2+} levels (Matsuda *et al.,* 2006).

Lastly, NCX is sensitive to different combinations of reducing-oxidizing (redox) agents (Reeves, Bailey and Hale, 1986; Amoroso *et al.,* 2000; Santacruz-Toloza *et al.,* 2000). In particular, stimulation of exchange activity requires the combination of a reducing agent (DTT, GSH, Fe^{2+}or superoxide) with an oxidizing agent (hydrogen peroxide (H_2O_2), GSSG, or Fe^{3+}). The effects of both agents are mediated by metal ions (e.g., Fe^{2+}). Interestingly, stimulation induced by the combination of $FeSO_4$ and DTT removed Na^+-dependent inactivation of NCX and activated the exchanger. H_2O_2 and the superoxide enzyme generating system, xanthine+xanthine oxidase, together stimulated NCX whereas the strong oxidant HOCl alone inhibited its activity. Superoxide generated by the same system, without H_2O_2, substantially enhanced NCX-mediated Ca^{2+} fluxes. In isolated ventricular myocytes, reactive oxygen species (ROS) generated from H_2O_2 and

xanthine + xanthine oxidase augmented NCX activity. NCX sensitivity to changes in the redox status may be significant during oxidative stress (Annunziato, Pignataro and Di Renzo, 2004). In this condition, the modulation of ROS could affect the Na^{+} and Ca^{2+} transport through the PM.

Inhibitors

NCX inhibitors are invaluable to understanding the role of NCX. The different types of inhibitors are categorized as inorganic ions, synthetic peptides, and organic compounds. The limitations of each of these inhibitors, especially the lack of selectivity, have hindered the characterization of NCX function in intact cells and tissues. Another complicating factor is the movement of Ca^{2+} both into and out of the cell by NCX, hence the need for mode specific inhibitors. In addition to helping to understand the physiological roles of NCX, mode specific inhibitors have high therapeutic potential.

Several transition heavy metals such as La^{3+}, Cd^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} have been reported to block NCX (Trosper and Philipson, 1983; Brommundt and Kavaler, 1987; Smith, Cragoe and Smith, 1987; Torok, 2007; Iwamoto and Shigekawa, 1998). Ni^{2+} and Co^{2+} discriminate between the NCX isoforms (NCX1-3) through direct interaction of its alpha repeats, however they are less potent relative to La^{3+} and Cd^{2+} , which already have relatively low affinity for NCX (IC_{50} for $La^{3+} > 0.5$mM) (Iwamoto and Shigekawa, 1998; Shimizu, Borin and Blaustein, 1997). They may also inhibit NCX by competing with Ca^{2+} for the external transport site. None of these transition metals, however, are selective for NCX since they block other Ca^{2+} transporters (Lansman, Hess and Tsien, 1986;

McFarlane and Gilly, 1998). Additionally, they may also be transported by NCX (Blaustein and Lederer, 1999; Shimizu, Borin and Blaustein, 1997).

As mentioned previously, XIP is an auto inhibitory segment located on the f-loop segment of NCX. The Exchanger Inhibitory Peptide was synthesized with the same 20 amino acid sequence as this region and potently inhibits both modes of NCX when applied intracellularly (Li *et al.,* 1991). If applied extracellularly, the peptide activates a nonspecific large resting inward current (Li *et al.,* 1991; Chin *et al.,* 1993). Furthermore, it does not readily pass through the cell membrane making it difficult to administer. FRCRCFamide is another potent and selective peptide inhibitor of NCX which is cell impermeable (Khananshvili *et al.,* 1995; Convery *et al.,* 1998). Recently, a new endogenous inhibitor known as NCX_{IF} has been found (Hiller *et al.,* 2000; Boyman *et al.,* 2005). It has no structural relation to XIP or phospholemman and has capacity to inhibit both modes of NCX (Hiller *et al.,* 2000; Shpak *et al,* 2004; Ahlers *et al.,* 2005).

The organic inhibitors include amiloride and its derivatives, benzamil and dichlorobenzamil. Using Ca^{2+} flux measurements, Kaczorowski *et al.,* (1985) have reported 50% inhibition with 1 mM amiloride in rat anterior pituitary cells. Amiloride is however, also not selective and inhibits the Na^+/K^+ ATPase, voltage operated Ca^{2+} channels (VOCC), Na^+ channel and Na^+/H^+ exchange (Torok, 2007). The amiloride analogue benzamil inhibited the Na^+_i dependent Ca^{2+} uptake in cardiac sarcolemmal vesicles and the Na^+_o dependent Ca^{2+} efflux in rat anterior pituitary cells (Kaczorowski *et al.,* 1989; Slaughter *et al.,* 1988). Another amiloride derivative is 3',4'-dichlorbenazamil and it preferentially blocks the inward Na^+/Ca^{2+} current (Watano *et al.,* 1996; Kleyman

and Cragoe, 1988). It also however, blocks several of the VOCC and Na^+ and K^+ channels, as well as the Na^+/H^+ exchanger (Kleyman and Cragoe, 1988; Garcia *et al.*, 1990; Plasman *et al.*, 1991; Shigekawa and Iwamoto, 2001). Phenylalkylamines such as verapamil and its methoxy-analog D-600, as well as the aminoglycoside antibiotic neomycin are similar non specific inhibitors of NCX (Motulsky *et al.*, 1983; Pancrazio *et al.*, 1991; Haworth, Goknur and Berkoff, 1989).

The isothiourea derivatives KB-R7943 and SN-6 have been described as relatively potent inhibitors of NCX in the Ca^{2+} entry mode (Shigekawa and Iwamoto, 2001; Iwamoto *et al.*, 2004; Kimura *et al.*, 2002). In aortic smooth muscle cells and cardiac NCX1 transfected CCL39 cells, the IC_{50} of KB-R7943 ranges from 0.3 to 3 μM, however, in the Ca^{2+} removal mode, the IC_{50} is above 30 μM (Watano *et al.*, 1996; Watano and Kimura, 1998; Iwamoto, Watano and Shigekawa, 1996). KB-R7943 has a 3 fold higher affinity for NCX3 relative to NCX1 or NCX2 (IC_{50}= 1.5, 4.9, and 4.1 μM respectively) (Iwamoto and Shigekawa, 1998; Iwamoto *et al.*, 2001). The alpha2 repeat is believed to be responsible for the different inhibitory effects (Iwamoto *et al.*, 2001). KB-R7943 is also far from selective. At higher concentrations, it blocks many channels and receptors, including several members of the transient receptor potential (TRPC) family (Kimura *et al.*, 2002; Watano *et al.*, 1999; Sobolevsky and Khodorov, 1999; Kraft, 2007; Matsuda *et al.*, 2001; Pintado *et al.*, 2000). KB-R7943 also inhibits store-operated Ca^{2+}-entry (capacitive Ca^{2+}-entry) making it very difficult to use as a tool to study the role of NCX (Arakawa *et al.*, 2000). SN-6 has a similar chemical structure to KB-R7943 (Niu *et al.*, 2007). This compound preferentially inhibits the Ca^{2+} uptake mode of all NCX

isoforms (NCX1, 2, and 3) (Iwamoto *et al.,* 2004). It also has cardio-protective effects against ischemia reperfusion with an oxygen radical scavenging effect (Matsuda *et al.,* 2001).

SEA 0400 is a more potent inhibitor of NCX mediated Ca^{2+} entry than KB-R7943 (EC_{50} = 5-92 nM) however, may also interfere with Ca^{2+} movement across the cell by other pathways (Matsuda *et al.,* 2001; Reuter *et al.,* 2002; Takahashi *et al.,* 2003). In addition, SEA 0400 demonstrates stronger isoform preference than KB-R7943 and is most selective for NCX1 (IC_{50} = 56nM) (Iwamoto *et al.,* 2004). It also inhibits the vascular isoform NCX1.3 more potently than the cardiac isoform (NCX1.1) and neuronal isoforms (NCX1.4, NCX2.1 and NCX3.3) (Iwamoto *et al.,* 2004; Iwamoto, Kita, Zhang *et al.,* 2004). SEA 0400 has protective effects against myocardial ischemia– reperfusion injuries (Takahashi *et al.,* 2003; Matsuda *et al.,* 2001; Magee *et al.,* 2003). In addition, long term treatment with SEA 0400 overcomes the development of salt-sensitive hypertension, vascular hypertrophy, and renal dysfunction in animal models (Iwamoto, Kita, Zhang *et al.,* 2004).

NCX is involved in a variety of pathological states such as ischemia-reperfusion, hypertension, and oxidative stress (discussed in pathophysiology section) (Matsuda *et al.,* 2001; Iwamoto, Kita, Zhang *et al.,* 2004; Unlap *et al.*, 2003). As a result, mode specific inhibitors are being investigated as pharmaceutical tools to counteract the changes that occur to NCX regulation during these pathological states.

Functional Properties

Several interesting aspects of NCX function are predominant in the literature. They include localization of NCX, Ca^{2+} extrusion versus Ca^{2+} entry, and regulation of the SER Ca^{2+} content. General properties of the modes (binding affinities to substrates, rate of ion movement, etc.) were previously described; however, each mode may have different physiological effects.

In vascular smooth muscle, astrocytes, and neurons, NCX appears to be confined to PM regions that are closely apposed to underlying junctional SER (Blaustein *et al.*, 1992; Goldman *et al.*, 1994; Juhaszova *et al.*, 1994; Juhaszova *et al.*, 1996; Moore *et al.*, 1993). This distribution contrasts with the uniform distribution of PMCA suggesting it has a different function than NCX (Juhaszova *et al.*, 1996). PMCA is a high affinity Ca^{2+} transporter which is important for regulation of Ca^{2+} at lower resting levels, i.e. maintaining low $[Ca^{2+}]$ in the bulk cytosol. NCX is useful for transporting large amounts of Ca^{2+} when the $[Ca^{2+}]_i$ levels are high. Its role is more suitable for regulating the small junctional space and indirectly modulating the Ca^{2+} content of the SER stores. This in turn would influence Ca^{2+} signalling and tension development (Blaustein, 1993; Bova *et al.*, 1990; McCarron *et al.*, 1994). In neurons, NCX is very abundant and expressed at high concentrations in presynaptic nerve terminals, where relatively large amounts of Ca^{2+} must be transported (Juhaszova *et al.*, 1996; Luther *et al.*, 1992; Reuter and Porzig, 1995). In this tissue, it may play a role in the modulation of Ca^{2+}-dependent neurotransmitter release as well as in Ca^{2+} homeostasis. NCX and PMCA also have different distributions at nerve terminals implicating different functions here. PMCA are

thought to be close to the synaptic vesicle docking sites, whereas NCX are further away from the release sites. In skeletal muscle, NCX, PMCA1, and the Na^+ pump may be confined to transverse tubules rather than the peripheral PM (Sacchetto *et al.,* 1996).

NCX works in a bidirectional manner to either remove cytosolic Ca^{2+} or allow its entry. Whether it functions in both modes during normal physiological activity is still unknown due to the lack of mode selective inhibitors (Blaustein and Lederer, 1999). NCX is thought to play an important role in extruding Ca^{2+} after periods of cell activity and when $[Ca^{2+}]_i$ is elevated and the driving force is large. It is well suited for this because of its capacity to extrude a large amount of Ca^{2+} rapidly under these conditions. Operation primarily in the Ca^{2+} extrusion mode is evident in cells where the Na^+ pump is not present and PMCA is generating the Ca^{2+} gradient which then drives NCX (Milanick and Frame, 1991; Parker, 1978). NCX also plays an important physiological role in excitation-contraction coupling in the cardiac myocyte. NCX is the primary extrusion mechanism in this tissue and thus its primary role is to extrude Ca^{2+} (Barry and Bridge, 1993; Bers, Lederer and Berlin, 1990; Bridge *et al.,* 1991). Moreover, NCX may influence $[Ca^{2+}]_i$ and indirectly yet profoundly modulate SER Ca^{2+} load. It can also trigger Ca^{2+} release from the SER by bringing in Ca^{2+} under certain conditions (Huang, Hove-Madsen, and Tibbits, 2008; Artman, 1992; Huang, Hove-Madsen and Tibbits, 2005). In smooth muscle and other tissues, the Ca^{2+} entry mode also is involved in re-filling the SER (Fameli, van Breeman, and Kuo, 2007; Graham and Trafford, 2007; van Breeman, Chen and Laher, 1995; Lee, Poburko, Sahota *et al.,* 2001; Nazer and van Breeman, 1998; Wu and Fry, 2001; Hirota, Pertens, and Janseen, 2007; Blaustein and Lederer, 1999; Matsuda *et al.,*

2006). This is expanded on in greater detail in sections 1.4 and 3.0. The Ca^{2+} entry mode is also implicated in pathological situations when ion distributions are deregulated (Annunziato *et al.,* 2007; Zhang *et al.,* 2007; Iwamoto and Kita, 2006; Munch *et al.,* 2006; Bers, Despa, and Bossuyt, 2006). A potentially harmful effect of NCX in this mode is the subsequent Ca^{2+} overloading in the cell. Inhibition of this entry by agents such as SEA 0400 and KB-R7943 may be therapeutically useful (Iwamoto, 2004).

The localization of NCX in PM microdomains that overly the SER may implicate that one of the functional roles of NCX is to help control signalling Ca^{2+} levels in the SER. The microdomains are known as caveolae, and localization of NCX here, along with other Ca^{2+} transporters, may lead to specialized regulation pockets. Caveolae are vesicular organelles (50- to 100-nm in diameter) that form invaginations of the plasma membrane (Razani, Woodman, and Lisanti, 2002; Williams and Lisanti, 2004; Daniel, El-Yazbi and Cho, 2006). They are present in a wide range of cardiovascular cells, including smooth muscle cells (Song *et al.,* 1996; Segal, Brett and Sessa, 1999; Thyberg, 2002; Thyberg *et al.,* 1997). They are a specialized subset of lipid rafts which are rich in cholesterol and sphingolipids. These properties have enabled efficient purification of caveolae-enriched membrane fractions by sucrose gradient centrifugation due to their reduced density (high buoyancy) and resistance to solubilization by non-ionic detergents (i.e. Triton X-100) at 4°C (Sargiacomo *et al.,* 1993; Lisanti, Scherer, Tang *et al.,* 1994; Lisanti, Tang, Scherer and Sargiacomo, 1995; Lisanti, Sargiacomo and Scherer, 1999). Caveolins are the structural proteins that are necessary and sufficient for the formation of caveolae. They are small proteins (18-24kDa) but can form higher-order oligomers made

up of 14-16 monomers (~350-400 kDa). One of the major hypothesized functions of caveolae is to serve as signalling platforms by concentrating signalling molecules (Lisanti, Scherer, Tang *et al.,* 1994). Signalling molecules bind caveolin's 'caveolin scaffolding domain' mediated by their own caveolin-binding motifs. The scaffolding domain is important for targeting and concentrating caveolin-1 to caveolae, and is necessary for the interaction between caveolin-1 and cholesterol (Okamoto, Schlegel, and Scherer, 1998; Schlegel and Lisanti, 2000; Murata *et al.,* 1995). NCX has been shown to contain caveolin binding motifs (Bossuyt *et al.,* 2002). Two motifs are within the endogenous XIP domain, a region of NCX1 involved in regulation of transport (Slaughter, Sutko and Reeves, 1983; Shannon, Hale and Milanick, 1994). The third motif is an alternative splicing exon found in only NCX1, and not other isoforms (Kofuji, Lederer, and Schulze, 1994). The precise physiological role of NCX in caveolae, especially in smooth muscle, is not yet clear.

Pathophysiology

NCX has been implicated in a wide variety of pathologies including oxidative stress, hypertension, hypoxia/anoxia, and ischemia reperfusion. Outside of the cardiovascular system, it may play a role in white matter degeneration after spinal cord injury, brain trauma, and optical nerve injury.

NCX appears to be sensitive to oxidative stress. When treated with AAPH (a peroxyl radical generator) or peroxynitrite, NCX activity was attenuated as a result of formation of high molecular weight aggregates and NCX fragmentation (Huschenbett,

Zaidi and Michaelis, 1998; Garcia, de Salamanca and Portoles, 1999). Furthermore, induction of oxidative stress by treatment with Fe-EDTA resulted in a 47 % reduction in exchange activity (Kaplan, Matejovicova, and Mezesova, 1997).

Selective NCX inhibitors and genetically engineered mice show that Ca^{2+} entry through NCX1 in arterial smooth muscle is involved in the development of salt-sensitive hypertension (Iwamoto, Kita, and Katsuragi, 2005; Iwamoto and Kita, 2006). Oxidative stress induced by treatment with H_2O_2 and peroxynitrite further impairs NCX activity in this salt sensitive rat model (Unlap *et al.,* 2003). The NCX inhibitor SEA 0400 selectively suppresses salt-dependent hypertension (Iwamoto, Kita, Zhang *et al.,* 2004). This is a unique antihypertensive profile that differs from that of Ca^{2+}-channel blockers, which lower blood pressure in most hypertensive models (Iwamoto, Kita, and Katsuragi, 2005).

Hypoxia has been proposed to inhibit NCX, however, this hypothesis has been challenged (Zhang *et al.,* 2007; Becker *et al.,* 2006). Meng, To and Gu (2008) recently found that NCX contributes to hypoxia-induced $[Ca^{2+}]_i$ elevation in PC12 cells. This was consistent with other studies in pulmonary artery smooth muscle suggesting NCX plays a role in hypoxia (Becker *et al.,* 2006; Zhang *et al.,* 2007).

The Ca^{2+} entry mode of NCX may also contribute to damage in white matter injury in spinal cord preparations caused by anoxia or trauma (Stys, Waxman and Ransom, 1992; Imaizumi, Kocsis, and Waxman, 1997; Li, Jiang and Stys, 2000). Inhibiting NCX with SEA- 0400 or bepridil appears to limit the extent of ischemic brain damage in different models of white matter injury (Stys 1998; Li, Jiang and Stys, 2000).

This mode of NCX has also been suggested to contribute to NMDA excitotoxic cell death (Kiedrowski, 1999; Czyz and Kiedrowski, 2002).

A number of conflicting reports have been published on the role played by NCX during ischemia (Andreeva *et al.,* 1991; Amoroso *et al.,* 1997; Amoroso *et al.,* 2000; Matsuda *et al.,* 2001; Schroder *et al.,* 1999; Takahashi *et al.,* 2003). In *in vitro* and *in vivo* models of anoxia and ischemia, stimulation of NCX activity was neuroprotective, and its pharmacological blockade was neurodamaging. The neuroprotective effect exerted by NCX during ischemic injury is due to NCX1 and NCX3; proteolytic cleavage of these isoforms may render increased susceptibility to ischaemic and even excitotoxic death (Pignataro *et al.,* 2004; Annunziato *et al.,* 2007).

An increase or upregulation of NCX1 in heart failure has also been reported from animal and human clinical studies, however there is no direct evidence linking the exchanger to this disease (Studer *et al.,* 1994; Flesch *et al.,* 1996; Hobai and O'Rourke, 2000; Pogwizd, 2000).

NCKX

Another type of bi-directional Na^+-Ca^{2+} exchanger found in the PM of mainly retina and brain is NCKX. Unlike NCX, however, NCKX co-transports one Ca^{2+} and one K^+ in exchange of four Na^+ (Dong *et al.,* 2006). Although the NCKX shares low sequence homology, its suggested membrane topology is similar to that of NCX (Chicka and Strehler, 2003). Five genes encoding NCKX have been cloned. We have not found any

evidence of this exchanger's activity in pig coronary artery therefore this protein will not be further discussed.

1.2.2 Ca^{2+} pumps

As mentioned previously, the Ca^{2+} pumps are generally considered to be high affinity Ca^{2+} removal systems. There are three types: PMCA, SERCA, and a secretory pathway Ca^{2+} ATPase in the Golgi (SPCA) (Missiaen *et al.*, 1991; Missiaen *et al.*, 2007). These proteins utilize the energy obtained from the hydrolysis of ATP to move Ca^{2+} against its concentration gradient (Carafoli *et al.*, 2001). The subsequent section focuses on the general properties, structural organization, regulation and importance of SERCA, followed with a brief description of PMCA.

Sarco/endoplasmic Reticulum ATPase (SERCA)

General Properties

SERCA is a member of the P-type Ca^{2+}-ATPase family responsible for maintaining a 10,000-fold Ca^{2+} gradient across the SER membrane (Periasamy and Kalyanasundaramm, 2007; Rossi and Dirksen, 2006). A notable feature of P-type ATPases is the transfer of a terminal (γ -) phosphate from ATP to a highly conserved aspartyl residue in the cytoplasmic domain. This transfer results in a reversible conformational change intimately linked with the ion translocation process (Periasamy and Kalyanasundaramm, 2007; Toyoshima and Inesi, 2004; Apell, 2004; Moller, Juul, le Maire, 1996). As mentioned above, SERCA utilizes the energy derived from ATP

hydrolysis to transport Ca^{2+} across the membrane. The mechanism of the coupling process is such that two Ca^{2+} ions are transported for each molecule of ATP hydrolyzed per reaction cycle (Periasamy and Kalyanasundaramm, 2007; Yu *et al.,* 1993; Martonosi and Pikula, 2003; Toyoshima and Inesi, 2004; Rossi and Dirksen, 2006).

The reaction cycle of SERCA is based on the cyclical changes between two main conformational states denoted as E_1 and E_2 (Olesen *et al.,* 2004; Olesen *et al.,* 2007; Toyoshima and Nomura, 2002). The E_1 conformation is obtained upon high affinity (between 0.1 - 2 μM, depending on the isoform), and cooperative binding of two Ca^{2+} from the cytoplasmic side of the membrane (2 Ca^{2+} -E_1) (Tada and Katz, 1982; Tanford, Reynolds and Johnson, 1987; Levy *et al.,* 1990). The Ca^{2+} activated SERCA then binds ATP, and the γ-phosphate of ATP is transferred to an aspartyl residue to form a high-energy covalent aspartyl-phosphate intermediate (2 Ca^{2+} -E_1P). This transition state with occluded Ca^{2+} is converted to the low Ca^{2+} affinity, low energy E_2P state. Accompanying this transition is the opening of the Ca^{2+} gate now facing the reticulum and subsequent movement of Ca^{2+} into the SER lumen. Two or three protons then bind the exposed cation-binding sites. This closes the Ca^{2+} translocation channel leading to the E_2P* transition state with occluded protons. The E_2P* state is then dephosphorylated to E_2, a pathway opens in which an exchange of protons with Ca^{2+} completes the reaction cycle (Olesen *et al.,* 2007).

Isoforms

In vertebrates, the SERCA protein is encoded by three distinct genes - SERCA1,

SERCA2 and SERCA3 (Burk *et al.,* 1989; Dode *et al.,* 1998; Grover and Khan, 1992). Additional SERCA isoform variants are generated as a result of alternative splicing of the primary gene transcripts. The SERCA1 isoform is found in fast-twitch skeletal muscle (Zhang *et al.,* 1995). The last two exons of this gene are alternatively spliced producing the SERCA1a and 1b gene products in adults and neocytes, respectively (Zhang *et al.,* 1995; Brandl *et al.,* 1987). Similarly, alternative splicing of the SERCA2 gene also results in production of two carboxyl terminal splice variants (SERCA2a and SERCA2b) as well as two relatively rare isoforms (SERCA2c and SERCA2d) (Eggermont *et al.,* 1990; Lytton and MacLennan, 1988; Gelebart *et al.,* 2003; Zarain-Herzberg and Alvarez-Fernandez, 2002). These SERCA2 splice variants differ only in their carboxyl terminus, described in detail in the subsequent section. The SERCA2a protein is expressed at high levels in cardiac, slow-twitch skeletal and neonatal skeletal muscles (Misquitta, Mack and Grover, 1999; Rossi and Dirksen, 2006; MacLennan *et al.,* 1985). SERCA2b protein is expressed in all tissues including muscle and non-muscle cells and is considered to be the housekeeping isoform (Lytton *et al.,* 1989; Lytton and MacLennan, 1988; Wuytack et al., 1992; Wu *et al.,* 1995). The only reported isoforms of SERCA in smooth muscle are SERCA2a and 2b (Dode *et al.,* 1998). Alternative splicing of the SERCA3 transcripts produces three protein isoforms (SERCA3a, 3b, or 3c) (Grover and Khan, 1992; Lytton *et al.,* 1989; Dode *et al.,* 1998; Poch *et al.,* 1998). These isoforms are expressed at high levels in the hematopoietic cell lineages, platelets, epithelial cells, fibroblasts, and endothelial cells (Anger *et al.*, 1993; Bobe *et al.,* 2005; Wuytack *et al.,* 1994). The developmental and tissue-specific expression pattern of the different SERCA isoforms

suggests that each isoform is adapted to cell-specific functions. Before getting into these details, the structure of SERCA will first be described.

Structure

The current structural model for SERCA contains three important regions designated as the cytoplasmic head, the stalk domain, and an SER transmembrane domain (MacLennan, Toyofuku and Lytton, 1992; Toyoshima and Inesi, 2004; MacLennan *et al.*, 1985). More than half of the total mass of SERCA is exposed on the cytoplasmic surface of the membrane. The cytoplasmic domain is subdivided into three distinct regions: the actuator/anchor domain, the nucleotide binding domain, and the phosphorylation domain (Martonosi and Pikula, 2003; Rossi and Dirksen, 2006). The actuator domain facilitates engagement of the gating mechanism and thereby regulates Ca^{2+} binding and release (Toyoshima and Inesi, 2004). The phosphorylation and nucleotide binding domains form the active site of ATP hydrolysis (Martonosi and Pikula, 2003; Misquitta, Mack and Grover, 1999). The γ-phosphate of ATP is transferred to an aspartate residue in the phosphorylation domain whereas the adenosine moiety binds to a site in the nucleotide domain. The phosphorylation domain also contains a conserved sequence called the 'hinge' region, which is essential to bring the phosphorylation site close to the bound ATP during the reaction cycle. Both the N- and C- terminals are located in the cytoplasm, however, as mentioned above, the SERCA2 isoforms differ in their carboxy termini. The variable part of the C-terminus consists of four amino acids in SERCA2a, which are replaced by 49 amino acids in SERCA2b and by 6 amino acids in the putative SERCA2c

protein (Campbell *et al.,* 1991; Gelebart *et al.*, 2003). The extended tail of SERCA2b may form an additional eleventh TM domain, locating its C-terminus on the lumenal surface (Lytton and MacLennan, 1988). Consequently, the C-termini of SERCA2a and SERCA2b are situated at opposite sides of the ER membrane (Campbell, Kessler and Famborough, 1992). The stalk domain connects the cytoplasmic head to the SER membrane.

According to a hydropathy plot, ten hydrophobic transmembrane helices (TM1–10) anchor SERCA to the lipid bilayer. A number of residues in TM 4, 5, 6 and 8 are essential for coordinating the cooperative binding of two closely spaced Ca^{2+} binding sites as well as forming a Ca^{2+} translocation channel in this region (Inesi *et al.,* 1995; Toyoshima, Sasabe, and Stokes, 1993; MacLennan *et al.,* 1985; Martonosi and Pikula, 2003; Rossi and Dirksen, 2006). Two Ca^{2+} bind with high affinity to these sites on the cytoplasmic side of the protein at the beginning of the reaction cycle (see above) and are ultimately released from the luminal side following ATP hydrolysis (Rossi and Dirksen, 2006; Yu *et al.,* 1993). The luminal linkers between the TM segments are short except for the loop between TM7 and 8.

SERCA structure has been characterized mainly using the adult fast-twitch skeletal muscle pump but the high degree of homology suggests similar tertiary conformations and transmembrane topologies for all SERCA isoforms (Periasamy and Kalyanasundaram, 2007). The molecular masses of all muscle isoforms are close to 110 kDa. Although they are monomers, SERCA proteins also have a tendency to oligerimize.

Regulation

A unique feature of all SERCA isoforms is their regulation by smaller proteins that associate with the pump and are in turn responsive to additional regulation such as that by kinase phosphorylation. The following section highlights the agents involved in either direct or indirect regulation of SERCA.

Phospholamban is the best studied regulator of SERCA2. This 52aa SER transmembrane protein, in its unphosphorylated form, inhibits SERCA by lowering its apparent Ca^{2+} affinity without altering its maximal pumping rate (Cantilina *et al.,* 1993; Vangheluwe *et al.,* 2005; Fujiii *et al.,* 1987). Phospholamban can exist as a monomer or as a pentamer and it is the latter form which appears to be responsible for the inhibition. Preferential inhibition of SERCA1a, 2a and 2b occurs upon phospholamban recognizing and interacting with a particular cytoplasmic sequence of these isoforms (Lalli *et al.,* 1997; Toyofuku *et al.,* 1994; Asahi *et al.,* 2003). Concerted and additive phosphorylation consequently disrupts phospholamban's interaction with and inhibition of SERCA (James *et al.,* 1989; Koss and Kranias, 1996; Kadambi and Kranias, 1997; Toyofuku *et al.,* 1994). The protein kinases that may be involved include cAMP- or cGMP-kinases and Ca^{2+}/calmodulin-kinase II (Koss and Kranias, 1996; Kadambi and Kranias, 1997; Morris *et al.,* 1991). Phospholamban can be dephosphorylated by protein phosphatases. Thus, phosphorylation and dephosphorylation of phospholamban may regulate SERCA activity in response to various stimuli and in a tissue-selective manner (Misquitta, Mack and Grover, 1999).

Another protein involved in direct protein-protein interaction and subsequent regulation of SERCA is the phospholamban homologue, sarcolipin (Odermatt *et al.,* 1998). It is a regulator of fast-twitch skeletal muscle, but is also found in slow-twitch and in cardiac muscle (Wuytack, Raeymaekers, and Missiaen, 2002). Sarcolipin thus interacts with and inhibits SERCA1a and 2a by lowering the apparent Ca^{2+} affinity. There remains some controversy as to how it affects SERCA with some reports saying it increases the V_{max} while others say sarcolipin lowers it (Hellstern *et al.,* 2001; Odermatt *et al.,* 1998; Asahi *et al.,* 2002). Interestingly, sarcolipin and phospholamban induce a synergistic effect on SERCA (Asahi *et al.,* 2002). The binding site of sarcolipin to SERCA1a is the same as phospholamban's, however, the latter has a stronger affinity (Asahi *et al.,* 2003). Unlike phospholamban, its interaction with SERCA is not modulated by phosphorylation of the inhibitor but by a change in its expression levels (Raeymaekers, and Missiaen, 2002).

Additional regulatory mechanisms of SERCA include its direct phosphorylation. As mentioned above, Ca^{2+}/calmodulin-kinase II phosphorylates phospholamban. The kinase also targets SERCA2 to increase its V_{max}, without altering its K_m (Xu, Hawkins and Narayanan, 1993; Grover, Samson and Narayanan, 1996). This effect is SERCA2 specific and may be attributed to a residue of a CaMKII consensus site absent in SERCA1 (Hawkins, Xu, and Narayanan, 1994). The physiological importance of SERCA direct phosphorylation still remains unclear (Vangheluwe *et al.,* 2005).

SERCA, functionally, can be easily distinguished due to its highly selective inhibitors, thapsigargin and cyclopiazonic acid (Missiaen *et al.,* 1991; Wuytack, Raeymaekers, and Missiaen, 2002).

Functional Comparison of SERCA isoforms

SERCA activity lowers $[Ca^{2+}]_i$ while at the same time raising that of the SER. The content of Ca^{2+} within the SER controls other processes such as the sensitivity of Ca^{2+} release and activity of PM Ca^{2+} entry channels. Consequently, SERCA activity has a very direct and dynamic effect on patterns of Ca^{2+} signalling and the cellular events these signals control. As described previously, SERCA gene transcripts are subject to alternative processing in a differential and tissue dependent manner. The Ca^{2+} pump isoforms thus generated may present different functional properties. The divergence in the carboxyl terminal of SERCA2a and 2b is responsible for their known functional differences as exemplified in their Ca^{2+} sensitivities. SERCA2b displays a two-fold higher apparent affinity for Ca^{2+} ($K_m = 0.17 \pm 0.01$ μM) relative to SERCA2a ($K_m = 0.31 \pm 0.02$ μM) (Vangheluwe *et al.,* 2005; Verboomen *et al.,* 1992; Bosch *et al.,* 1992). It also has a two-fold lower maximal pumping rate (Verboomen *et al.,* 1992). In addition, there are observed differences in the partial reaction steps of the isoforms. SERCA2b has a ten-fold lower rate of Ca^{2+} dissociation from the high affinity Ca^{2+} binding site (C1), a two-fold lower rate of conversion to the ADP-insensitive phosphointermediate, and lastly, a three-fold lower rate of dephosphorylation (Vangheluwe *et al.,* 2005). Removal of the last 12 amino acids causes SERCA2b to acquire the same properties as SERCA2a

(Verboomen *et al.*, 1994). SERCA2c has a lower affinity for $Ca^{2+}{}_i$ than SERCA2a and 2b, which could be due to its specific C-terminus (Dally *et al.*, 2006; Gelebart *et al.*, 2003). The physiological relevance of SERCA2c is yet to be defined (Periasamy and Kalyanasundaram, 2007). Very little is known about the functional properties of the SERCA3 isoforms. SERCA3 when expressed in COS cells show a 5-fold lower apparent affinity for Ca^{2+} and an altered pH optimum (pH 7.2–7.4 vs. pH 6.8 –7.0) compared to the other two isoforms (Dode *et al.*, 2002; Misquitta, Mack, and Grover, 1999). Furthermore, the 3b and 3c isoforms show a significantly lower Ca^{2+} affinity than SERCA3a (Misquitta, Mack, and Grover, 1999).

The sensitivity of SERCA2a and SERCA2b towards thapsigargin inhibition appears to be identical. In addition, both SERCA2 isoforms show a similar extent of inhibition when co-expressed with phospholamban (Bosch *et al.*, 1992). Phospholamban does not affect the SERCA3 isoform because it lacks the putative domain for its binding (see SERCA Regulation above) (Periasamy and Kalyanasundaram, 2007).

Redox agents are also known to modulate SERCA. SERCA contains 25 cysteine residues, but only 1 or 2 are essential for enzyme action. Thus, the redox state of these sulfhydryl groups can determine its activity. Reagents that oxidize thiols inhibit pump activity, whereas reducing agents (i.e. DTT and GSH) protect SERCA from this inhibition (Grover and Samson, 1988; Grover *et al.*, 1995). SERCA activity is also sensitive to oxidative stress. This is described in greater detail in the Pathophysiology section below.

Pathophysiology

Several studies show that SERCA is sensitive to oxidate stress (Grover and Samson, 1988; Grover *et al.,* 1995; Grover, Samson, and Fomin, 1992). ROS such as H_2O_2, superoxide ($O_2^{\cdot-}$), peroxynitrite ($ONOO^-$), and hydroxyl radical ($OH^{\cdot}$) generation increases rapidly in myocardial ischemia reperfusion injury (Kaneko *et al.,* 1994; Lefer and Granger, 2000). $O_2^{\cdot-}$ and H_2O_2/$OH^{\cdot}$ inhibit Ca^{2+} uptake into the SER resulting in an increase in $[Ca^{2+}]_i$ (Grover and Samson, 1988; Grover *et al.,* 1995; Rowe *et al.,* 1983; Kukreja *et al.,* 1988; Grover, Samson and Misquitta, 1997; Xu, Zweier, Becker, 1997). The Ca^{2+} transport is tightly coupled to ATP hydrolysis and inhibition of ATPase activity will ultimately decrease the Ca^{2+} pumping rate. H_2O_2/ $OH^{\cdot}$ inactivates cardiac and skeletal muscle SERCA by directly interfering with the ATP binding site (Xu, Zweier, Becker, 1997). Furthermore, ROS cause inhibition of SERCA function by uncoupling Ca^{2+} uptake activity from ATP hydrolysis (Rowe *et al.,* 1983).

Despite the fact that there is 90% amino acid homology between all SERCA isoforms, they exhibit different sensitivities to ROS. The cardiac SERCA2a isoform is more sensitive to H_2O_2/ $OH^{\cdot}$ than to $O_2^{\cdot-}$ and SERCA3 is more resistant than SERCA2b to peroxides (Rowe *et al.,* 1983; Grover, Samson and Misquitta, 1997; Grover, Kwan, and Samson, 2003). NO does not appear to affect SERCA activity by S-nitrosylation of cysteine residues directly; however $ONOO^-$ was found to increase SERCA2 activity (Stoyanovsky *et al.,* 1997; Eu *et al.*, 2000; Adachi *et al.,* 2004). During pathological conditions that are associated with increases in ROS production, this mechanism of NO signaling is likely to be abolished as a result of irreversible oxidation of sulfhydryl groups

(Adachi *et al.*, 2004).

Changes in SERCA expression have been associated with heart failure in humans and with animal models of heart disease, hypertension, diabetes, and aging (Wu, Bungard, and Lytton, 2001). An increased SERCA/phospholamban ratio promoted SER Ca^{2+} reuptake in a mouse model of congestive heart failure (Vafiadaki *et al.*, 2009). Moreover, changes in the expression of SERCA genes in smooth muscle have been reported in response to platelet-derived growth factor (Magnier *et al.*, 1992).

PMCA

PMCA have ten transmembrane domains, six cytosolic domains, and five putative extracellular domains. There are four PMCA genes with PMCA 1 and 4 being widely expressed, while PMCA 2 and 3 are more tissue specific (Carafoli, 2002). Pig coronary artery smooth muscle cells (PCSMC) express both PMCA1 and PMCA4 (Szewczyk *et al.*, 2007). Various PMCA splice variants are regulated differently by calmodulin, protein kinases and acidic phospholipids (Guerini, 1998; Pande and Grover, 2005; Stauffer, Guerini, and Carafoli, 1995; Strehler and Zacharias, 2001). Our laboratory has developed PMCA specific and isoform selective inhibitors called caloxins (Pande *et al.*, 2008; Szewczyk, Pande, Grover, 2008; Pande and Grover, 2005).

ROS can substantially inhibit both Ca^{2+} transport and ATPase activity of PMCA (Kaneko, Beamish, and Dhalla, 1989; Kaneko, Elimban and Dhalla, 1989). These effects of ROS occur through oxidation of sulfhydryl groups and peroxidation of membrane phospholipids. Furthermore, $ONOO^-$ irreversibly inhibited PMCA in rat brain

synaptosomes, suggesting that a similar effect may occur in cardiac tissue (Gutierrez-Martin *et al.,* 2002).

1.3 Mechanisms of elevating cytosolic Ca^{2+}

1.3.1 Ca^{2+} Entry Channels

Extracellular Ca^{2+} can enter through the opening of voltage operated Ca^{2+} channels or non-voltage operated Ca^{2+} channels, or through the Ca^{2+} entry mode of NCX, all of which are in the PM (Kamm and Stull, 1989; Missiaen *et al.,* 1991; van Breeman and Saida, 1989; Hai and Murphy, 1989; Stull *et al.,* 1991). VOCC are activated by membrane depolarization and are associated with excitable cells such as muscle and neuronal cells. They are classified as either high voltage activated channels (L, N, P/Q, R type) or low voltage activated channels (T type) (Carafoli, 2002; Bootman *et al.,* 2001; Marin *et al.,* 1999). The former require strong depolarization, while the latter require weak depolarization for their activation. The L-type (long-lasting type) VOCC are considered to be a major Ca^{2+} influx pathway in most cells, especially in smooth muscle (Lipskaia and Lompre, 2004; Marin *et al.,* 1999). These channels supply the bulk of Ca^{2+} required to activate the myofilaments and are therefore the target of pharmaceutical interventions directed at for example, hypertension (Floyd and Wray, 2007). There is evidence for the expression of T-type Ca^{2+} channels in a variety of smooth muscles (Young, Smith and McLaren, 1993; Sui, Wu, and Fry, 2003).

Receptor operated Ca^{2+} channels (ROCC), and store operated Ca^{2+} channels (SOCC) are the two major subtypes of non-voltage operated Ca^{2+} channels. ROCC are

non-specific cation channels with a certain preference for divalent cations (Yamada *et al.*, 1992). These channels are activated by second messengers generated by receptor activation. The TRPC channels can form Ca^{2+} permeable channels and may function as ROCC (Hofmann *et al.*, 2000; Clapham, Runnels, and Strubing, 2001; Montell, Birnbaumer, and Flockerzi, 2002).

SOCC open in response to a depletion of SER Ca^{2+} stores and are permeable to Ca^{2+} and Na^{+} (Sweeney *et al.*, 2002; Trepakova *et al.*, 2001; Dietrich *et al.*, 2007; Cioffi *et al.*, 2005). The coupling mechanisms by which the SER signals the SOCC are unclear; two hypotheses include the involvement of a diffusible messenger and a physical interaction between the PM and SER. These mechanisms are further explained in section 1.4. Depletion of the SER stores subsequently leading to activation of SOCC and store operated Ca^{2+} entry (SOCE) may also be caused with pharmacological agents such as thapsigargin or cyclopiazonic acid, which inhibit SERCA (Dong *et al.*, 2006). SOCC may be one of the most ubiquitous PM Ca^{2+} channels (Bootman *et al.*, 2001; Carafoli, 2002; Marin *et al.*, 1999). The prototypic and best characterized type is the Ca^{2+} release activated Ca^{2+} (CRAC) channel; however, it is not universal to all cells that exhibit SOCC activity (Hoth and Penner, 1992; Lewis and Cahalan, 1990; Prakriya and Lewis, 2003).

1.3.2 Ca^{2+} Release Channels

Increasing $[Ca^{2+}]_i$ may also occur through the release of Ca^{2+} from internal stores such as the SER. The SER is the primary Ca^{2+} storage organelle that maintains its free Ca^{2+} around 1 mM. There are two types of Ca^{2+} release channels in the SER membrane

which are not voltage dependent: Ca^{2+}-induced Ca^{2+}-release channels (CICR, also termed ryanodine receptors) and inositol 1,4,5-trisphosphate (IP_3)- induced Ca^{2+} release channels (IP_3 receptors) (Berridge, Lipp and Bootman, 2000; Boron and Baulpaep, 2003; Lipskaia and Lompre, 2004; Marin *et al.,* 1999). The CICR are activated typically upon entry of Ca^{2+} through VOCC. IP_3 is generated during the inositol phosphate calcium signaling pathway and readily diffuses to encounter the IP_3 receptors in the SER. The binding of IP_3 to its receptor changes the IP_3 receptor conformation to open an integral channel to allow the release of Ca^{2+} stored in the SER. The rapid, transient release of Ca^{2+} from these channels results in a depleted SER Ca^{2+} pool. This signal functions as a primary trigger for a message that is returned to the PM, resulting in the relatively slow activation of SOCC and subsequent entry of extracellular Ca^{2+} (Putney and McKay, 1999; Berridge, 1995; Putney and Ribeiro, 2000; Putney *et al.,* 2001). This process is key to mediating longer term $Ca^{2+}{}_i$ signals and replenishing the SER Ca^{2+} pool (Putney *et al.,* 2001; Venkatachalam *et al.,* 2002; Parekh and Putney, 2005; Gill *et al.,* 1996).

1.4 Mechanisms of Refilling the SER

As mentioned above, receptor activation stimulates the release of Ca^{2+} from the SER ultimately leading to a depleted Ca^{2+} pool. Depletion of the SER activates SOCC in the PM causing a slow and sustained entry of $Ca^{2+}{}_o$ which then enters the SER through SERCA. This process, known as capacitative Ca^{2+} entry or SOCE, is key to mediating longer term cytosolic Ca^{2+} signals and replenishing the SER Ca^{2+} pool. Recent advances in capacitative Ca^{2+} entry have focused on the role of stromal interacting molecule

(STIM1) and Orai1 proteins, as well as the Ca^{2+} entry mode of NCX.

Two models have been proposed for transmitting the signal from the SER to the PM during capacitative Ca^{2+} entry. The first is the involvement of a diffusible chemical messenger and the second is a physical interaction between the PM and SER (Berridge, 1995; Su *et al.,* 2001; Randriamampita and Tsien, 1993; Thomas and Hanley, 1995; Xie *et al.,* 2002). There is controversy in the literature as to what the diffusible messenger is; candidates include cyclic GMP, cytochrome P450 products, and an unidentified small molecule called the Ca^{2+} influx factor (CIF) (Xu *et al.,* 1994; Alvarez, Montero, Garcia-Sancho, 1992; Graier, Simecek, and Sturek, 1995; Randriamampita and Tsien, 1993; Bolotina and Csutora, 2005). The physical interaction model may include mechanisms involving exocytotic insertion of channels into the PM and a process termed conformational coupling. Spatial coupling of the PM and SER suggesting a close organization of the two membranes, as opposed to a direct molecular interaction, has also been proposed (Venkatachalam *et al.,* 2002). The concept of an SER Ca^{2+} sensor that interacts directly with PM channels is currently the leading idea for SOCC regulation (Putney, 2009). The sensor, STIM1, is a single pass transmembrane protein located both in the PM and SER (Dziadek and Johnstone, 2007). It is expressed and functional in vascular smooth mucle cells (Dietrich *et al.,* 2007; Takahashi *et al.,* 2007; Berra-Romani *et al.,* 2008). Following depletion of Ca^{2+} stores, STIM1 molecules aggregate and accumulate into puncta in the subplasma membrane region within 10-25 nm of the PM (Smyth *et al.,* 2008; Wu *et al.,* 2006; Liou *et al.,* 2005; Zhang *et al.,* 2005). Whether formation occurs at predetermined sites in lipid rafts or distinct from them is under

investigation and is explored further in section 10.5 of the Discussion (Smyth *et al.*, 2008; DeHaven *et al.*, 2009). Co-immunoprecipitation experiments show conflicting results on whether STIM1 directly interacts with the pore forming subunits of the CRAC channels, Orai1, in puncta (Yeromin *et al.*, 2006; Feske *et al.*, 2006; Vig *et al*,. 2006). However, Orai1 was recruited to sites of Stim1 puncta formation and Ca^{2+} entry indicating the two proteins communicate over short distances (Luik *et al.*, 2006). The generation of a secondary message (i.e. CIF) downstream of STIM1 which then acts on Orai is an alternative theory (Bolotina and Csutora, 2005; Csutora *et al.*, 2008). There is great interest in the literature to discern the mechanisms involved in the activation of SOCE and interaction between STIM1 and Orai1.

Several lines of evidence suggest NCX may also play a role in refilling the SER during capacitative Ca^{2+} entry. First, the Ca^{2+} entry mode of NCX is important in refilling the SER after asynchronous Ca^{2+} oscillations in vascular smooth muscle (Fameli, van Breeman, and Kuo, 2007). The junctional space between the SER and PM may be important for this (van Breemen, Chen, Laher, 1995; Lee *et al.*, 2001; Nazer and van Breemen, 1998). Furthermore, Arnon and colleagues (2000) proposed that activation of SOCC in such junctions could generate the $[Na^+]$ required to mediate Ca^{2+} entry by NCX. This mechanism was shown to efficiently refill the SER following IP_3-receptor mediated Ca^{2+} release in airway smooth muscle (Dai *et al.*, 2005). Wu and Fry (2001) also demonstrated that Na^+-dependent entry of Ca^{2+} through NCX could contribute to the overall pool of intracellular Ca^{2+} in guinea pig detrusor smooth muscle. This mode of NCX may provide a source of Ca^{2+} for store refilling following agonist-induced Ca^{2+}

mobilization (Hirota, Pertens, and Janseen, 2007). In addition, it may also contribute to maintaining a longer and stronger cardiac contraction (Graham and Trafford, 2007).

2.0 CORONARY ARTERY

Function

Coronary arteries deliver oxygenated blood and nutrients to the heart (Cox and Vatner, 1982). Any damage to the artery or obstruction in coronary blood flow may cause serious physiological effects such as development of atherosclerosis and other cardiovascular diseases (Kralios *et al.,* 1998). An important factor in regulating coronary blood flow is the vascular tone.

Anatomy

A coronary artery is constructed of three different tissue layers: adventitia, media, and intima. The outermost layer, the adventitia, is a thin layer of connective tissue made up of elastic and collagen fibers and functions as mechanical support (Missiaen *et al.*, 1991; Kamm and Stull, 1989; van Breeman and Saida, 1989). The media consists of elastic fibers and most importantly, smooth muscle cells. The elastic fibers in the smooth muscle cells allow the artery to stretch or expand in response to small increases in pressure without tearing. The innermost layer, or the intima, consists of endothelial cells which function as a barricade between the blood and the underlying tissues (Walia *et al.,* 2000; Malhorta *et al.,* 2003). Both endothelial cells and smooth muscle cells are pivotal in

altering vascular tone and subsequently are responsible for the regulation of blood flow and blood pressure Missiaen *et al.*, 1991; Kamm and Stull, 1989; van Breeman and Saida, 1989).

Smooth muscle

Smooth muscle is a type of non-striated muscle composed of elongated and spindle shaped mono-nucleate cells. Each cell is connected by gap junctions and contains loose bundles of thick, intermediate, and thin filaments within the cytoplasm (Kamm and Stull, 1989; Hai and Murphy, 1989; Murphy, 1989; Stull *et al.*, 1991; Craig and Woodhead, 2006). Contraction of smooth muscle occurs via cross-bridges between the thick and thin filaments which form as a result of interaction between their primary structural components, myosin and actin, respectively (Kamm and Stull, 1989; Hai and Murphy, 1989; Murphy, 1989; Stull *et al.*, 1991). The contraction mechanism is initiated upon a rise of $[Ca^{2+}]_i$, but may also occur by Ca^{2+} independent pathways (Murphy, 1989; Stull *et al.*, 1991; Horowitz *et al.*, 1996).

3.0 EVIDENCE OF NCX & SERCA IN CORONARY ARTERY AND OTHER VASCULATURE

NCX and SERCA may demonstrate different activity levels, isoforms, functional properties and regulation in various types of tissue; hence, it is important to reassess these transporters and their interactions within each model. In the coronary artery, NCX and

SERCA are important in controlling the levels of $Ca^{2+}i$ which in turn regulates the coronary tone. Outlined below is the evidence of NCX and SERCA in vascular tissue with a particular focus on smooth muscle found in the literature, as well as the evidence for a linkage between the two.

NCX expression and activity

Experiments from our laboratory demonstrate cultured pig coronary artery smooth muscle cells express NCX1.3, NCX1.7, as well as phospholemman mRNA (RT-PCR) and NCX1 protein (Western blot) (Szewczyk *et al.,* 2007). Furthermore, we found that NCX1 is the isoform largely responsible for the NCX-mediated Na^+-dependent $^{45}Ca^{2+}$ entry activity in these cells. This NCX-mediated Ca^{2+} entry was inhibited by KB-R7943, SEA0400, and by depletion of the Na^+ gradient with monensin, but not by the Na^+-H^+ exchanger inhibitor cariporide (Szewczyk *et al.,* 2007; Davis *et al.,* 2008). Further evidence for NCX activity in the Ca^{2+} entry mode in vascular smooth muscle has been examined with electrophysiology, monitoring $[Ca^{2+}]_i$ with fluorescence probes, and vascular contractility experiments (Raina, Ella, and Hill, 2008; Slodzinski and Blaustein, 1998; Slodzinski, Schweda, Kramer and Kurtz, 2001; Bova *et al.,* 1990; Borin, Tribe and Blaustein, 1994).

SERCA expression and activity

Our laboratory has examined SERCA in pig coronary artery extensively (Barnes, Samson and Grover, 2000; Elmoselhi *et al.,* 1995; Grover and Samson, 1986; Grover,

Samson and Lee, 1985; Grover *et al.*, 1996; Khan *et al.*, 2000; Pande *et al.*, 2006). We have demonstrated that cultured pig coronary artery smooth muscle cells express SERCA2 mRNA (RT-PCR) and the SERCA2 protein (Western blot). In addition, we have extensively measured SERCA activity in these cells. Other studies also show vascular smooth muscle tissue predominantly expresses the SERCA2b splice variant.

NCX and SERCA linkage

There is also evidence for a linkage between the two transporters. The SER is classified according to its location as superficial or deep, with distinct functions being attributed to the superficial SER (Lee *et al.*, 2002). In domains where the superficial SER apposes the PM, a narrow space known as the junctional space is created (Lee *et al.*, 2002). This space is thought to present an imperfect barrier to diffusion of small molecules and ions, in particular, Ca^{2+} and Na^{+} (Lee *et al.*, 2002). The actions of NCX and SERCA have been proposed to be linked through a limited junctional cytoplasmic space model in which NCX mediated Ca^{2+} entry creates a local increase in $[Ca^{2+}]_i$ which then is sequestered into the reticulum by SERCA (Lee *et al.*, 2001; Li and van Breeman, 1995; Nazer and van Breeman, 1998; Nazer and van Breeman, 1998). Evidence for this model is based on several key theories.

The first theory linking NCX and SERCA assumes that the PM containing NCX and the SER membrane containing SERCA are in close proximity. Moore *et al.*, (1993) report that NCX is largely co-distributed with the Na^{+}/K^{+} pump on unique regions of the PM in accord with, and close to, calsequestrin-containing regions of the SER in toad

stomach smooth muscle cells. Immunofluorescence microscopy in cultured arterial myocytes indicates that NCX molecules are organized in reticular patterns over cell surfaces, similar to SERCA distributions (Juhaszova *et al.,* 1994). Additional immunocytochemical studies reveal that NCX in smooth muscle appears to be restricted primarily to PM regions that are adjacent to junctional SER (Blaustein *et al.,* 1992; Moore *et al.,* 1993; Juhaszova *et al.,* 1996). This localization may imply that a major role of the exchanger in smooth muscles is to modulate the Ca^{2+} content of the SER stores and thereby influence Ca^{2+} signaling and tension development (Blaustein, 1993; Bova *et al.,* 1990; McCarron *et al.,* 1994; Blaustein and Lederer, 1999). Finally, recent advances in imaging techniques have allowed for a direct visualization of co-localization of NCX and the superficial SER (Lyashkov *et al.,* 2007).

A second theory linking the two Ca^{2+} transporters is that the inhibition of one protein affects the other without affecting other proteins such as PMCA. In a study based on $[Ca^{2+}]_i$ measurements in rabbit inferior vena cava smooth muscle, when NCX was arrested by removing both external Na^+ and Ca^{2+}, Ca^{2+} released from the SER was re-sequestered (Nazer and van Breeman, 1998). However, when both NCX and SERCA were blocked, the Ca^{2+} released from the reticulum was then extruded from the cells by PMCA. Results from our laboratory with pig coronary artery smooth muscle are also consistent with this theory. First, SERCA inhibition with thapsigargin diminished the NCX-mediated $^{45}Ca^{2+}$ entry (Davis *et al.,* 2008). Second, preloading with the Ca^{2+} chelator BAPTA enhanced NCX- mediated $^{45}Ca^{2+}$ entry and thapsigargin had no effect in

these cells (Davis *et al.*, 2008). Chelation allows for maintenance of the Ca^{2+} gradient and thus eliminates the need for a functional linkage with SERCA.

The concept of a linkage between NCX and SERCA in smooth muscle was also confirmed by the similar distribution of NCX1 and SERCA2 proteins when detergent treated microsomes were fractionated by flotation on sucrose density gradients (Davis *et al.*, 2008). Both proteins strongly correlated with caveolin, the protein localized to specialized lipid rafts known as caveolae. The data, although preliminary, are consistent with a spatial linkage between SERCA2, caveolin and NCX in a model proposed based on electron microscopy studies (Fameli *et al.*, 2007). Caveolin-1 and caveolin-3 are present in vascular smooth muscle cells with caveolin-1 expressed in arteries and veins (Song *et al.*, 1996). Little is known about the physiological involvement of caveolins in vascular smooth muscle cell function. There is some evidence showing NCX is enriched in caveolae in smooth muscle. In cardiac sarcolemmal vesicles composed of smooth muscle cells, endothelial cells, and myocytes, NCX1 and caveolin-3 co-precipitated and disruption of caveolae with methyl-β-cyclodextran diminished the co-precipitation (Bossuyt *et al.*, 2002). In the same model, NCX1 also co-fractionated with caveolin-3 on sucrose density gradients. Altogether, the above observations confirm the presence of a functional linkage, as well as possibly a spatial linkage, between NCX and SERCA. The spatial linkage may involve caveolae, however this has yet to be fully elucidated in smooth muscle.

The third theory supporting the NCX-SERCA linkage is that it may serve some physiological purpose (see section 1.4 for more detail). 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 regenerative Ca^{2+} release (van Breemen, Chen, Laher, 1995; Lee *et al.,* 2001; Nazer and van Breemen, 1998; Fameli, van Breemen and Kuo, 2007). This underlies agonist induced asynchronous Ca^{2+} oscillations in vascular smooth muscle (Fameli, van Breemen and Kuo, 2007). The literature supports the proposal that the NCX-SERCA linkage in SMC is to regulate the SER Ca^{2+} levels (Blaustein and Lederer, 1999).

Overall, there is sufficient evidence for a linkage between NCX and SERCA; however, other processes may also participate. For instance, store operated Ca^{2+} channels may also be associated with NCX activity, especially in the Ca^{2+} entry mode (Arnon, Hamlyn and Blaustein, 2000; Zhang *et al.,* 2005).

4.0 EXPERIMENTAL MODEL

Our laboratory has used pig as a model animal since 1983. We have extensive experience using this model, particularly in Ca^{2+} related studies. This model is a good model to use because the cardiovascular anatomy and physiology is similar to humans. In addition, large numbers of fresh arteries and replicates can be obtained, unlike in a human model, because of routine availability from a large abattoir (Maple Leaf Meats, Burlington, ON).

5.0 OVERALL OBJECTIVE

The primary function of vascular smooth muscle is the generation of tone which regulates blood flow. An increase in $Ca^{2+}{}_i$ leads to smooth muscle contraction, therefore its level has to be tightly regulated. Ca^{2+} transporters play a key role in this regulation. The high affinity pumps SERCA and PMCA keep $[Ca^{2+}]_i$ levels low. NCX may work to expel Ca^{2+} or bring it into the cell. The exchanger has been proposed to play a role in refilling the SER Ca^{2+} pool. We have previously shown that NCX and SERCA are functionally linked in pig coronary artery smooth muscle (Davis *et al.*, 2008). Although the nature of the linkage has yet to be identified, we believe this linkage is crucial to smooth muscle Ca^{2+} regulation. The function of NCX and SERCA may be influenced by their subcellular localization. The overall objective of this thesis was to determine whether NCX and SERCA proteins were co-localized in pig coronary artery smooth muscle. The membrane invaginations termed caveolae (lipid rafts containing caveolin) have been suggested to play a role in directing Ca^{2+} movements during cell signalling. Thus, our hypothesis was that NCX is localized on caveolae which brings the PM in close proximity to the superficial SER (Figure 2). To test this hypothesis, NCX and SERCA co-migration in detergent treated membranes using sucrose density gradient centrifugation was examined.

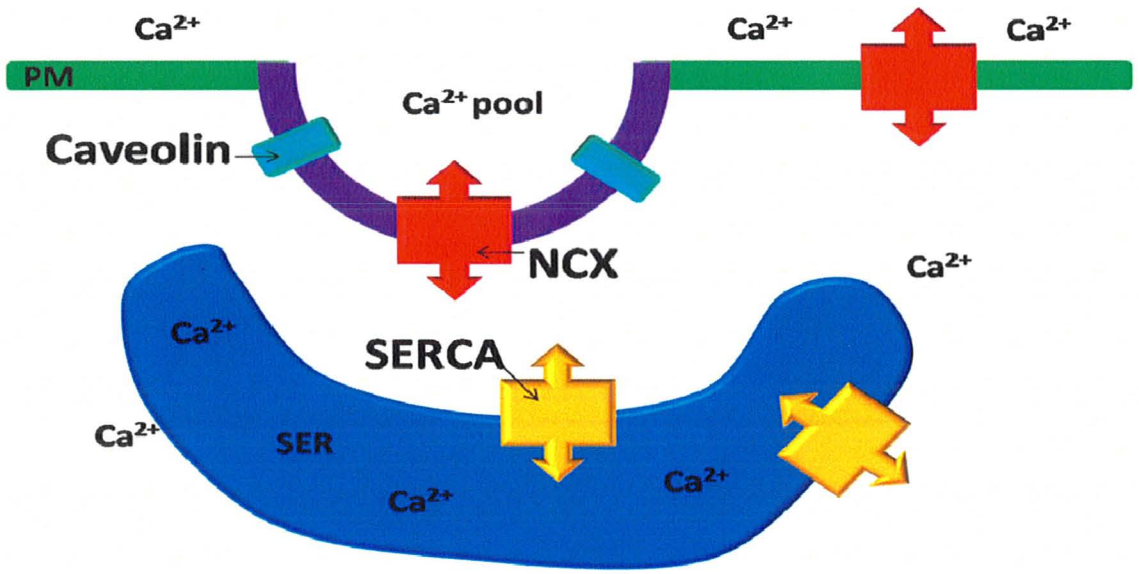

Figure 2. Proposed model for linkage between SERCA, NCX and caveolin. NCX: Na^{+}-Ca^{2+}-exchanger, SERCA: sarco/endoplasmic reticulum Ca^{2+} pump, PM: plasma membrane, SER: sarco/endoplasmic reticulum

6.0 METHODS

6.1 Materials:

Bovine Serum Albumin (BSA), imidazole, ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA), ethylenediamine tetra-acetic acid (EDTA), dithiothreitol (DTT), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), phenylmethylsulfonylfluoride (PMSF), N,N,N',N'-Tetramethyl-1-,2-diaminomethane (TEMED) and TWEEN®20 (P5927) were obtained from Sigma Aldrich (St Louis, Missouri, USA). Polyvinylidene fluoride (PVDF) membranes and nitrocellulose membranes were purchased from Pall Life Sciences (Pensacola, FL, USA). All other chemicals were purchased from the standard commercial sources. Sources of antibodies are listed in Table 4.

6.2 Preparation of Pig Coronary Arteries and Isolation of Smooth Muscle

Fresh pig hearts (typically 60) were obtained from the abattoir and immediately placed in an ice cold physiological salt solution (PSS; see Table 2 for buffer composition). The left anterior descending coronary artery was dissected from each heart and placed in a HEPES-Krebs' solution on ice (Table 2). The endothelium, fat, cardiac muscle and adherent connective tissue were then removed and the artery was placed in a beaker of homogenization solution (Table 2) on ice until the next stage. The smooth muscle was removed from the artery and placed in fresh homogenization solution. The smooth muscle was finely chopped and run through the sieve-press twice using first 0.5 mm and then 0.3 mm pore sizes. The tissue was next suspended in a small volume of the

homogenization solution and homogenized using a motor driven teflon-glass homogenizer and again with a homogenizer with a clearance of 0.1-0.3 mm.

6.3 Crude Microsome Isolation and Cellular Subfractionation

Crude microsomes were isolated from smooth muscle as described previously with minor adjustments (Grover, Samson and Lee, 1985). Briefly, the ground tissue (Methods 6.2) was transferred to 50 ml centrifuge tubes and centrifuged at 10,000 x g for 10 min at 4°C to remove the nuclear and mitochondrial fragments. The supernatant (postnuclear and postmitochondrial) was saved and the pellet from this step was re-extracted using the motor driven homogenizer to obtain another supernatant. The two supernatants were pooled and filtered through a cheese cloth. To prevent aggregation of microsomes, this supernatant was mixed with more KCl to give a final concentration of 700 mM and stirred at 4°C for 15 min. Then, the sample was centrifuged for 30 min at 388,000 x g at 4°C. The supernatant was discarded and the pellet (microsomes) was suspended in a solubilization buffer (Table 2). The suspension underwent a second microsomal spin (388,000 x g for 20 min). The resulting pellet (microsomes) was suspended in a minimal volume (<1ml) of the solubilization buffer. A specified amount of suspended microsomes (MIC) was used for further fractionation (Methods 6.5) and the remainder was aliquoted and stored at -20°C until needed.

6.4 Solubilization of Microsomes with Detergent or High pH and Ultrasonication

MIC (Methods 6.3) of specified concentrations were either solubilized with a non-

ionic detergent (Triton X-100) or with high pH and ultrasonication. Control MIC were not treated with either method. For detergent solubilization, MIC were mixed by gentle rotation for 1 h at 4°C with a buffer containing 150 mM NaCl, 15 mM imidizole-HCl (pH = 7.0) as well as 10 mg/ml of Triton X-100. Solubilization with the second method consisted of treating the MIC with 250 mM sodium carbonate (pH = 11) and subjecting them to three cycles of sonication at 80 % power in 20 s bursts (on ice) using a Fisher Scientific Model 550 sonicator (Pittsburgh, PA, USA). In between each cycle, samples were put on ice for a 1 min break. These samples were then neutralized with 15 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer containing 88 mM NaCl (pH = 6.5). The treated and control MIC were then centrifuged at 388,000 x g for 30 min and the supernatant and pellet were collected and further characterized for protein distributions. In both methods, the pellets were resuspended in sample buffer (without bromophenol blue; Table 2) in the same volume MIC were solubilized in prior to centrifugation. In subsequent optimization of the detergent method, the ratio of Triton X-100 to microsomal protein was varied.

6.5 Sucrose Density Gradient Centrifugation

MIC (Methods 6.3) were diluted to a final concentration of 12 mg protein/ml with Triton X-100 (final 1.5 mg/ml) and mixed by gentle rotation for 30 min at 4°C. Then, 600 µl of 60 % sucrose (weight/weight) was added to 300 µl of the treated MIC to obtain a uniform suspension in 40 % sucrose (w/w). This was pipetted into the bottom of a swinging bucket rotor gradient tube and a gradient of sucrose concentrations were layered gently atop in the following order: 37, 34, 31, 29, 25, 20, and 15% (w/w). The gradients

were centrifuged at 278,000 x g for 20 h at 4°C in a swinging bucket rotor with gentle acceleration and deceleration. Fractions were collected starting from the top and protein and sucrose (measured with a refractometer) concentrations of each were then determined. The remainder was aliquoted and stored at -20°C for further characterization.

Table 2. Buffer Compositions

BUFFER	FINAL COMPOSITIONS (mM)
HEPES-Krebs'	116 NaCl, 11 glucose, 11 HEPES, 2 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4 and 1.7 $CaCl_2$, pH = 6.4
PSS	134 NaCl, 6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose, pH = 6.4 at 25°C
Reaction Buffer	100 KH_2PO_4 (pH = 7.4), 50 NaCl, 5 cholic acid, 0.1% Triton X-100
Running Buffer	25 TRIS, 192 glycine (volume/volume), 0.1% sodium dodecyl sulfate (SDS; v/v), pH = 8.3
Sample Buffer	125 TRIS-HCl (pH = 6.8), 7.5% SDS (v/v), 25% glycerol (v/v), dash of Bromophenol Blue
Solubilization Buffer	150 NaCl, 15 imidazole-HCl (pH = 7.0), 0.05 EGTA and 0.25 DTT
Homogenization Solution	8% sucrose (w/w), 1 PMSF, 2 DTT, 100 KCl, 0.05 EGTA
Transfer Buffer	10% methanol, 25 TRIS, 192 glycine (v/v), 0.1% SDS, pH = 8.3
TRIS Buffered Saline (TBS)	10 TRIS-HCl (pH = 7.4), 140 NaCl
TBS Tween	10 TRIS-HCl (pH = 7.4), 140 NaCl, 0.1% Tween®20 (v/v)
Working Solution	0.15 Amplex Red reagent, 1 U/mL HRP, 1 U/mL cholesterol oxidase and 0.2 U/mL cholesterol esterase, made in Reaction Buffer

6.6 Electrophoresis and Western Blots

Protein samples were diluted with 4X sample buffer (Table 2). Equal amount of protein from each gradient fraction and specified amounts of control MIC were then separated on SDS gels containing 7.5 % polyacrylamide separating gel (Table 3). For caveolin-1 detection, 12 % gels were used and samples were boiled at 95 °C for 10 min before separation. Both gels had a 5 % stacking gel component (Table 3). The gels were run at 80 V for ~3 h. The Spectra^{TM} Multicolor Broad Range Protein Ladder (Fermentas, Burlington, ON) was also loaded on each gel as a molecular weight marker. Proteins were then transferred electrophoretically (100 V) to PVDF membranes in Transfer Buffer (Table 2) for 2.5 h.

Table 3. Gel Components

GEL TYPE	FINAL COMPOSITIONS
7.5% Separating Gel	7.5% Bis-acrylamide, 750 mM TRIS-HCl (pH= 8.8), 0.1 % SDS (v/v), 0.15 % TEMED, 0.07 % ammonium persulfate
12% Separating Gel	12% Bis-acrylamide, 750 mM TRIS-HCl (pH= 8.8), 0.1 % SDS (v/v), 0.15 % TEMED, 0.07 % ammonium persulfate
5% Stacking Gel	5% Bis-acrylamide, 125 mM TRIS-HCl (pH= 6.8), 0.1 % SDS (v/v), 0.3 % TEMED, 0.06 % ammonium persulfate

Membranes were blocked for 1 h in 3% (w/v) non-fat dry milk in filtered TBS-Tween (Table 2) at 25°C, and then incubated with the appropriate primary antibody for 1

h at 25°C. Sources and selectivity of the various antibodies are in Table 4. Following 1 h of washing every 10 min with filtered TBS-Tween, blots were then incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse immunoglobulin G (IgG) (Sigma, St. Louis, Missouri, USA) for 1 h at 25°C at a 1/20,000 dilution. HRP conjugated donkey anti-rabbit IgG (Sigma, St. Louis, Missouri, USA) at a 1/10,000 dilution was used for detection of anti-myosin. After another 1 h of washing every 10 min, the peroxidase activity was then visualized with a femto-kit (Pierce Chemical Company, Rockford) and an enhanced chemiluminescence detection system - LAS-3000 mini Luminescent Image Analyzer (Fujifilm Life Sciences, Stamford, CT).

6.7 Protein Estimation

Protein estimation was carried out with Bradford reagent (BioRad, Mississauga, ON). In a 96 well plate (Sarstedt, Montreal, Canada), 10 µl of the respective sample 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 min and the absorbancy at 595 nm determined with a 96 well plate reader. The concentration of the protein was estimated by interpolation of a standard curve using 0.5 – 4.0 µg bovine serum albumin.

6.8 Cholesterol Estimation

Cholesterol content of samples was determined fluorometrically using an Amplex Red Cholesterol Assay Kit (A12216) following instructions of the manufacturer

(Invitrogen, Burlington). In a 96 well black plate (Sarstedt, Montreal), 50 µl of the respective sample containing 0.5 µg protein was placed. In each well, 50 µl of a 2X working solution (Table 1) was added. After approximately 5-10 min, the fluorescence was monitored (excitation at 530/525 nm and emission at 590/595 nm). The concentration of the cholesterol was estimated by interpolation of a standard curve using 0.2-4 µM cholesterol. Reaction Buffer with no cholesterol was used to correct for background fluorescence. All samples and solutions were made or diluted with Reaction Buffer (Table 1).

Table 4. Sources and Selectivity of Primary Antibodies Used

Protein	Antibody	Mono/Poly-clonal (M/P)	Immuno-globulin Type	Dilution	Source
Actin	mouse anti-actin, α smooth muscle (1A4)	M	IgG2a	1/5000	Sigma, St. Louis, Missouri, USA
Caveolin-1	mouse anti-caveolin-1 (2297)	M	IgG1	1/1000	BD Biosciences, Mississauga, Canada
Clathrin	mouse anti-clathrin heavy chain (23)	M	IgG1	1/2000	BD Biosciences, Mississauga, Canada
Flotillin-2	mouse anti-flotillin-2/ESA (29)	M	IgG1	1/2000	BD Biosciences, Mississauga, Canada
Myosin	rabbit anti-smooth muscle myosin heavy chain 2	P	IgG	1/500	Abcam Inc., Cambridge, MA, USA
NCX1	mouse anti-NCX1 (R3F1)	M	IgG1	1/1000	Swant Swiss Antibodies, Bellinzona
PrP	mouse anti-prion protein (6H4)	M	IgG1	1/1000	Prionics AG, Switzerland
SERCA2	mouse anti-SERCA2 (IID8)	M	IgG1	1/2000	Affinity Bioreagents, Golden Co., USA

6.9 Gangliosides Estimation

For ganglioside GM1 estimation, 5 µl (containing 0.2 and 0.5 µg protein replicates) of each sample including control MIC was spotted onto a nitrocellulose sheet. The sheet was allowed to dry and then kept at -20°C until further processed. The blot was blocked with 3% (w/v) non-fat dry milk in filtered TBS-Tween for 1 h. The blot was then

incubated with Cholera Toxin, Subunit B, conjugated to HRP (Sigma, St. Louis, Missouri, USA) at a 1/15,000x dilution for 1 h. The membrane was washed with filtered TBS-Tween for 1 h, every 10 min. Dot blots were visualized with a femto-kit and a LAS-3000 mini Luminescent Image Analyzer.

7.0 Data and Statistical Analysis:

Relative band (pixel) intensities from Western blots and dot blots were quantified using Multi Gage v3.0 software (Fujifilm Life Sciences, Stamford). The relative abundance of each protein amongst the sucrose gradient fractions are presented as percentages of the specific protein activity (Intensity obtained in specific fraction/ maximum intensity obtained * 100). The Western and dot blotting was repeated 2-4 times for each marker and the mean ± SEM values are shown for one representative experiment. From 5 different experiments with fresh microsomes isolated each time, the averaged relative intensities from the Western blots were pooled for each pair of markers (i.e. NCX1 to SERCA2, NCX1 to caveolin-1, SERCA2 to caveolin-1, etc). Pearson's product-moment correlation coefficient and Spearman's rank correlation coefficient between these paired values were then analyzed for covariation/correlation using GraphPad InStat (San Diego, USA). If the obtained two-tailed P value was < 0.05, the correlations were considered significantly different than zero.

8.0 RESULTS

The overall objective of this thesis was to determine whether NCX, SERCA and caveolin proteins were co-localized in pig coronary artery smooth muscle. This was investigated by examining the co-migration of these proteins in detergent treated microsomal membranes upon sucrose density gradient centrifugation.

8.1 Initial experiments for sucrose density gradients

Initial experiments were conducted to resolve two issues: the appropriateness of antibodies to be used and the conditions to be used for sucrose density gradient fractionation.

8.1.1. Determining linearity of antibody signals

We tested the linearity between signal intensities of selected antibodies to the amount of MIC protein loaded in Western blots. A major band at the expected molecular weight was obtained for NCX1 (116 kDa), SERCA2 (110 kDa), and caveolin-1 (24 kDa) (Fig. 3). The three antibodies also gave less prominent minor bands (Fig. 3). The signal intensities of the major bands and the amount of protein increased linearly with an r^2 = 0.997 for NCX1, r^2 = 0.970 for SERCA2 up to 2 μg protein, and r^2 = 0.996 for caveolin-1 up to 10 μg protein. Linearity with the amount of protein loaded was also tested for other markers (flotillin-2, clathrin, myosin, prion protein) to be used in Western blots (Appendix A1), and for assays for cholesterol and gangliosides (data not shown). In all

further experiments, the protein amounts used were in the linear range for the intensities of the signal obtained and only main bands were analyzed.

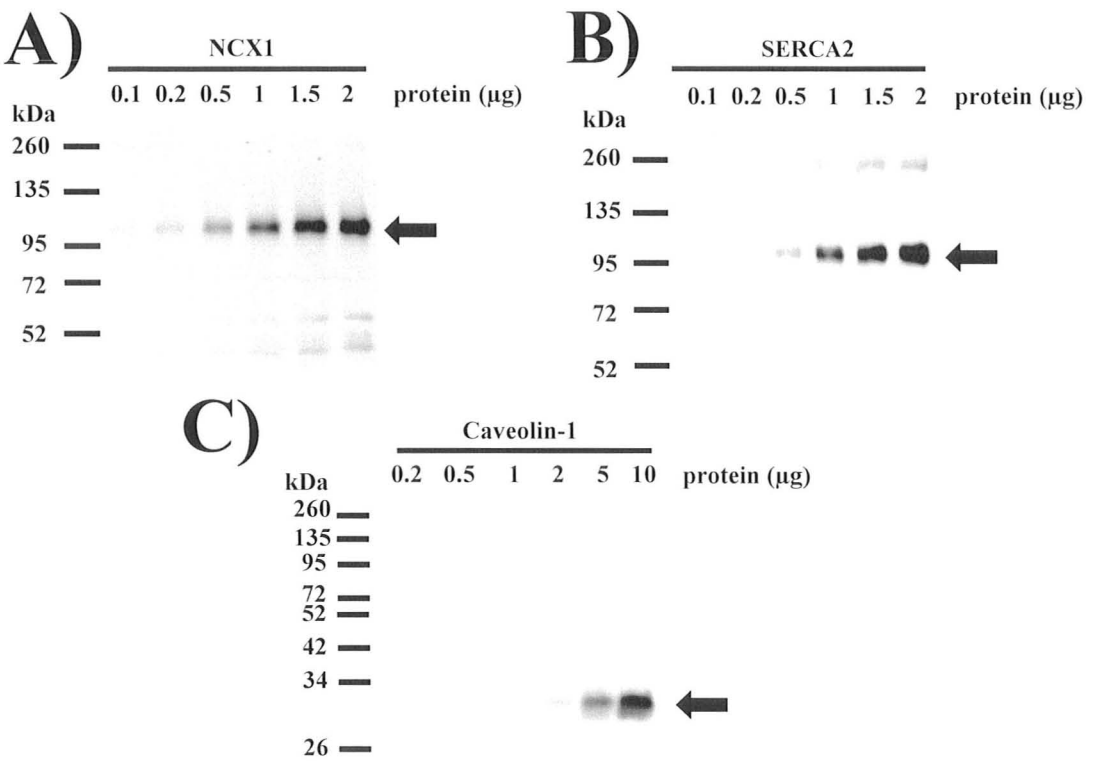

Figure 3. Protein concentration dependence for NCX1, SERCA2, and caveolin-1 detection. Western blots from full gels are shown for A) NCX1, B) SERCA2, and C) caveolin-1 with specified amounts of MIC protein (µg) loaded in different lanes. Molecular weights in kDa are shown on the left of each blot. The signal intensity of the main bands (marked by black arrows) increased linearly with the amount of protein loaded with an $r^2 = 0.997$ for NCX1, $r^2 = 0.970$ for SERCA2, and $r^2 = 0.996$ for caveolin-1.

8.1.2. Justification of using detergent as method of solubilization

In literature, two main methods have been used to examine caveolin distribution with sucrose density gradients: high pH with ultrasonication and non-ionic detergents.

Since the distributions of NCX1, SERCA2 and caveolin-1 were of highest interest, testing was done to determine if detergent or ultrasonication would affect these proteins in the microsomes (Methods 6.4). High pH and ultrasonication did not alter the NCX1 or caveolin-1 proteins, but caused oligomerization of SERCA2 when 2 mg protein/ml MIC was used (Fig. 4). All three proteins remained mainly insoluble with this method. Alternatively, treatment with 10 mg/ml of the non-ionic detergent Triton X-100 solubilized most of NCX1, SERCA2 and caveolin-1 (Fig. 4). When MIC were not treated with either detergent or ultrasonication, the three proteins remained insoluble (Appendix A3). Similar results were obtained with 4 and 1 mg protein/ml MIC as well as when other markers were tested (Appendix A2). The high pH and ultrasonication method was thus deemed unsuitable and the detergent method was used in all subsequent experiments. Next, we optimized the detergent to amount of microsomal protein ratio to use.

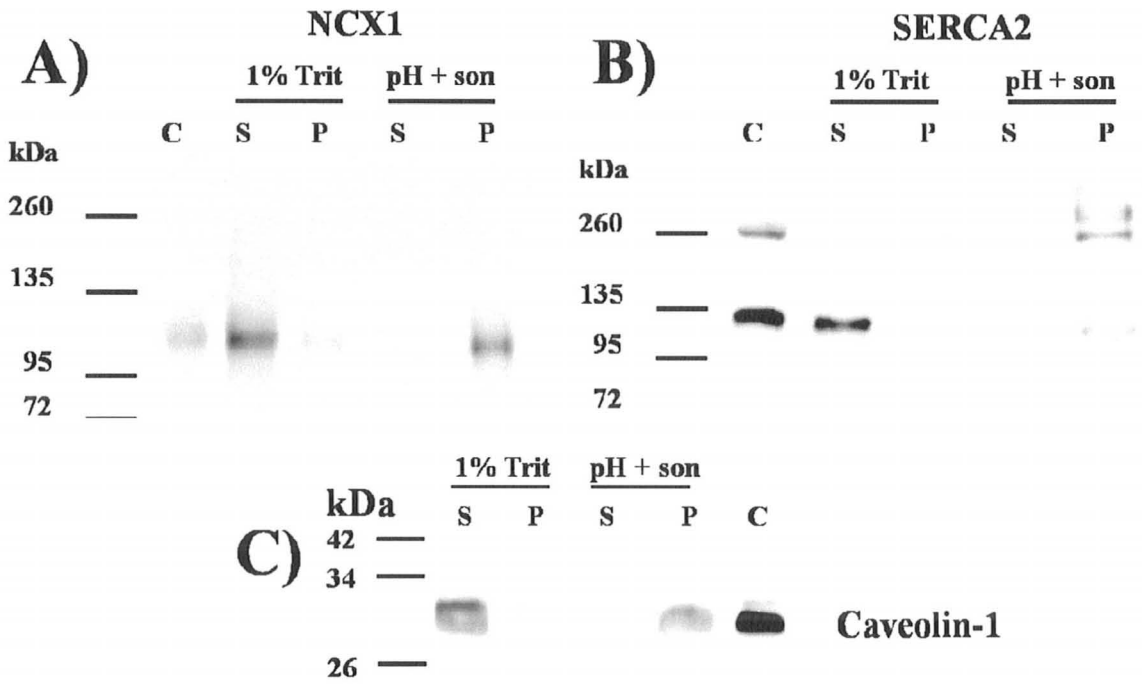

Figure 4. Effect of detergent treatment or high pH and ultrasonication on NCX1, SERCA2, and caveolin-1. 2 mg protein/ml MIC were treated with 10 mg/ml Triton X-100 or high pH and ultrasonication. The effects on A) NCX1, B) SERCA2, and C) caveolin-1 are shown. High pH and ultrasonication did not solubilize either NCX1, caveolin-1 or SERCA2, but did cause oligomerization of SERCA2. 10 mg/ml Triton X-1 solubilized most of NCX1, caveolin-1 and SERCA2. Equal volumes of the supernatant (S) and pellet (P) were loaded in each lane. The amount of control (C) MIC protein loaded was 20 μg for NCX1, 7 μg for SERCA2, and 10 μg for caveolin-1. Molecular weights in kDa are shown on the left of each blot.

8.1.3. Optimization of detergent to microsomal protein ratio

In order to use the detergent solubilization method prior to sucrose density gradient centrifugation, the ratio of Triton X-100 to microsomal protein was optimized (following Method 6.4). Extremely high detergent to protein ratios solubilized most of NCX1 and SERCA2 and very low concentrations did not solubilize either marker

(Appendices A3-A4). Thus, the solubility of NCX1 was similar to SERCA2 in these experiments (Appendices A3 and A4). Alternatively, caveolin-1 was not solubilized even with high detergent to protein ratios (Appendices A3-A4). When microsomes were treated without detergent as a control, all three markers remained in the pellet hence were insoluble (Appendix A4). Half of NCX1 was solubilized when treated for 30 min at 1.5 mg/ml Triton X-100 at a final MIC concentration of 12 mg protein/ml (Fig. 5). This ratio was used in all subsequent experiments.

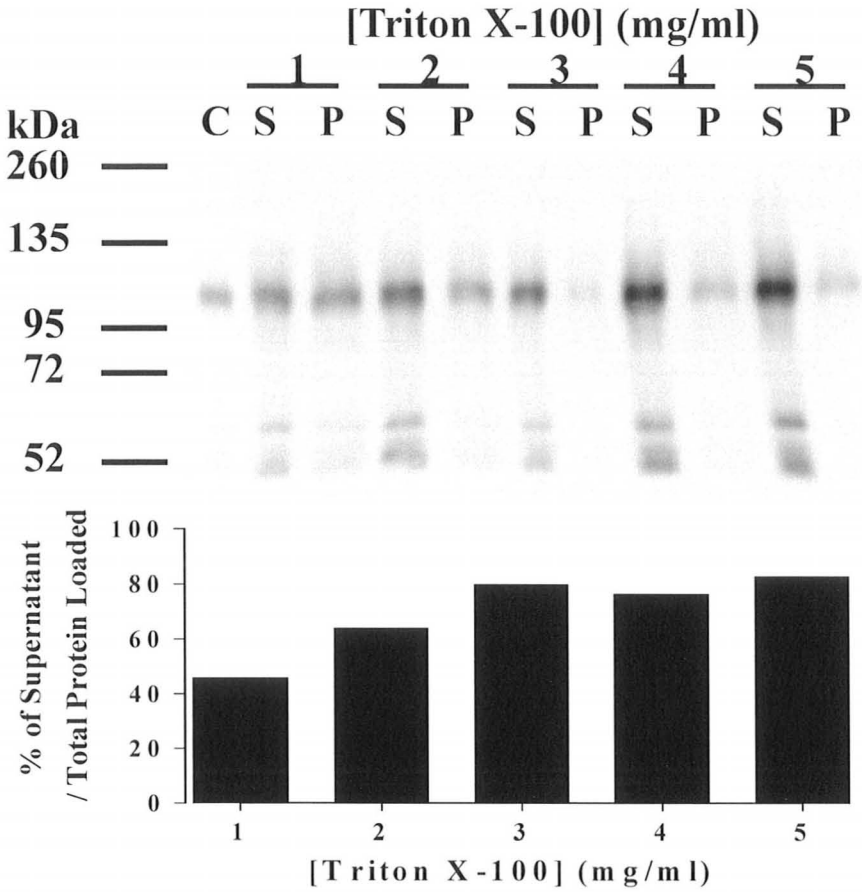

Figure 5. Triton X-100 solubilization of NCX1. The effect of treating MIC (12 mg protein/ml) with various concentrations of Triton X-100 (indicated above lanes) for 30 min on NCX1 is shown in the Western Blot. Equal volumes of the supernatant (S) and pellet (P) were loaded in each lane. The protein amount of control (C) MIC loaded was 15 μg. Molecular weights in kDa are shown on the left of each blot. The graph shows the amount of protein solubilized as a percent of the total protein loaded (S/(S+P)*100). Approximately 50 % of NCX1 was solubilized with 1.5 mg/ml Triton X-100.

8.2 Distribution of NCX1, SERCA2 and markers in sucrose density gradient fractions

In each experiment, MIC were solubilized and separated into fractions by flotation in a sucrose density gradient. The fractions were collected from the top (lowest sucrose concentration) and examined for concentrations of sucrose, protein and cholesterol. The fractions were examined in Western blots for relative amounts of NCX1 and SERCA2 along with caveolin-1 and lipid raft markers flotillin-2 and prion protein. Non-lipid raft proteins actin, myosin and clathrin were also examined. Ganglioside amounts were determined in dot blots. Fig. 6 shows the characterization of the fractions from one representative experiment. The top fraction that had the lowest sucrose concentration had the lowest protein concentration. The bottom fraction, which had the highest sucrose concentration, also had the highest protein concentration. Various parameters were determined with each using a fixed amount of protein from individual fractions. In this determination, cholesterol content was the highest in the fraction with the lowest sucrose concentration and it decreased monotonically with the increasing sucrose concentration. Similar to cholesterol, the other lipid raft markers caveolin-1, flotillin-2, gangliosides, and prion protein were more abundant in the fractions containing low sucrose concentrations (20-27 %) than in those with the higher sucrose concentrations. In contrast, the negative markers actin, clathrin and myosin, were most abundant in the bottom fractions (35-44 % sucrose). NCX1 and SERCA2 appear to be the most abundant in the fractions containing 20-27 % sucrose, consistent with the lipid raft markers. Similar results were obtained when the experiment was replicated on five different days with fresh MIC isolated for each experiment.

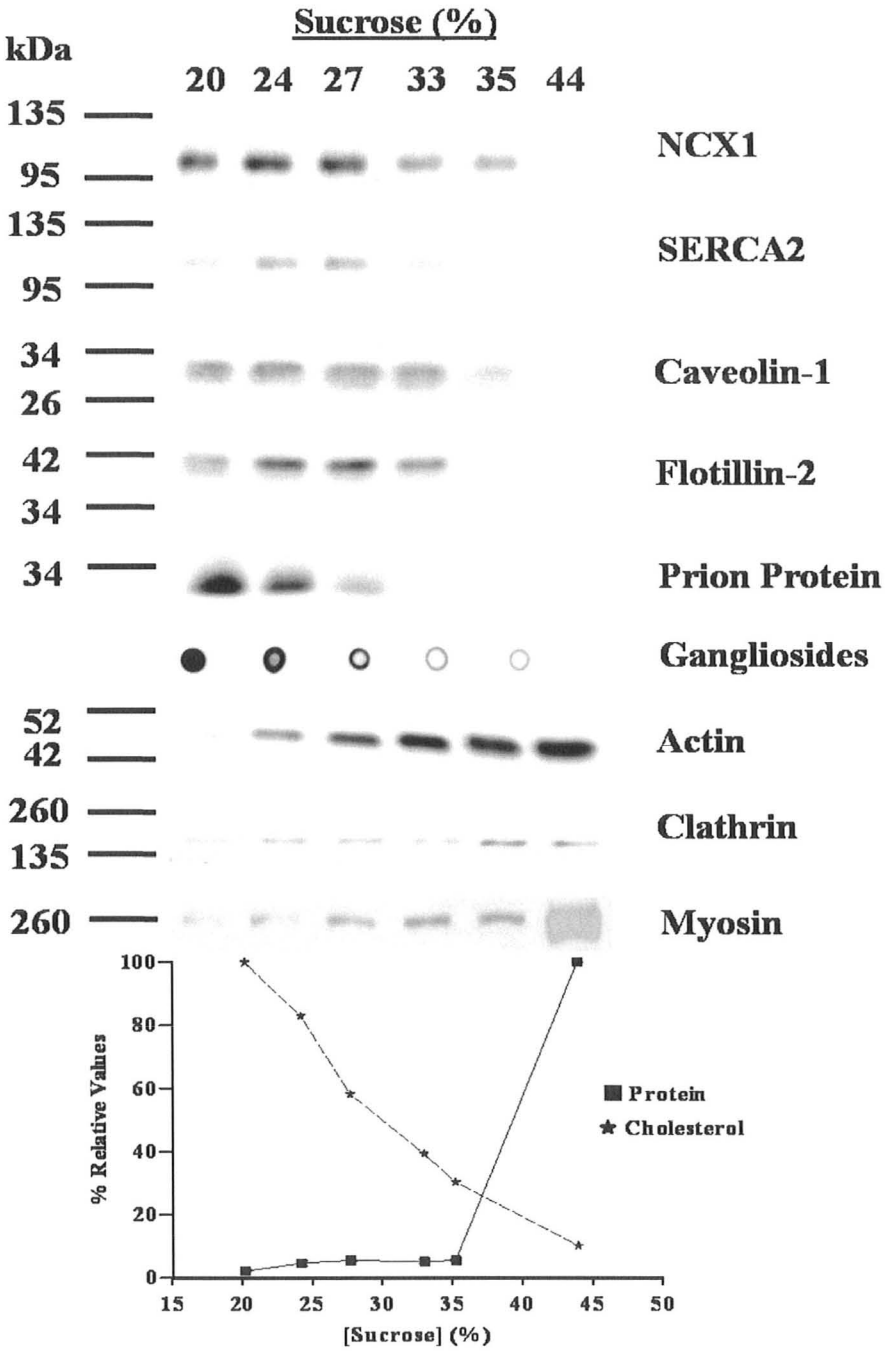

Figure 6. Characterization of different markers in gradient fractions. For Western blots, the amounts of protein loaded in μg/well was 2 for NCX1, and myosin detection; 1 for SERCA2 and caveolin-1; 0.5 for flotillin-2 and prion protein; 0.2 for actin and clathrin. Only the main bands for each of the proteins are shown with molecular weights in kDa on the left. Dot blots with 0.2 μg protein/spot were used for ganglioside detection. In all the instances, the protein amounts used were in the linear range for the intensities of the signal obtained. The graph shows protein and cholesterol amounts in the sucrose density gradient fractions. Relative amounts of protein concentration/ fraction were determined taking the value in the last fraction as 100 %. Relative amounts of cholesterol/μg protein were determined taking the value in the first fraction as 100 %.

The data from Fig. 6 were plotted to show the relative density distributions of NCX1, SERCA2 and the various markers in fractions of different sucrose densities. For each protein, the values are expressed as percent of the fraction with the highest intensity (% specific activity). There was a considerable overlap in the distribution of NCX1 and SERCA2; both proteins had the highest relative activity in fractions containing low sucrose (24-27 %) (Fig. 7A). The NCX1 distribution partially overlapped with caveolin-1 and flotillin-2 and less so with gangliosides, cholesterol and prion protein (Fig. 7B, C). Caveolin-1 distribution was more widespread peaking in fractions containing 27-33 % sucrose, while the other lipid raft markers distinctively peaked in the 20% sucrose fraction. There was very little overlap between the NCX1 distribution and the following proteins: actin, myosin and clathrin (Fig. 7D). These markers had the highest relative distributions in the bottom fractions containing 44% sucrose.

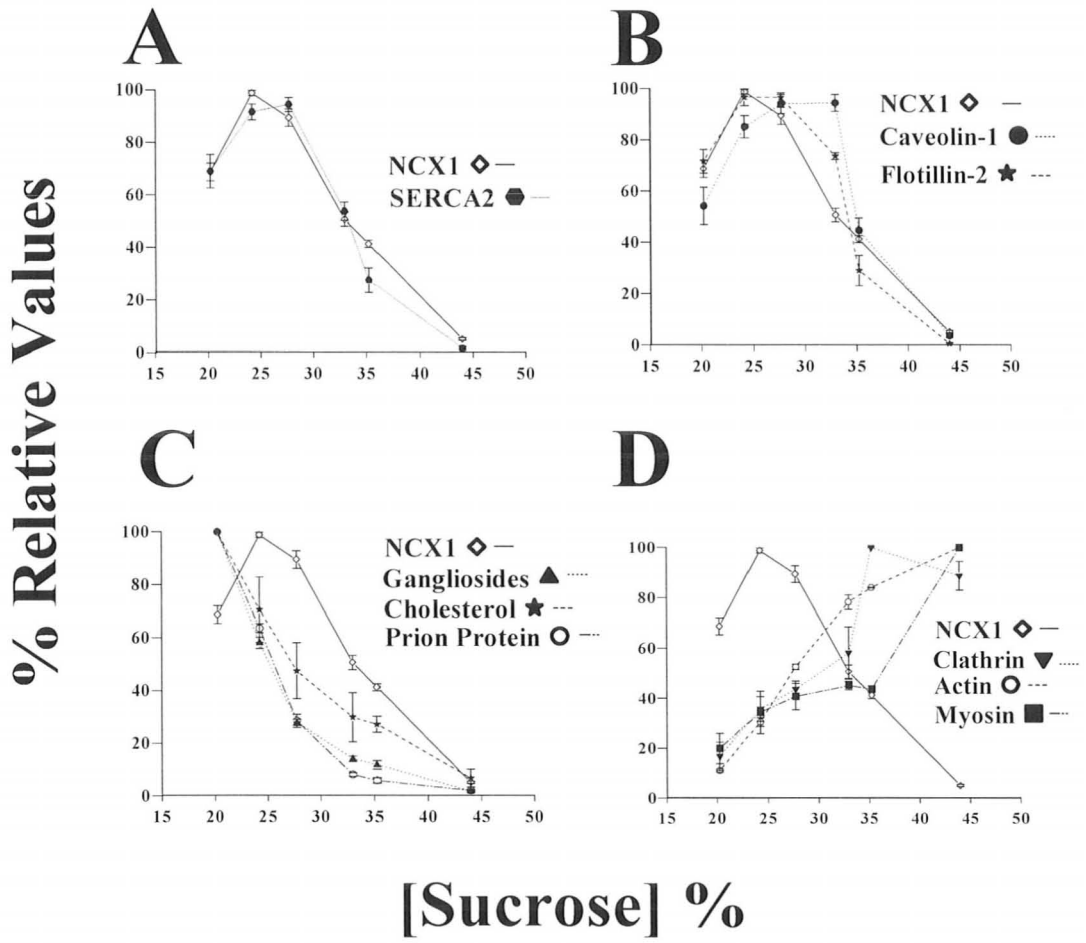

Figure 7. Analysis of distribution of immunoblot intensities in sucrose density gradients. A) NCX1 and SERCA2, B) NCX1, caveolin-1 and flotillin-2, C) NCX1, gangliosides, prion proteins and cholesterol, and D) NCX1, actin, clathrin, and myosin. The values are expressed as % specific activity. The amounts of protein loaded (μg) per well were the same in all fractions in each gel. Between gels, the amounts ranged from 1-2 μg/well for NCX1, 0.5-1 for SERCA2, 0.8-1 for caveolin-1, 0.5 for flotillin-2 and prion protein, 2-3 for myosin, 0.2 for actin, and 1-4 for clathrin. For cholesterol and gangliosides determination, 0.5 μg/well and 0.2 μg/spot were used, respectively. The Western and dot blotting was repeated 2-4 times for each marker and mean ± SEM values are shown.

8.3 Correlation between distributions of markers in sucrose density gradient fractions

Fig. 8 summarizes the Pearson's correlation coefficient (r) of the NCX1 distribution as % specific activity against the various markers pooled from five separate experiments. The correlation (r) was positive and significant for SERCA2 (0.710), as well as the following markers caveolin-1 (0.785), flotillin-2 (0.922), gangliosides (0.600) and cholesterol (0.707) ($p < 0.05$ for all pairs). The correlation was negative and significant for actin (- 0.574) and myosin (- 0.453) ($p < 0.05$). There was no significant correlation between NCX1 and prion protein (0.385) or clathrin (- 0.162) distributions ($p > 0.05$). Similar results were obtained when analyzing for Spearman correlation, except for prion protein which had a positive but significant correlation (Appendix A5).

Pearson and Spearman correlations for the distributions of all other markers relative to each other were also examined, i.e. SERCA2 with caveolin-1, flotillin-2, actin, etc. For comparison between these observed correlations and for simplicity, the correlations for each marker were ranked from 1 through 9, with 1 being the most positive value. Rank orders derived from the Pearson correlations are in Table 5 while those derived from Spearman correlations are in Table 6. Actual values are given in Appendices A6 and A7. The rank orders of NCX1, SERCA2, and caveolin-1 distributions (as % specific activity) to all other markers were the same with both types of correlation analysis, further validating the above results. From Fig. 8 and Tables 5 and 6, the distribution of NCX1 correlated strongest with the following proteins: flotillin-2 > caveolin-1> SERCA2. Flotillin-2 ranked the highest with that of NCX1, second with caveolin-1, and third with cholesterol. Interestingly, SERCA2 distribution correlated

strongest with caveolin-1 > NCX1 > flotillin-2. Caveolin-1 was the strongest with SERCA2, followed by flotillin-2, then NCX1. The other lipid raft markers gangliosides, prion protein and cholesterol correlated strongly with each other and with flotillin-2, and less so with caveolin-1. Actin, myosin, and clathrin also strongly and positively correlated with each other. These markers negatively correlated with all other proteins including NCX1, SERCA2, and caveolin-1.

Overall, the results showed that NCX1 co-migrated with SERCA2 and caveolin-1 but the correlations were not perfect. This indicates that NCX1 may be localized in domains close to the SER and caveolae but also in additional regions in the PM.

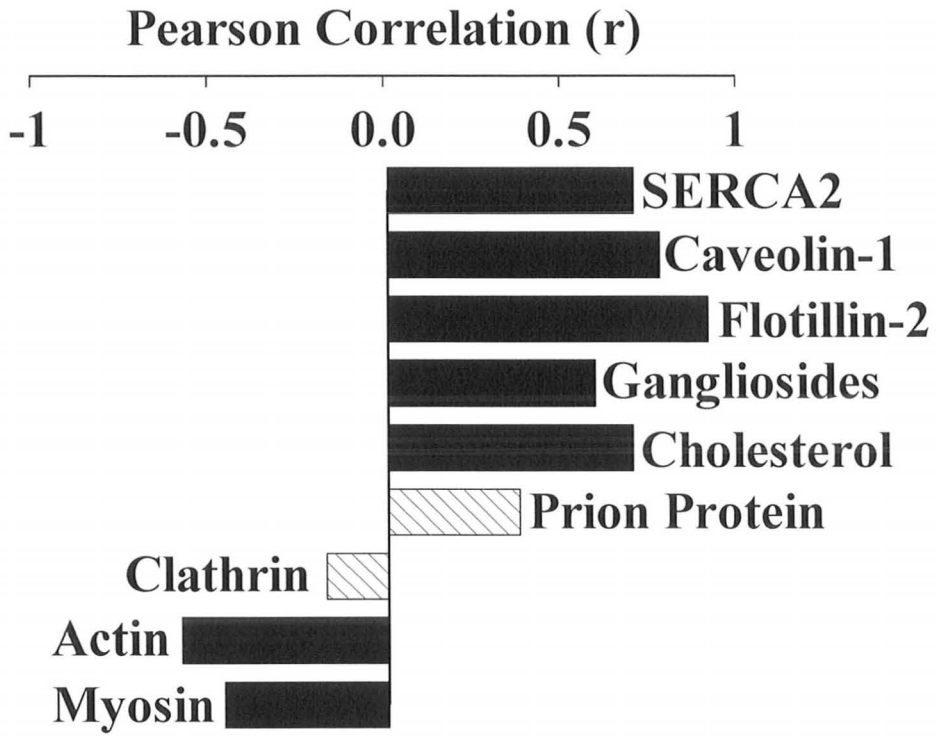

Figure 8. Pearson's correlation between distributions (as % specific activity) of NCX1 and various proteins in the sucrose density gradient experiments. A total of 40 paired values of intensities of NCX1 and SERCA2 were obtained in 5 separate experiments similar to Fig. 7 and the Pearson's correlation (r) was determined. Similarly, NCX1 data were also paired with other proteins and analyzed (34 pairs for myosin, 18 for prion protein, and 40 pairs for all other markers). Solid bars indicate that the correlation was significantly different than zero ($p < 0.05$).

Table 5. Rank order of marker distributions (as % specific activity) according to Pearson correlations

	NCX	SERCA	Cav	Flot	Gang	Chol	PrP	Clath	Actin	Myo
NCX1	--	3	2	1	5	4	[6]	[7]	9	8
SERCA2	2	--	1	3	4	[5]	[6]	[7]	9	8
Caveolin-1	3	1	--	2	4	5	[6]	[7]	9	8
Flotillin-2	1	5	2	--	4	3	6	7	9	8
Gangliosides	4	6	5	3	--	2	1	7	9	8
Cholesterol	4	[6]	5	3	2	--	1	7	9	8
Prion Protein	[4]	[6]	[5]	3	2	1	--	7	8	9
Clathrin	[4]	[3]	[5]	6	7	8	9	--	2	1
Actin	4	3	5	6	7	8	9	2	--	1
Myosin	4	3	7	6	5	8	9	2	1	--

- indicates a negative correlation

[#] - indicates correlation was not significant ($p > 0.05$)

[#] - indicates negative correlation was not significant ($p > 0.05$)

Table 6. Rank order of marker distributions (as % specific activity) according to Spearman correlations

	NCX	SERCA	Cav	Flot	Gang	Chol	PrP	Clath	Actin	Myo
NCX1	--	3	2	1	5	4	6	[7]	9	8
SERCA2	2	--	1	3	4	5	[6]	[7]	9	[8]
Caveolin-1	3	1	--	2	4	5	[6]	[7]	9	8
Flotillin-2	1	6	2	--	5	3	4	7	9	8
Gangliosides	4	6	5	3	--	2	1	8	9	7
Cholesterol	4	6	5	3	2	--	1	7	9	8
Prion Protein	4	[6]	[5]	3	1	2	--	7	8	9
Clathrin	[3]	[4]	[5]	6	8	7	9	--	1	2
Actin	5	3	4	6	7	8	9	2	--	1
Myosin	4	[3]	7	5	6	8	9	2	1	--

- indicates a negative correlation

[#] – indicates correlation was not significant ($p > 0.05$)

[#] – indicates negative correlation was not significant ($p > 0.05$)

9.0 DISCUSSION

9.1 Overview

Biochemical fractionation was used to examine the spatial linkage between NCX1, SERCA2 and caveolin-1 in pig coronary artery smooth muscle using sucrose density flotation of detergent solubilized membranes. The results indicate there was a significant correlation in the distribution of NCX1 and SERCA2, NCX1 and caveolin-1, and SERCA2 and caveolin-1 in the flotation; however the migration was not perfect. This section focuses on validations of the methods used, comparison of the results with those in literature and their contribution to smooth muscle physiology, as well as proposals for future experiments.

9.2 Validation of solubilization method used

In literature, two main methods have been used to examine caveolin distribution with sucrose density gradient fractionation: high pH with ultrasonication and non-ionic detergents (Cavalli *et al.*, 2007; Silva *et al.*, 1999; Smart *et al.*, 1995; Babiychuk and Draeger, 2006; Gaus *et al.*, 2005; Sargiacomo *et al.*, 1993; Lisanti, Scherer, Tang *et al.*, 1994; Lisanti, Tang, Scherer and Sargiacomo, 1995; Lisanti, Sargiacomo and Scherer, 1999). In my initial experiments, the first method caused oligomerization of SERCA2 (Fig. 4). Oligomerization of SERCA2 protein has also been shown upon treatment with the reactive oxygen species, peroxynitrite and hydrogen peroxide which affected SERCA activity (Grover, Kwan, and Samson, 2003). Since it is not known how oligomerization influences the NCX- SERCA linkage, this method was not used further for fractionation experiments.

The second method relies on the resistance of lipid rafts to solubilization due to their distinct lipid composition and high buoyancy. This property is exploited by treating membranes with non-ionic

detergents, subfractionating them based on their densities, and then examining protein (including lipid raft and non-lipid raft markers) distributions within the fractions. This method has been used widely in the literature for co-localization studies (Sargiacomo *et al.,* 1993; Lisanti, Scherer, Tang *et al.,* 1994; Cavalli *et al.,* 2007; Lisanti, Tang, Scherer and Sargiacomo, 1995; Lisanti, Sargiacomo and Scherer, 1999). In my experiments, the detergent to protein ratio was optimized since too much detergent also solubilized lipid rafts (Appendix A3). This could potentially hinder the ability to separate lipid raft rich domains from other types of membrane domains using sucrose density gradient centrifugation. Moreover, too much of the detergents may also affect the potential NCX-SERCA linkage. In literature, the solubilization and consequent distribution of proteins in flotation experiments using Triton X-100 has been suggested to be dependent on the concentration chosen and may be artefactual (Babiychuk and Draeger, 2006; Lichtenberg, Goni, Heerklotz, 2005). Therefore, we decided to use conditions which will solubilise 50% of the NCX protein from smooth muscle microsomes. The optimal condition was when microsomes (12 mg protein/ml) were treated with 1.5 mg/ml of Triton X-100 (Fig. 5). It has been recently shown that detergent-resistant membranes can be isolated at physiological temperatures (37 ° C) and not only at low temperatures (4 ° C) with non-ionic detergents (Chen *et al.,* 2009). Thus, distributions and co-migration of proteins in this proposal are not due to artefacts induced by the use of Triton X-100, and are not due to the use of an inappropriate detergent concentration.

9.3 Validation of method of analysis

The relative abundance of each protein amongst sucrose gradient fractions was presented as percent specific activity. Considering that the total amount of protein in each fraction differed with the highest amount present in the last fraction (Fig. 6), another way to analyze the data was as percentage

of total for each marker protein. Initially, this was done according to the equation (Intensity per 1 μg protein loaded * total μg protein per fraction) / (sum of total μg protein in all fractions) * 100. A key observation was that the overlaps of marker distributions obtained were independent of whether the specific activities of the markers or their total amounts were compared. There was, unfortunately, an inherent error with calculating the latter method since the specific activity of several proteins, including NCX1, SERCA2, and caveolin-1, was very small in the last fraction (intensity was close to background). Another measure of total marker activity would be obtained if equal volumes of each fraction were resolved in SDS-PAGE, rather than equal protein amounts from each fraction. Unfortunately, the amount of material obtained was limited thus the alternative measurement could not be done in this manner. Before I started, an experiment using slightly different protein to detergent ratios had been conducted in the lab. In this, equal volumes of each fraction (rather than equal amount of protein) were analyzed in Western blots. The co-migration results of NCX1 with SERCA2 were consistent with those obtained in my study.

Marker distributions obtained in sucrose density gradient fractions were further examined by covariation/correlation analysis. The direction and magnitude of the correlation was quantified by the correlation coefficient, r, by two different methods. The first was the Pearson's product-moment correlation coefficient. This method is based on the assumption that the two variables examined are sampled from a population that follows a Gaussian distribution, at least approximately. Due to uncertainties in the Gaussian distribution, the second method, the Spearman's rank correlation coefficient, was used. This method determines a non-parametric correlation. It basis its calculations on ranking of the two variables and so makes no assumptions about the distribution of the values. To account for prep to prep variability, correlation analysis was determined on the paired values of

markers pooled from five similar experiments, each of which had fresh microsomes isolated. Pearson and Spearman correlation analysis gave the same results when comparing NCX1, SERCA2, and caveolin-1 distributions to each other as well as to all other markers (Fig. 8, Tables 5 and 6, Appendix A5). The distributions of the other markers to each other did not produce the same results between these two methods. This further validated the observed results and confirms that the correlations observed were not dependent on the method of analysis.

9.4 Distribution and co-migration of proteins

The lipid raft rich membrane domains were localized in low density fractions while cholesterol, caveolin-1, and the other lipid raft markers flotillin-2, gangliosides, and prion protein were localized in fractions containing less than 30 % sucrose (Figures 6, 7B and 7C). Based on the correlation of the lipid raft marker distributions, not all markers co-migrated together (Tables 5 and 6). Caveolin-1 correlated strongly with flotillin-2 and less so with gangliosides, prion protein and cholesterol. Conversely, the latter markers correlated strongly with each other and flotillin-2 and less with caveolin-1. This indicates the existence of caveolar and non-caveolar lipid rafts in smooth muscle microsomes. Similar results were obtained by Yao *et al.,* (2009) where the caveolae domains were rich in caveolin-1 and flotillin-2, while the other domains were also enriched with cholesterol and sphingolipids. Other studies have also demonstrated that lipid rafts with distinctly different protein and/or lipid components coexist within the PM (Madore *et al.,* 1999; Drevot *et al.,* 2002; Schuck *et al.,* 2003; Roper, Corbeil, Huttner, 2000; Drobnik *et al.,* 2002; Pike, 2004; Babiychuk and Draeger, 2006). The difference in protein compositions may imply that membrane microdomains may have different functions (Yao *et al.,* 2009). In fact, lipid rafts mediate many different yet crucial functions including regulation of signalling by PM

receptors, regulation of ion pump and channel activity, and mediation of vesicle trafficking and fusion (Rajendran and Simons, 2005; Hanzal-Bayer and Hancock, 2007).

The distributions of several cytoskeletal proteins were also examined. Actin, clathrin, and myosin were most abundant in the bottom fractions containing 44 % sucrose, opposite of the lipid raft markers (Fig. 6 and 7D). Moreover, they did not co-migrate with any of the lipid raft markers but did strongly with each other, as expected (Tables 5 and 6). Therefore, our method of separating lipid raft rich membrane domains from lipid raft poor domains was validated. Actin, myosin, and clathrin did not co-migrate with NCX1, SERCA2, or caveolin-1 (Fig. 8, Tables 5 and 6). The actin cytoskeleton has been suggested to modulate the activity of NCX1 and may be linked with caveolae so a partial co-migration between actin and these proteins was somewhat expected. The role of the cytoskeleton in the NCX and SERCA linkage is explored in greater detail in the Discussion, section 10.5. In retrospect, it would have been useful to monitor the migration of a non-lipid raft transmembrane protein such as the transferrin receptor (Persaud-Sawin, Lightcap, and Harry, 2009; Pani *et al.,* 2008).

The distributions of the two proteins of interest, NCX1 and SERCA2, were next examined. Both proteins had overall similar distributions and were in the lipid raft rich domain fractions (Fig. 6 and 7A). There was a positive and significant correlation indicating that these two proteins co-migrated (Fig. 8, Tables 5 and 6, Appendix A5). However, the correlation was not perfect (not 1) suggesting that the proteins were not always co-localized. In literature, there is also evidence for close associations between NCX and SERCA in a variety of cells. In rabbit aortic endothelial cells, ryanodine receptors on the SER are in close functional proximity to NCX near the PM (Liang *et al.,* 2004). Moreover, Brading *et al.,* (2002) suggested that NCX activity took place at the locations where the PM and SER membranes made contact. The close proximity of NCX and SERCA is important for NCX playing a

role in SER refilling, which has been shown previously (see sections 1.4 and 3.0 of the Introduction).

In addition to co-migrating together, NCX1 and SERCA2 co-migrated with caveolin-1 and flotillin-2 more so then they did with the other lipid raft markers, cholesterol, gangliosides, and prion protein (Fig. 8 and Tables 5 and 6). Caveolin associates with or binds to other proteins due to the presence of two related binding motifs in its scaffolding domain (Okamoto, Schlegel, and Scherer, 1998). It appears to be important for trapping receptors in caveolae to form efficient signalling microdomains (Couet, Sargiacomo, and Lisanti; 1997; Liu, Rudick, and Anderson, 2002). Therefore, for a protein to associate directly with caveolins, it must contain this motif. Interestingly, NCX contains three potential caveolin-binding motifs, as described in the Introduction (functional properties of NCX). In rat C6 glioma cells null of caveolin-3, NCX1 co-immunoprecipitated and co-localized with caveolin-1 (Cha *et al.,* 2004). Co-localization was shown with confocal microscopy as well as co-migration in sucrose density gradient fractionation in that study. Immunoflorescence and co-localization analysis concluded that caveolin-3 and NCX1 are co-localized at the surface of the cell in ventricular myocytes and caveolin-1 and NCX are co-localized in smooth muscle and interstitial cells of Cajal of the mouse intestine (Scriven *et al.,* 2005; Cho and Daniel, 2005). Association of NCX1 and caveolin-3 shown through fractionation and immunoprecipitation experiments was also evident in cardiac myocytes (Bossuyt et al., 2002; Bossuyt, Taylor, James-Kracke, and Hale, 2002). Therefore, the co-migration of NCX1 and caveolin-1 in sucrose density gradient fractions can be justified. Identification of caveolin binding motifs in SERCA2 has not yet been documented. However, in a model based on electron microscopy and transmission electron microscopy tomography, caveolae, the superficial SER, and mitochondria may be linked (Gherghiceanu and Popescu, 2007; Gherghiceanu and Popescu, 2006). In this model, approximately 80% of caveolae established close contacts with the SER. The results in my

study were consistent with this since the co-migration of SERCA2 was strongest with caveolin-1, and vice versa (Tables 5 and 6). Localization of caveolin-3 to SER vesicles derived from rabbit skeletal muscle has also been shown with immunofluorescence and sucrose density gradient fractionation studies (Fameli *et al.,* 2007). Thus, NCX1 and SERCA2 may be co-localized in similar types of membrane microdomains, which are rich in lipid rafts and include caveolae.

9.5 NCX and SERCA physical linkage

Although the spatial linkage between NCX and SERCA in pig coronary artery smooth muscle has been examined in this proposal, it is unknown whether the two proteins are physically linked. Immunoprecipitation and immunoblotting experiments were used to isolate and characterize protein complexes from solubilized smooth muscle microsomes to test this possibility. However, these experiments led to several artefacts, possibly due to non-specific binding of membrane proteins to antibodies and beads.

It is not known if the cytoskeleton or other unknown scaffolding protein(s) allow for contact between NCX and SERCA. In smooth muscle cells, disruption of the cytoskeleton with Calyculin A inhibited the phenylephrine-induced generation of asynchronous Ca^{2+} waves maintained by NCX and SERCA (Lee *et al.,* 2005; Lee, Kuo, Dai *et al.,* 2005). Cytochalasin D also disrupts the cytoskeleton by inhibiting filamentous actin (F-actin) polymerization. This compound has also been shown to alter NCX1 activity in cardiac myocytes (Ohmori, Toyama and Toyama, 1992; Condrescu *et al.,* 1995). In transfected CHO cells, NCX1.1 interacted with the F-actin cytoskeleton at cell surface regions and this interaction modulated allosteric Ca^{2+} activation of NCX activity (Condrescu and Reeves, 2006). Further evidence is that SERCA2 co-immunoprecipitates with NCX in neurons and astrocytes (Lencesova *et*

al., 2004). The effect of cytoskeleton disruption on the co-localization of NCX and SERCA may aid in identifying the structures maintaining their linkage. In human platelets, Ca^{2+} store depletion caused by SERCA inhibition with thapsigargin activated the Ca^{2+} entry mode of NCX. This activation was due to Na^{+} entry through the SOCC, TRPC1, and required a functional actin cytoskeleton (Harper and Sage, 2007). However, the literature provides conflicting results on the role of the actin cytoskeleton on SOCC suggesting mechanisms of activation may be cell or method pecific (Holda and Blatter, 1997; Wang, Gregory, and Barritt, 2002; Kunzelmann-Marche, Freyssinet, and Martinez, 2001; Bakowski, Glitsch, and Parekh, 2001; Sabala *et al.,* 2002).

SERCA, NCX and SOCC all play a role in refilling the SER Ca^{2+} pool, although their contributions to this process may vary. The concept of an SER Ca^{2+} sensor, STIM1, which interacts directly with PM channels, is currently the leading idea for SOCC regulation. The sensor communicates over short distances (approx. 10-25 nm) to Orai1, the pore forming subunit of a macromolecular SOCC complex in the PM (see section 1.4 in the Introduction for more detail) (Hogan and Rao, 2007; Vig and Kinet, 2007; Liao *et al.,* 2008; Alicia *et al.,* 2008). The distance these two proteins communicate is in accordance to the junctional space apposing the PM and superficial SER, of which NCX and SERCA reside, respectively. It would be interesting to investigate whether STIM1 and/or Orai1 are thus the scaffolding proteins which bring NCX and SERCA together. Using real-time fluorescence resonance energy transfer (FRET), it was shown that upon store depletion with thapsigargin, STIM1 aggregates at the ER and then associates with SERCA2A in transfected HEK293 cells (Sampieri *et al.,* 2009). Co-immunoprecipitation confirmed the association of STIM1 and SERCA2A was in a thapsigargin-dependent manner. When the constitutively active mutant STIM1(D76A) was used, thapsigargin had no effect on the SERCA2 association. The observed STIM1-

SERCA2A association followed the assembly of Orai at the PM and then Ca^{2+}-selective, whole cell inward currents developed. Another recent study shows that agonist stimulation increases the association of STIM1 to SERCA3 in human platelets (Lopez *et al.,* 2008). Furthermore, knocking down STIM1 with RNAi reveals that SOCC appear to be in close proximity to SERCA and silently refilling the ER (Jousset, Frieden, Demaurex, 2007). Lipid rafts may also play a role in the interactions between STIM1, Orai1, and TRPC1 (Pani *et al.,* 2008; Alicia *et al.,* 2008). The corresponding events and proteins involved in the coronary artery smooth muscle remain to be investigated.

9.6 Summary and Proposed Model

The results altogether suggest that NCX1 and SERCA2 co-migrate to similar types of membrane domains in pig coronary artery smooth muscle. These domains are rich in lipid rafts and include caveolae. The results also indicate that although a spatial interaction exists between these proteins, co-localization may not always be present. The observed NCX1 and SERCA2 spatial linkage are consistent with the functional linkage results obtained previously, as well as models obtained from other studies (Davis *et al.,* 2008; Floyd and Wray, 2007; van Breemen, Chen, and Laher, 1995; Poburko *et al.,* 2004; Lee *et al.,* 2002; Fameli, van Breemen, and Kuo, 2007; Moore *et al.,* 1993; Nazer and van Breemen, 1998; Juhaszova *et al.*, 1994; Daniel, El Yazbi, and Cho, 2006). A model based on the aforementioned results and previous work is thus proposed below (Fig. 10). This model is also consistent with the recent observations using immunoflourescence microscopy by Rajneet Kuner in our lab. In this model, the PM contains non-lipid raft membranes with planar lipid raft domains and invaginated domains (caveolae). NCX1 is proximal to caveolae but may also be present in other domains in the PM. SERCA2 is present in the superficial SER and is associated with NCX1 and

caveolae here. However, SERCA2 may also continue into the deep SER. NCX1 may supply Ca^{2+} for refilling the SER pool when active in the Ca^{2+} entry mode. This Ca^{2+} is brought into the limited junctional space between the superficial SER and PM. NCX may not be solely responsible for this process, however. Since caveolae are Ca^{2+} stores, and are in close proximity to SERCA2 and NCX1, they may also contribute to SER refilling. Overall, the linkage between NCX, SERCA, and caveolae may be important for restoring the SER Ca^{2+} pool as well as maintaining levels of $[Ca^{2+}]_i$. Although not presented in this model, the role of PMCA should also be considered since it is another key regulator of $[Ca^{2+}]_i$.

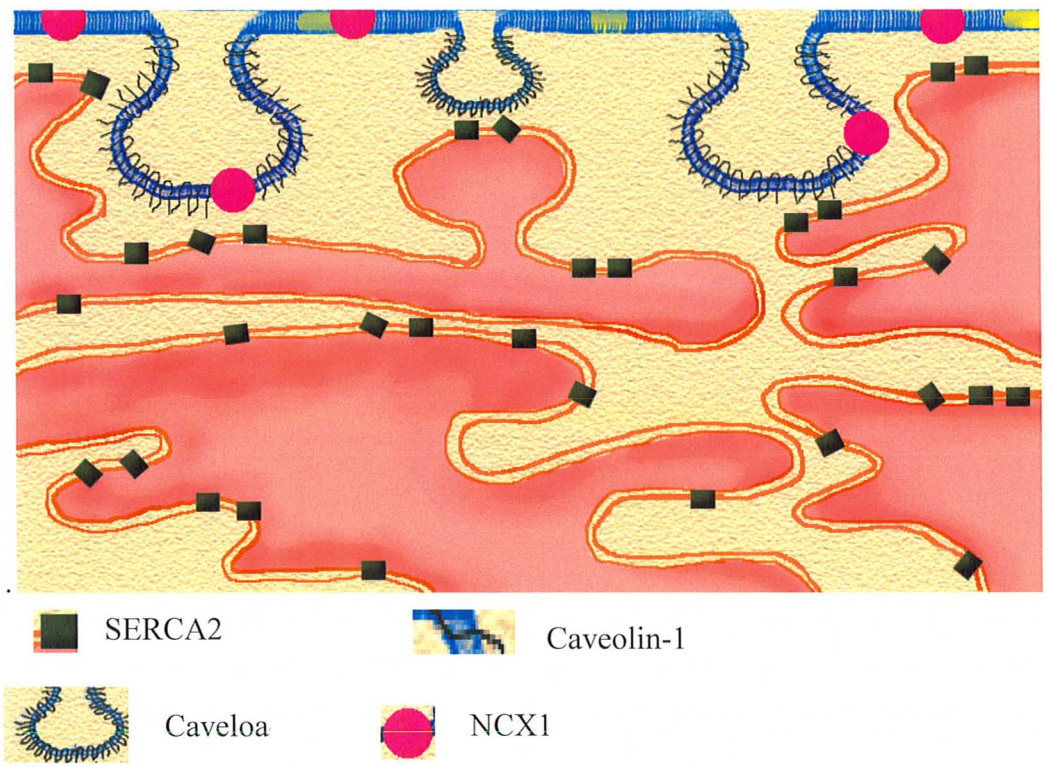

Figure 9. A 2-Dimentional schematic representation of NCX1, caveolin-1 and SERCA2 localization in pig coronary artery smooth muscle.

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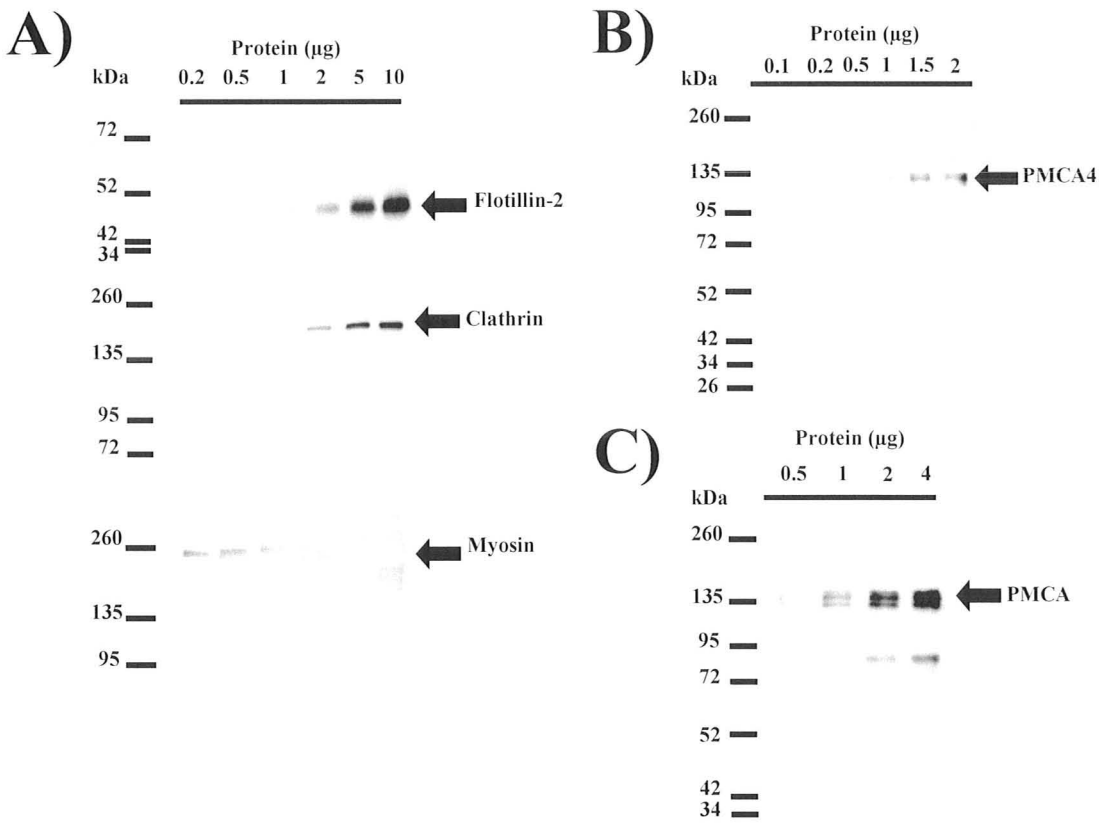
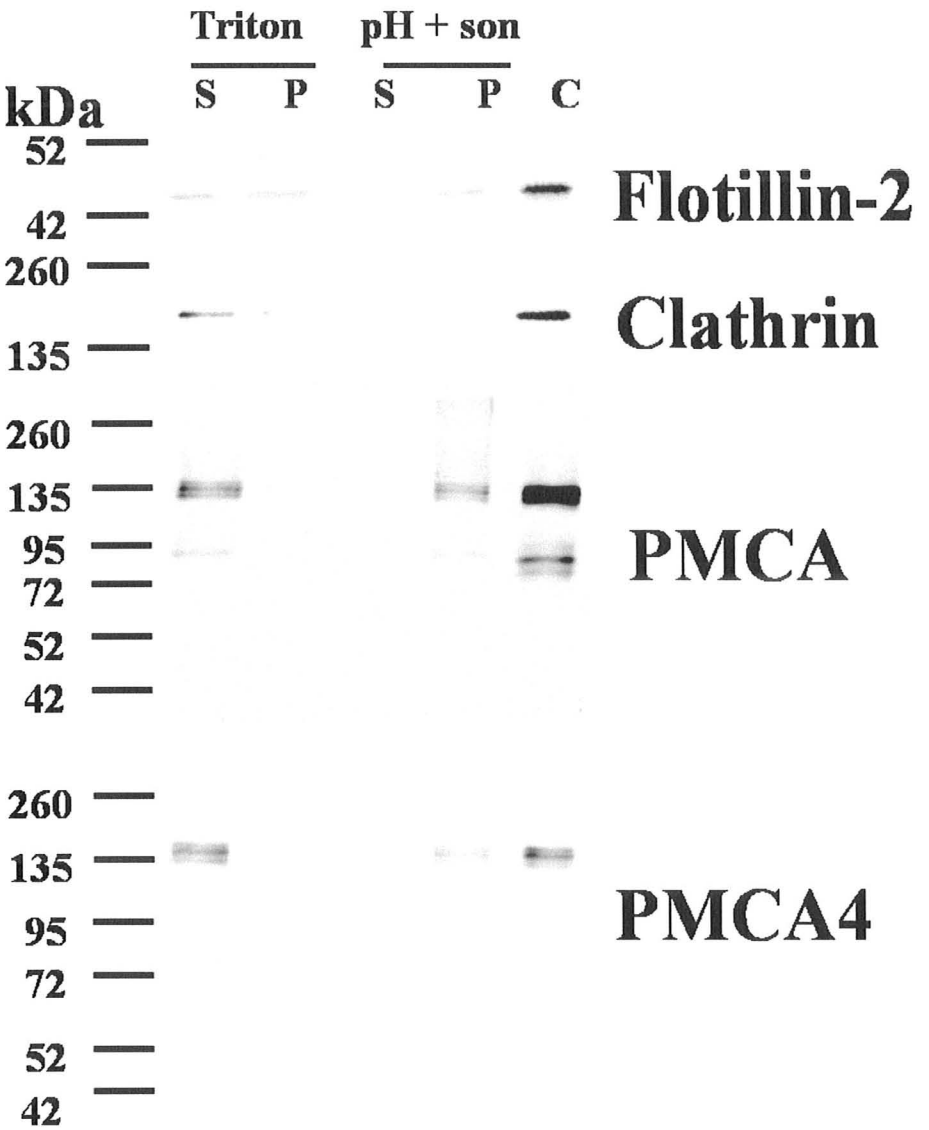
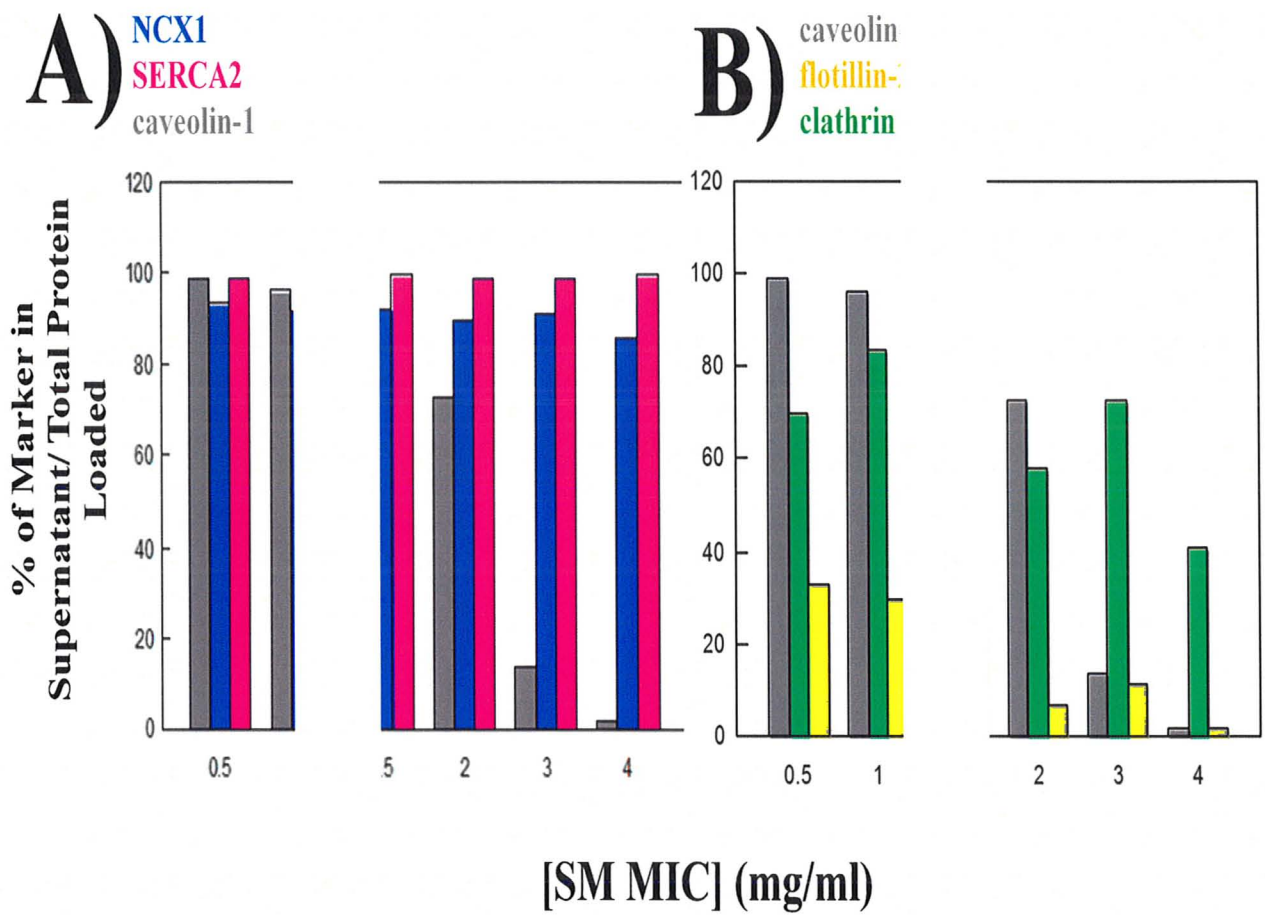
APPENDIX



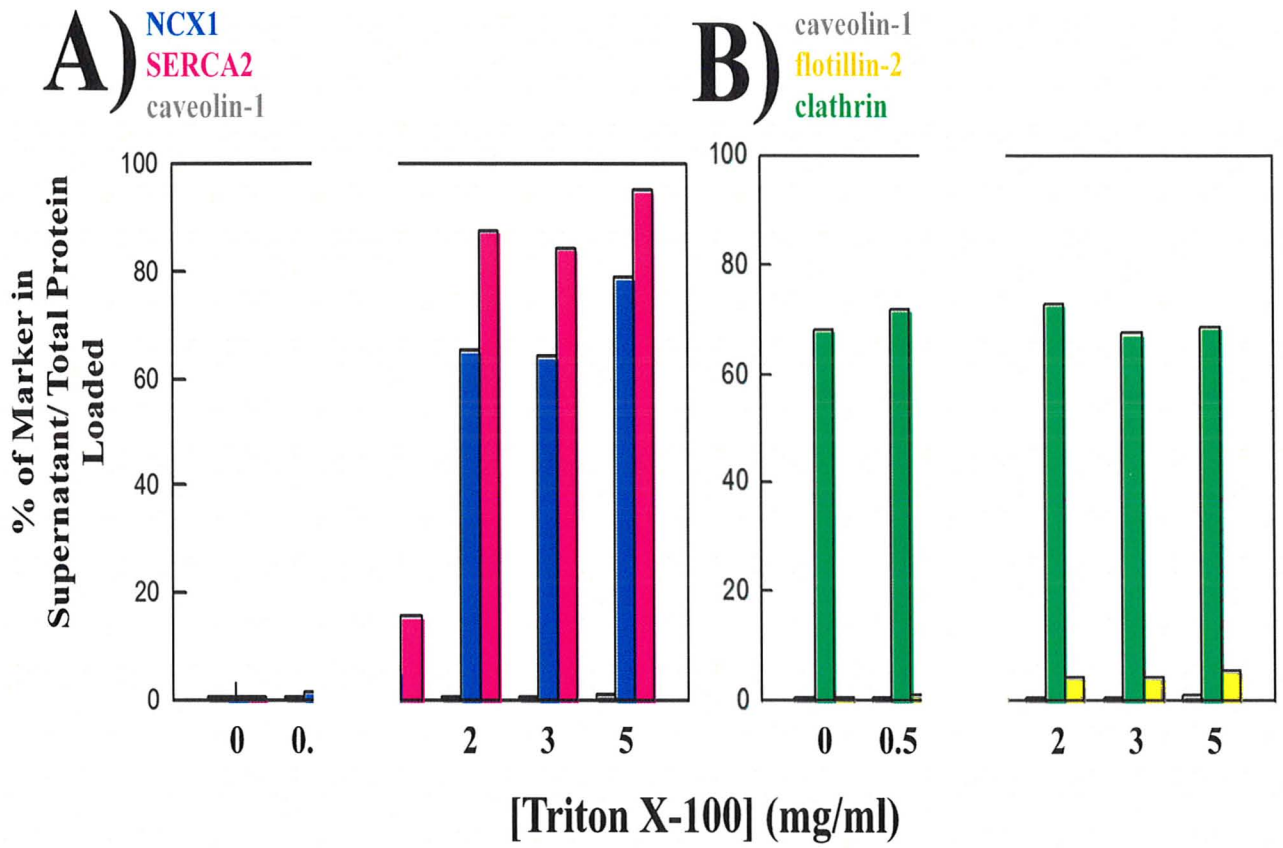
A1. Protein concentration dependence for detection of other markers. Western blots from gels are shown for A) flotillin-2, clathrin, and myosin; B) PMCA4; and C) PMCA with specified amounts of protein from smooth muscle microsomes loaded in different lanes. The signal intensity of the main bands (marked by black arrows) increased linearly with the amount of protein loaded with an r^2 = 0.9975 for flotillin-2, 0.9967 for clathrin (up to 5 µg), and 0.9505 for myosin (up to 2 µg). The r^2 for PMCA4 = 0.9923, and for PMCA = 0.9882.



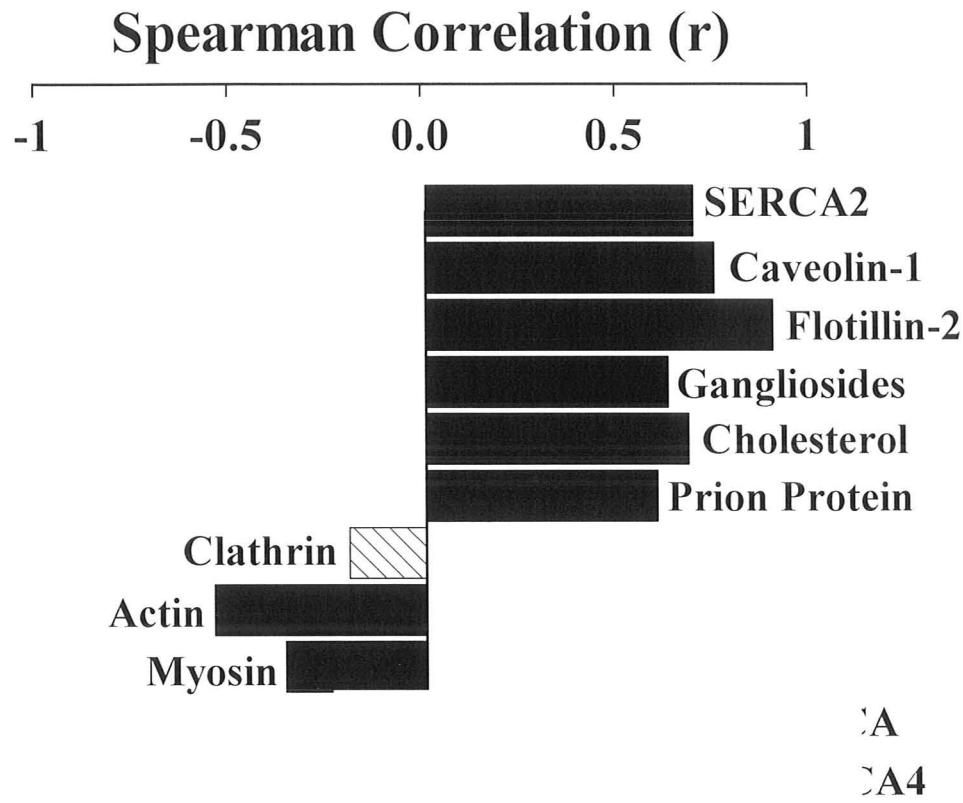
A2. Effect of detergent treatment or high pH and ultrasonication on various proteins. 2 mg protein/ml MIC were treated with a high concentration (10 mg/ml) of the non-ionic detergent Triton X-100 or high pH and ultrasonication. Equal volumes of the supernatant (S; detergent soluble fraction) and pellet (P; detergent insoluble fraction) were loaded. Triton mostly solubilized flotillin-2, PMCA, and PMCA4, but only partially solubilized clathrin. High pH and ultrasonication did not solubilize any of the proteins except clathrin. The amount of control (C) MIC loaded was 20 μg for PMCA, and 7 μg for PMCA4, clathrin, and flotillin-2. Molecular weights in kDa are to the left of each blot.



A3. Effect of treating smooth muscle microsomes with 10 mg/ml Triton X-100 on solubility of markers. Different concentrations of smooth muscle microsomes (SM MIC) were treated with 10 mg/ml of the non-ionic detergent, Triton X-100, for 1 hr and the effects of this treatment on the solubility of A) NCX1, SERCA2, and caveolin-1 and B) caveolin-1, flotillin-2, and clathrin proteins is shown. The graphs show the amount of each marker solubilized as a percent of the total protein loaded (S/(S+P)*100), where S is the detergent soluble fraction and P is the detergent insoluble fraction (equal volumes were loaded). Even at low microsomal protein concentrations, Triton completely solubilized NCX1 and SERCA2. Triton reduced its ability to solubilize caveolin-1 and flotillin-2 with increasing microsomal protein concentrations.



A4. Effect of treating 4 mg/ml smooth muscle microsomes with Triton X-100 on solubility of markers. Smooth muscle microsomes (4 mg protein/ml) were treated with different concentrations of the non-ionic detergent, Triton X-100, for 1 hr and the effects of this treatment on the solubility of A) NCX1, SERCA2, and caveolin-1 and B) caveolin-1, flotillin-2, and clathrin proteins is shown. The graphs show the amount of each marker solubilized as a percent of the total protein loaded (S/(S+P)*100), where S is the detergent soluble fraction and P is the detergent insoluble fraction (equal volumes were loaded). All markers except clathrin were insoluble when Triton was not added. Both NCX1 and SERCA2 start to solubilize between 1-2 mg/ml Triton, whereas caveolin-1 and flotillin-2 stay insoluble even up to 5 mg/ml Triton.



A5. Spearman's correlation between distributions (as % specific activity) of NCX1 and various proteins in the sucrose density gradient experiments. A total of 40 paired values of intensities of NCX1 and SERCA2 were obtained in 5 experiments similar to Fig. 7 and the Spearman's correlation (r) was determined. Similarly, NCX1 data were also paired with other proteins and analyzed (34 pairs for myosin, 18 for prion protein and 40 pairs for all others). Solid bars indicate that the correlation was significantly different than zero ($p < 0.05$)

Appendix A6. Pooled Pearson Correlations (r) of Distributions of Markers (as % specific activity)

	NCX	SERCA	Cav	Flot	Gang	Chol	PrP	Clath	Actin	Myo
NCX1	/	0.505	0.616	0.851	0.360	0.500	0.148	0.026	0.329	0.205
	/	0.711	0.785	0.922	0.600	0.707	0.385	-0.162	-0.574	-0.453
	/	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.115	0.319	0.0001	0.007
SERCA2	0.505	/	0.684	0.481	0.100	0.082	0.0001	0.006	0.124	0.116
	0.711	/	0.827	0.693	0.316	0.287	-0.007	-0.074	-0.352	-0.340
	<0.0001	/	<0.0001	<0.0001	0.047	0.073	0.977	0.648	0.026	0.049
Caveolin-1	0.616	0.684	/	0.677	0.201	0.194	0.019	0.066	0.345	0.286
	0.785	0.827	/	0.823	0.449	0.440	0.136	-0.257	-0.588	-0.535
	<0.0001	<0.0001	/	<0.0001	0.004	0.005	0.590	0.110	<0.0001	0.001
Flotillin-2	0.851	0.481	0.677	/	0.498	0.511	0.354	0.113	0.349	0.254
	0.922	0.693	0.823	/	0.706	0.715	0.595	-0.336	-0.591	-0.504
	<0.0001	<0.0001	<0.0001	/	<0.0001	<0.0001	0.009	0.034	<0.0001	0.002
Gangliosides	0.360	0.100	0.201	0.498	/	0.697	0.781	0.202	0.359	0.227
	0.600	0.316	0.449	0.706	/	0.835	0.884	-0.449	-0.600	-0.477
	<0.0001	0.047	0.004	<0.0001	/	<0.0001	<0.0001	0.004	<0.0001	0.004
Cholesterol	0.500	0.082	0.194	0.511	0.697	/	0.835	0.256	0.524	0.318
	0.707	0.287	0.440	0.715	0.835	/	0.914	-0.506	-0.724	-0.564
	<0.0001	0.073	0.005	<0.0001	<0.0001	/	<0.0001	0.001	<0.0001	0.001
Prion Protein	0.148	0.0001	0.019	0.354	0.781	0.835	/	0.565	0.571	0.573
	0.385	-0.007	0.136	0.595	0.884	0.914	/	-0.752	-0.756	-0.757
	0.115	0.977	0.590	0.009	<0.0001	<0.0001	/	0.0001	0.0001	0.004
Clathrin	0.026	0.006	0.066	0.113	0.202	0.256	0.565	/	0.394	0.539
	-0.162	-0.074	-0.257	-0.336	-0.449	-0.506	-0.752	/	0.628	0.734
	0.319	0.648	0.110	0.034	0.004	0.001	0.0001	/	<0.0001	<0.0001
Actin	0.329	0.124	0.345	0.349	0.359	0.524	0.571	0.394	/	0.728
	-0.574	-0.352	-0.588	-0.591	-0.600	-0.724	-0.756	0.628	/	0.853
	0.0001	0.026	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	/	<0.0001
Myosin	0.205	0.116	0.286	0.254	0.227	0.318	0.573	0.539	0.728	/
	-0.453	-0.340	-0.535	-0.504	-0.477	-0.564	-0.757	0.734	0.853	/
	0.007	0.049	0.001	0.002	0.004	0.001	0.004	<0.0001	<0.0001	/

Top # - r^2 Middle # - Pearson correlation (r) Bottom # - p value

Red indicates the correlation was not significant ($p > 0.05$)

Data pooled from 5 different experiments: 34 pairs for myosin, 18 for prion protein, and 40 pairs for all other markers

Appendix A7. Pooled Spearman Correlations (r) of Distributions of Markers (as % specific activity)

	NCX	SERCA	Cav	Flot	Gang	Chol	PrP	Clath	Actin	Myo
NCX1	/	0.703	0.756	0.909	0.637	0.690	0.609	-0.186	-0.535	-0.352
	/	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.007	0.251	0.0004	0.041
SERCA2	0.703	/	0.841	0.633	0.391	0.370	0.196	-0.199	-0.368	-0.224
	<0.0001	/	<0.0001	<0.0001	0.013	0.019	0.437	0.218	0.020	0.203
Caveolin-1	0.756	0.841	/	0.758	0.479	0.460	0.330	-0.245	-0.513	-0.384
	<0.0001	<0.0001	/	<0.0001	0.002	0.003	0.181	0.128	0.001	0.025
Flotillin-2	0.909	0.633	0.758	/	0.720	0.743	0.735	-0.364	-0.578	-0.366
	<0.0001	<0.0001	<0.0001	/	<0.0001	<0.0001	0.001	0.021	<0.0001	0.033
Gangliosides	0.637	0.391	0.479	0.720	/	0.844	0.911	-0.527	-0.625	-0.381
	<0.0001	0.013	0.002	<0.0001	/	<0.0001	<0.0001	0.001	<0.0001	0.026
Cholesterol	0.690	0.370	0.460	0.743	0.844	/	0.849	-0.495	-0.756	-0.558
	<0.0001	0.019	0.003	<0.0001	<0.0001	/	<0.0001	0.001	<0.0001	0.001
Prion Protein	0.609	0.196	0.330	0.735	0.911	0.849	/	-0.656	-0.838	-0.954
	0.007	0.437	0.181	0.001	<0.0001	<0.0001	/	0.003	<0.0001	<0.0001
Clathrin	-0.186	-0.199	-0.245	-0.364	-0.527	-0.495	-0.656	/	0.592	0.564
	0.251	0.218	0.128	0.021	0.001	0.001	0.003	/	<0.0001	0.001
Actin	-0.535	-0.368	-0.513	-0.578	-0.625	-0.756	-0.838	0.592	/	0.902
	0.000	0.020	0.001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	/	<0.0001
Myosin	-0.352	-0.224	-0.384	-0.366	-0.381	-0.558	-0.954	0.564	0.902	/
	0.041	0.203	0.025	0.033	0.026	0.001	<0.0001	0.001	<0.0001	/

Top # - Spearman r Bottom # - p value

Red indicates the correlation was not significant ($p > 0.05$)

Data pooled from 5 different experiments: 34 pairs for myosin, 18 for prion protein, and 40 pairs for all other markers