MODULATION OF Ca^{2+} TRANSPORT CHARACTERISTICS WITH LIPIDS IN ISOLATED SARCOPLASMIC RETICULUM VESICLES

BY

PENELOPE P. RAMPERSAD

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Department of Physiology, and
The Division of Stroke and Vascular Disease
University of Manitoba
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ABSTRACT

Omega-3 polyunsaturated fatty acids (n-3 PUFA) reduce the arrhythmogenic potential of the myocardium during ischaemia. Since disturbances in intracellular Ca^{2+} regulation are arrhythmyogenic, we examined n-3 PUFA effects on sarcoplasmic reticulum (SR) Ca^{2+} handling. Concentration-dependent responses of n-3 PUFAs on SR Ca^{2+} uptake were measured using stopped flow rapid kinetic fluorimetric assay of (a) sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) catalytic activity and (b) Ca^{2+} sequestration. Under conditions favouring Ca^{2+}-dependent ryanodine receptor (RyR) activation, n-3 PUFAs significantly improved functional coupling of net Ca^{2+} transport. Subsequent studies showed improvements in functional coupling were due to effects upon both SERCA and RyR proteins. In ionomycin-permeabilised vesicles, n-3 PUFAs stimulated Ca^{2+}-dependent activation of SERCA catalytic activity. However, in the same concentration range, n-3 PUFAs reduced [^{3}H]-ryanodine binding significantly. In addition, the n-3 PUFA, docosahexaenoic acid, markedly diminished Ca^{2+}-induced Ca^{2+} release in active Ca^{2+}-transport studies. Overall these observations indicate that n-3 PUFAs promote Ca^{2+} uptake mechanisms partly by improvements in catalytic activity and partly by inhibiting Ca^{2+} release.

Since impaired Ca^{2+} handling by cardiac sarcoplasmic reticulum (CSR) is often implicated in ischaemia-induced arrhythmias, we hypothesised that RyR-mediated Ca^{2+} leaks from CSR in a model of arrhythmia would be improved by feeding flaxseed, an enriched source of the n-3 PUFA alpha linolenic acid. Male NZW rabbits were fed one of four diets for 8 weeks: regular chow, or regular chow supplemented with 10% flaxseed, or 0.5% cholesterol, or 10% flaxseed and 0.5% cholesterol. Hearts were excised, rapidly frozen or

subjected to an ischaemia-reperfusion protocol then frozen. CSR vesicles were isolated and assayed for SERCA activity, Ca^{2+} transport, and membrane lipid compostition. Gas chromatography revealed an increase in n-3 PUFA content of CSR in both flax-fed groups (ALA, $p<0.001$). Fura-2 fluorescent measurements of CSR Ca^{2+} transport showed no effect of diet in pre-ischaemic controls. However, after ischaemia, CSR from flax-fed animals transported Ca^{2+} at higher rates than CSR from all other groups ($p<0.001$). These data suggest increased n-3 PUFA content of CSR membrane confers protection against ischaemia-induced impairment of SERCA-mediated Ca^{2+} transport. This protection may account for some of the anti-arrhythmic effects observed from n-3 PUFA enriched diets.

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DEDICATION

This manuscript is dedicated to my family, with love.

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LIST OF ABBREVIATIONS

CaM	Calmodulin
CaMKII	Calmodulin Kinase Two
CF	0.5 % Cholesterol / 10% Flax Diet
CPA	Cyclopiazonic acid
CSR	Cardiac Sarcoplasmic Reticulum
DHPR	Dihydropyridine Receptor / L-type Calcium Channel
ECC	Excitation Contraction Coupling
FX	10 % Flax Diet
HSR	Heavy Sarcoplasmic Reticulum
I/R	Ischaemia-Reperfusion
IP	Ischaemic-Preconditioning
IP_3R	Inositol Triphosphate Receptor
LCC	L-type Calcium Channel / Dihydropyridine Receptor
MgATP	Magnesium Adenosine Triphosphate
n-3 PUFA	Omega-3 Polyunsaturated Fatty Acid
NCX	Sodium Calcium Exchanger
OL	0.5% Cholesterol Diet
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PL	Phospholipid
RG	Regular Rabbit Chow
RyR	Ryanodine Receptor Calcium Release Channel

SERCA	Sarco-endoplasmic reticulum Calcium ATPase
SL	Sarcolemma
SR	Sarcoplasmic Reticulum
Tg	Thapsigargin
TM	Transmembrane
VGSC	Voltage-gated sodium channel
Xc	Xestospongin

I. REVIEW OF THE LITERATURE

THE ROLE OF THE SARCOPLASMIC RETICULUM IN STRIATED MUSCLE EXCITATION CONTRACTION COUPLING

"Of the ions involved in the intricate workings of the heart, calcium is considered perhaps the most important. It is crucial to the very process that enables the chambers of the heart to contract and relax, a process called excitation-contraction coupling. It is important to understand in quantitative detail exactly how calcium is moved around the various organelles of the myocyte in order to bring about excitation-contraction coupling if we are to understand the basic physiology of heart function. Furthermore, spatial microdomains within the cell are important in localizing the molecular players that orchestrate cardiac function."

Donald M. Bers[1]

Preliminary studies of muscle contraction by Sidney Ringer in 1883 revealed that calcium was required for contraction of an isolated frog heart. The significance of this observation remained relatively obscure until Heilbrunn (1940) proposed an essential role for calcium in contraction. He showed that calcium diffusing into cut ends of muscle fibres caused them to contract: Kamada et al (1943) then confirmed these observations. At the same time Bailey (1942) began to deduce that calcium regulated contraction through the myosin ATPase. By 1950 Sandow coined the term *Excitation-Contraction Coupling*, the hypothesis that calcium released from terminal regions of muscle fibres activates the myosin ATPase. Ernesto Carafoli provides an account of the history and importance of calcium in muscle contraction in a recent review [2].

The sarcoplasmic reticulum (SR) is a specialized intracellular calcium store that is integral to the regulation of muscle contraction in eukaryotes. Analagous to the endoplasmic reticulum, it forms an intramembraneous structure that associates with both the plasma membrane and myofilaments. The SR acts as both a source and sink of Ca^{2+} in the cell. Ca^{2+} is released and sequestered respectively by ryanodine receptor Ca^{2+}-release channels (RyRs) and Sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) located in the SR membrane

bilayer. The movement of Ca^{2+} is synchronised in the myocyte so that an electrical signal coordinates its release into the cytosol from the SR resulting in contraction and its subsequent removal by the SR causing relaxation. The following is an overview of excitation-contraction coupling in both cardiac and skeletal muscle.

CARDIAC MUSCLE

The purpose of excitation-contraction coupling (ECC) in cardiac muscle is to transduce an electrical or chemical signal into physical muscle tension. Electrical coordination of myocytes pumps blood out of the heart in a synchronous manner. Calcium-induced calcium release (CICR) is the most widely held postulate on how ECC occurs in the heart. Publications by Endo, Ford and Podolsky, and Fabiato and Fabiato in the early 1970s gave insight into the mechanism of CICR [3-5]. CICR begins at the level of the myocyte's transverse tubule (t-tubule). These invaginations of the sarcolemma (SL) propagate an action potential that is usually initiated at the sinoatrial node. Activation of DHPRs by depolarization of the SL initiates calcium current (I_{Ca}) that is functionally coupled to RyRs in the SR. I_{Ca} is often labelled "trigger calcium" because it potentiates its own release from the SR by activating RyRs; hence the term calcium-induced calcium release. The result is an increase in cytosolic Ca^{2+} from 8 x 10^{-8} M to 1-3 x 10^{-7} M [6]. In fact 50–100 μM Ca^{2+} is needed to increase cytosolic Ca^{2+} from 100 nM to 1 μM, since buffering occurs in the cytosol during contraction [7]. Elevated cytosolic calcium then binds troponin C allowing the contractile filaments to interact and contract the cell.

Involvement of the SR in CICR begins with RyR activation in the junctional cleft. RyRs are located in the terminal cisternae of the SR and extend into the diadic cleft between the SR and t-tubules [6]. Electron-microscopy reveals that junctional complexes present in

the cleft are approximately 100-200 nm in diameter, 10-15 nm high and 300-2000 nm apart [6-8]. These complexes are composed of up to 100 RyRs in association with the SR accessory proteins junctin, triadin, and calsequestrin [9]. As well, a number of regulatory proteins can be associated with the RyR.

Ca^{2+} release from the SR is by couplon activation. A couplon consists of 10-25 DHPRs and 100 RyRs [1]. Thus four to nine RyRs are coupled to a single DHPR depending on the species [8]. The high ratio of RyRs to DHPRs ensures sufficient Ca^{2+} release at the junction and Ca^{2+} propagation in response to Ca^{2+} entry via DHPRs [1]. Other potential mechanisms of RyR activation by Ca^{2+} include the Na^{+}/Ca^{2+} exchanger (NCX) and inositol triphosphate receptor (IP_3R).

SR Ca^{2+} release is triggered in the first 10 ms of an action potential and peaks at 5-10 μM Ca^{2+} within 2-5 ms of the action potential upstroke [7, 10]. The Ca^{2+} transient or twitch is comprised of approximately 10 000 concerted sparks, the fundamental release unit of Ca^{2+} from the SR [7, 11]. A spark is the summation of 6-20 RyRs opening [6, 7, 12-16] and results in a large increase of cytosolic Ca^{2+} in a concentrated area [2]. They appear close to Z lines where diads or couplons are expected [6]. In every junction 1 spark produces 2-3 $x10^{-19}$ mol Ca^{2+}, which is equivalent to 12 000-18 000 Ca^{2+} ions/ spark [7].

Ca^{2+} sparks can function as local events, since a spark is the simultaneous opening of several RyRs from one DHPR. The theory of local control [17, 18] states SR Ca^{2+} release is mediated by the intensity of I_{Ca} [19]. Spontaneous distinct local events occur only within a given junction usually, except in Ca^{2+} overload where propagation of the spark results in Ca^{2+} waves [1]. Sparks have a low probability, less than 10^{-4} in the resting cardiomyocyte, but increase substantially during ECC [7]. Sparks also amplify Ca^{2+} signals and spread by additional channel activation [20].

The process of CICR is terminated when calcium is re-sequestered leading to relaxation. To maintain a homeostatic state in the cell total Ca^{2+} content must remain constant and be returned to its respective stores. There are four known methods of extruding calcium from the sarcoplasm after contraction [7]. In rabbit and human the sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) accounts for approximately 70% of Ca^{2+} efflux while the SL sodium/calcium exchanger (NCX) accounts for 28 % [1, 7]. The plasma membrane Ca^{2+}-ATPase (PMCA) and mitochondrial Ca^{2+} uniporter each represent about 1% of total Ca^{2+} removed [1]. In comparison SERCA represents 90% of Ca^{2+} removal activity in rat and mouse [7]. The SR sequesters most of the Ca^{2+} due to the large number of SERCAs in its tubular region in all species [6].

SKELETAL MUSCLE

Skeletal muscle is similar to cardiac muscle in that a process of Ca^{2+} release from RyRs activates contraction of the myofilaments. However, a process termed depolarization-induced Ca^{2+} release (DICR), rather than CICR, primarily regulates ECC in skeletal muscle. DICR is initiated in skeletal myofibrils by a motor neuron-induced depolarisation of t-tubules. DHPRs act as voltage-sensors and undergo a physical change in response to the action potential. This conformational change is mechanically transferred to RyRs by an unknown mechanism[6], so RyR activation is independent of I_{Ca} [21].

As compared to cardiac muscle, skeletal RyRs differ in their arrangement. A triad, like the cardiac diad, consisting of the t-tubule and two juxtaposed terminal cisternae is formed in skeletal muscle. Every other RyR is linked to a tetrad of DHPRs allowing for tighter regulation of RyR activation than in cardiac muscle [6, 22]. In skeletal muscle ECC is coupled between RyR1 and DHPR[23]. It has been deduced that loop II-III of DHPR

activates RyR1 [2, 23]. RyR1, like RyR2, is also regulated by Ca^{2+} and shows Ca^{2+}-dependent activation *in situ* and *in vitro*. Since all RyR1s are not coupled to DHPRs, it is hypothesised that the function of CICR in skeletal muscle is to amplify the DICR response [23].

The mechanism by which Ca^{2+} release is activated also differs between cardiac and skeletal muscle in some respects. During excitation-contraction coupling in cardiac muscle the RyR2 is generally thought to interact with the L-type Ca^{2+} channel to initiate CICR. In contrast RyR1 in skeletal vertebrates interacts with a DHPR that acts as a voltage sensor and not a Ca^{2+} conducting channel. RyRs exist as RyR1 and RyR3 isoforms in skeletal muscle [21]. Coupling of DHPRs to RyRs is thought to occur through RyR1 while it is suggested that RyR3 accounts for the CICR component in skeletal muscle [21].

Although CICR is one pathway that can induce Ca^{2+} release from the SR in skeletal muscle, DICR is the dominant mechanism. While both CICR and DICR are thought to play a role in both cardiac and skeletal muscle they are thought to contribute to the process of muscle twitch to differing extents [6].

SARCOPLASMIC RETICULUM CONSTITUENTS

The sarcoplasmic reticulum is organised into longitudinal and terminal cisternae regions. Longitudinal SR is enriched in Ca^{2+}-ATPase pumps and encases myofilaments. Terminal cisternae are the junctional regions of SR, so they contain the majority of Ca^{2+} release channels. Two families of Ca^{2+} release channels, RyRs and IP_3Rs, are known to function in the SR as well as an ATP-dependent Ca^{2+} pump. A variety of proteins coordinate the efforts of these main affectors including calsequestrin, FKBP 12, and phospholamban all to be discussed in this section.

CALSEQUESTRIN

Calsequestrin (CSQ) is a soluble low affinity high capacity Ca^{2+} binding protein found in the lumen of the SR in skeletal, cardiac and smooth muscles [24]. Initially characterised by David Maclennan as calsequestrin in 1971, it was described as an acidic 44 kDa protein, the major Ca^{2+} storage protein in the lumen of the SR [24]. It comprises 7% of SR protein and it is a monomer in aqueous solution [24]. CSQ selectively binds Ca^{2+} and carries a net negative charge [25]. Approximately 37% of CSQ amino acid residues are glutamate or aspartate [24]. These acidic amino acids bind 43 mol Ca^{2+} / mol protein at pH 7.5, $K_D = 4 \times 10^{-5}$ [24]. CSQ undergoes conformational changes in response to Ca^{2+} binding due to increased alpha-helical content and decreased surface hydrophobicity [25]. In the absence of Ca^{2+}, electrostatic repulsion between carboxyl groups prevents its folding [25].

CSQ hydrophobically binds the internal face of the SR junction [24, 26, 27]. CSQ is linked to RyR [28] through the SR proteins triadin, 95 kDa, [29, 30] and junctin, a 26 kDa protein that shares some homology with triadin [31]. Triadin and junctin both have single transmembrane domains in the SR lipid bilayer that bind CSQ to their basic regions [2]. One physiological function of CSQ may be to interact with RyRs in the terminal cisternae [26]. Structural changes in CSQ due to pH- and Ca^{2+}-dependent conformations may affect RyR P_o [32-34]. In addition it may complex with the SR protein triadin to produce RyR effects [9].

Another major function of CSQ is to maintain Ca^{2+} homeostasis [2]. CSQ may buffer up to 20 mM Ca^{2+} in the SR [24]. CSQ reduces free luminal Ca^{2+} concentrations to prevent reverse-mode SERCA activity and formation of Ca^{2+} precipitates [2]. Compared to other binding proteins CSQ is larger, contains more binding sites, has a larger dissociation

constant and is bound to the membrane instead of free [24]. All of these properties may contribute to its unique function.

INOSITOL TRIPHOSPHATE RECEPTOR

There are two families of calcium release channels that can be found in the sarcoplasmic reticulum, IP_3R and RyR [2, 23]. RyRs are found predominantly in skeletal muscle while IP_3R are found in most other tissues including smooth muscle cells, plasma membrane, nucleus, secretory vesicles, and golgi apparatus [2]. However, IP_3R are also present in striated muscle as well [35].

Some similarities are shared between the IP_3R and the RyR, the largest ion-channels known [35]. Both channels are tetramers composed of large subunits of 290 kDa and 550 kDa for IP_3R and RyR respectively [2, 35]. They exhibit homology near the carboxy-terminus that forms the transmembrane channel region of each receptor [2]. These channels carry large conductances of up to 100 pS in comparison to 10 pS in a DHPR [2]. The IP_3R channel exists in at least 3 isoforms. IP_3R's symmetrical central pore shares homology with the RyR [2, 23, 36, 37]. In contrast, RyRs are activated by voltage mediated changes, calcium, and nicotinamide adenine dinucleotide [38, 39] while IP_3Rs are primarily activated by the second messenger inositol 1,4,5 triphosphate, IP_3. Ca^{2+} transients arising from RyRs are termed sparks whereas IP_3Rs produce puffs, large increases in cytosolic Ca^{2+} in a concentrated area [2, 40]. Recent cryo-electron microscopic images have shown that IP_3Rs and RyRs are structurally different despite their molecular similarity [41]. The IP_3R has been described as an uneven dumbbell reaching 170 Å and spanning 100 Å and 150 Å laterally at either end [41]. "The distinguishing functional attributes of each RyR or IP_3R channels likely underlie the spatiotemporal complexity of intracellular Ca^{2+} signaling in cells"[23].

A number of modulators of IP_3R function have been identified [2]. The ligand IP_3 is formed in addition to diacylglycerol as a by-product of phosphotidyl inositol bisphosphate hydrolysis by phospholipase C in the inner leaf of the plasma membrane. Phospholipase C is activated by a number of primary messengers including acetylcholine, adrenaline, vasopression, thrombin, ATP, platelet-derived growth factor and endothelial growth factor acting through G-proteins and tyrosine kinases. Other modulators of the channel include both luminal and cytosolic calcium, calmodulin, ATP, protein kinase A, cGMP-dependent protein kinase, Ca^{2+}/CaM-dependent protein kinase (CaMKII), protein kinase C, tyrosine kinases, NADH, calcineurin, FKBP12, heparin, and xestospongin [2]. It has been hypothesised that IP_3Rs are biphasically regulated by Ca^{2+}-sensitive intrinsic activatory and inhibitory sites, which indicates that IP_3Rs are responsive to changes in cytosolic Ca^{2+} [42]. Similar mechanisms of modulation are proposed for the RyR below.

RYANODINE RECEPTOR

Structure and Function

The RyR functions as a Ca^{2+}-release channel during ECC in both skeletal and cardiac SR. It also has a role to play in non-muscle cells. First cited by Fleischer *et. al* in 1985, ryanodine's effects on Ca^{2+} accumulation in crude preparations of both longitudinal SR and terminal cisternae were examined [43]. Since those preliminary studies [44-48] detailed understanding of RyR structure/function has been hampered (or hindered) by its intracellular location and size. This has made it difficult to crystallize for x-ray analysis and study *in vivo*. Nevertheless, this protein has attracted an enormous interest and much is now known from controlled studies *in situ* and *in vitro*. A relatively brief overview of RyR isoforms, morphology and arrangement, and conductance properties is presented here.

RyRs exists in at least 3 isoforms in mammals. These three genes are located on separate chromosomes and can differ by splice variation as well [49-52]. In mammalian striated muscle RyR1 is predominantly found in skeletal muscle and brain, RyR2 in cardiac muscle and brain, and RyR3 is at relatively low levels in brain, diaphragm and jurkat T-lymphocytes [2, 23]. RyR2 and RyR3 respectively share 66% and 70% identity with RyR1 [50, 53].

The RyR channel is a 2.3 MDa holoreceptor [35]. RyRs are symmetrically shaped as tetramers, so the size of each monomer is about 550 kDa [35]. The cytoplasmic domain is approximately 29 nm x 29 nm x 12 nm while the transmembrane (TM) domain extends approximately 7 nm into the SR lipid bilayer [2, 35]. Approximately $4/5^{th}$ of the channel is cytoplasmic with the N-terminus oriented in that direction [35]. There are at least ten distinct domains within the loosely packed cytoplasmic portion of the channel that contains 2-4 Ca^{2+} binding sites [2, 7, 35, 54]. The remaining $1/5^{th}$ of the channel creates the TM domain with its C-terminus [35]. The number of TM spanning segments in RyRs is actively disputed. Anywhere from 4 to 12 TM domains have been proposed [50, 52]. The TM domain forms a 2 nm central hole between the monomers thereby creating the Ca^{2+}sensitive gate [55]. The width of the RyR2 channel pore is approximately 3? while the length of the voltage drop is 10.4 ? , a relatively short permeation pathway consistent with its rapid ionic conduction [23, 56].

The arrangement of RyRs differs according to isoform and tissue. In skeletal systems the cytosolic domains of RyR1s closely align themselves with DHPRs filling the junctional cleft. Fast-twitch skeletal muscle is configured so that every other RyR corresponds to a DHPR, for every 2 DHPR there is 1 RyR [57]. The direct interaction between the DHPR loop and RyR1 in skeletal muscle allows for rapid signal transduction, since the action potential is only 2 ms [23]. In contrast, the order of arrangement in cardiac muscle is 5-10

RyRs for every DHPR [57]. This lax organization is functional because the action potential (~100 ms in rat) is significantly slower than skeletal muscle [23].

All RyR isoforms exhibit high unitary conductance of up to 500 pS in monovalent cation solutions and 100 pS in divalent solutions [58, 59], in comparison to DHPRs (20 pS) or maxi-K_{Ca} (250 pS) channels [60, 61]. The ion selectivity of the RyR is relatively poor. It is predominantly a calcium channel but it also conducts K^+, Mg^{2+} and Na^{2+} [23]. RyRs select for divalent over monovalent cations, but do not distinguish between similarly charged ions [23]. Perhaps this is because its high conductance does not allow it time to "discriminate" between ions [23].

The RyR is named after the alkaloid ryanodine that binds to it from the plant *Ryania speciosa*. Ryanodine binds near the C-terminus, which forms the central pore, consequently interfering with RyR permeation characteristics by altering the diameter of the pore[62-64]. Generally, ryanodine maintains RyRs in a slow-gating subconductance state where the current becomes 1/3 to ½ control [64]. The concentration-dependent response of RyRs to ryanodine is similar across all three isoforms [23]. Relatively low concentrations of ryanodine, 10 nM, increase RyR open probability (P_o) while 1 μM ryanodine locks the channel open and decreases its conductance [65]. Even higher concentrations of ryanodine, 100 μM, lock the channel in a closed state [66]. The varied response of RyR to the alkaloid may be due to cooperativity at high (K_d~50 nM) and low (K_d~1 μM) affinity binding sites [67-69]. Furthermore the binding of ryanodine to the high affinity site may depend upon the conformation of the channel [67].

Mechanisms of Activation and Inactivation

Aside from pharmacological agents like ryanodine, there are a number of physiological affectors that regulate the ryanodine receptor. These include, but are not limited to molecular ligands such as Ca^{2+}, ATP, Mg^{2+}, and reactive oxygen species (ROS), as well as protein ligands like DHPR, NCX, PKA and CaMKII, CaM, and FKBP. As highlighted earlier, RyRs are physiologically activated during ECC. The process by which RyRs are inactivated will be discussed.

Calcium

Cytosolic Ca^{2+} is known to regulate RyR P_o. Within 1 ms of DHPR activation Ca^{2+} concentrations can reach 10-15 μM in the junctional cleft [70, 71]. The amount of trigger Ca^{2+} that enters the cell is proportional to the amount of Ca^{2+} released from the SR. Varying I_{Ca} shows this dependence and indicates that a larger I_{Ca} may increase Ca^{2+} release by recruiting more RyRs [7, 72, 73]. It has also been suggested that I_{Ca} may affect the amplitude of Ca^{2+} released [7, 74]. Generally, cytosolic Ca^{2+} activates RyRs between 1-10 μM Ca^{2+} and inhibits RyRs by 1-10 mM Ca^{2+} [23].

Lumenal Ca^{2+} simultaneously modulates the RyR with cytosolic Ca^{2+}. Under physiological conditions total Ca^{2+} within the SR is approximately 1 mM [23], of which 60-130 μM Ca^{2+} is free [75-77]. Above 40 μM $[Ca^{2+}]_{SR,\ free}$ there is an exponential relationship between I_{Ca} and the amount of Ca^{2+} released from the SR [73, 78-80]. RyR P_o and the frequency and amplitude of Ca^{2+} sparks vary directly with SR Ca^{2+} [11, 81-88]. One postulated mechanism of how lumenal Ca^{2+} regulates these effects is that it may activate the cytosolic side of the RyR [86, 87]. Another group of evidence suggests that SR Ca^{2+} acts on the lumenal side of the RyR [88]. Activating and inactivating lumenal sites may exist on the

RyR that alter the sensitivity of RyR to agonists [89]. Lumenal Ca^{2+} also binds lumenal proteins like calsequestrin and junctin [90, 91].

ATP and Mg^{2+}

ATP and Mg^{2+} have opposing actions on RyRs. Under physiological conditions free cytosolic ATP (300 μM ATP) sensitises the RyR to Ca^{2+} [2, 23, 92]. Under equivalent conditions 0.5-1mM free Mg^{2+} desensitises RyRs possibly by competing with Ca^{2+} at RyR activation and inactivation sites [7, 23, 93]. The significance of the equilibrium that must exist between these two affectors is seen during ischaemia when increased cytosolic Mg^{2+}, due to decreased buffering by ATP, may delay the onset Ca^{2+}-overload by decreasing RyR Ca^{2+}sensitivity [94].

Reactive Oxygen Species

The oxidation state of the RyR is also thought to alter its activity [95]. Each RyR monomer has 80-100 cysteine residues some of which can potentially be oxidized [50, 53]. Oxidation may inhibit Mg^{2+} and calmodulin effects [96, 97]. Nitric oxide (NO) likely alters the redox status of RyRs as well [98].

DHPRs

DHPRs are located at the junctional clefts of myocytes and interact with RyRs in the terminal cisternae [2, 99]. DHPRs transmits the action potential signal to the RyRs through an unknown direct or protein interaction [2]. Triadin, a junctional SR protein may be a potential candidate [2]. Cardiac muscle is generally regulated by a Ca^{2+}-mediated DHPR-RyR signal while skeletal muscle has a more direct linkage through regions II-III in the DHPR alpha cytoplasmic loop [2, 7, 23]. This is puzzling since there is relative homology

between DHPRs in skeletal and cardiac muscle. The physical distribution of the two channel types and their relative distribution may account for these different mechanisms of activation [99, 100].

NCX

The NCX can potentially activate the RyR by two means [7]. A sharp rise in Na^+ after the action potential may activate the NCX in the outward direction and create a source of trigger Ca^{2+} to activate SR Ca^{2+} release [101-103]. However, many argue that such a mechanism is not feasible [104-106] because NCXs are not present in junctional clefts [107]. The other mechanism of activating the RyR via the NCX may be by direct activation of the exchanger upon depolarisation [108, 109]. Still, the proximity of NCXs to RyRs and the significantly larger DHPR I_{Ca} oppose the idea that the NCX is a physiological activator of the RyR. The role of the NCX may be to potentiate SR Ca^{2+} release or trigger its release in the absence of DHPR activation [7].

PKA and CaMKII

Differential results have been obtained in response to kinases PKA and CaMKII acting on RyRs. PKA has been shown to both activate and inactivate RyR1 and RyR2 [7, 23]. Phosphorylation of RyR by PKA decreased the P_o at 100nM Ca^{2+}, but P_o was increased with the rapid application of trigger Ca^{2+} [110]. Studies by Marx *et. al* provide evidence that P_o RyR is increased due to FKBP 12.6 dissociation by phosphorylation at Ser2809 [111]. In comparison to the above lipid bilayer studies, intact cells show PKA has no effect on resting Ca^{2+} sparks [83]. Still, PKA may mediate the RyR DHPR interaction through sorcin, a 22 kDa SR accessory protein [112, 113]. Sorcin decreases Po and Ry binding which is reversed by PKA phosphorylation [114].

Experiments conducted on RyRs response to CaMKII yielded similar responses as to PKA. According to Marx, CaMKII phosphorylates the same serine residue as PKA [115]. Likewise to PKA, CaMKII has variable effects on RyR P_o [115-117]. A possible explanation may be that CaMKII potentiates Ca^{2+} release from RyRs after their activation [7], since phosphatases depress ECC gain and inhibition of CaMKII prevents Ca^{2+}-dependent SR Ca^{2+} release in a concentration-dependent manner [118, 119].

Calmodulin

Calmodulin (CaM) binds RyRs 10 nm from the entrance to the TM pore [120]. It activates RyRs at concentrations less that 2 μM, and inhibits them in greater amounts. RyR1 binds 4-16 CaM inversely to [Ca^{2+}] [121]. Contrary to RyR1, RyR2 binds 4 CaM at high Ca^{2+}, 200 μM, and binds only 1 CaM at low Ca^{2+}, 100 nM [7]. While there is debate over how many CaM bind to a tetramer there is consensus that Ca^{2+} increases the affinity of CaM for RyR2 [7]. Effects of CaM on Ca^{2+}-dependent activation of RyR are not well defined. RyR1 and RyR3 may be activated by CaM at low Ca^{2+} and inhibited by it at high Ca^{2+} whereas RyR2 is only inhibited by CaM [122, 123]. Alteration of RyR oxidation states by CaM and interactions with DHPRs could play a role in its action [97, 124].

FK506 Binding Proteins

Immunophilin FK506 binding proteins (FKBPs) are 12 kDa proteins that associate with RyRs in heart and skeletal muscle. FKBP12 and FKBP12.6 respectively bind RyR1 and RyR2 preferentially due to their relative cytosolic abundancy. Each FKBP stoichiometrically binds a RyR subunit, so four FKBPs bind per RyR tetramer [125-127]. The binding site for FKBP is about 10 nm from the channel opening [120]. General observations indicate that FKBP acts to stabilize RyRs in an open or closed state [2, 125]. FKBP removal from RyR1

results in activation of the channel [128]. Its role in RyR2 function isn't as clear. In channel studies where FKBP is removed, subconductance states are observed and normal conductance is restored with its reintroduction [125, 128-130]. FKBP gene knockout studies result in embryonic lethality due to impaired heart, brain but not skeletal muscle function [131].

The function of FKBPs may be to coordinate the RyR tetramer and neighbouring channels [7, 132], or mediate DHPR-RyR coupling [133]. FKBPs could potentially inhibit channel adaptation by increasing RyR Ca^{2+} sensitivity [7]. A role for FKBP has been suggested in heart failure. PKA phosphorylation of FKBP12.6 leads to its dissociation from RyR which could result in defective RyR regulation [111]. In support of this hypothesis, the FKBP antagonist FK506 increases Ca^{2+} spark frequency and decreases SR Ca^{2+} [134, 135].

RyR Inactivation

Three likely mechanisms have been proposed for terminating Ca^{2+} release from RyRs: RyR inactivation and adaptation, SR Ca^{2+} depletion, and stochastic attrition [1]. Fabiato proposed that a process of negative-feed back must exist for RyRs, if not CICR would be a continuously amplifying process [136]. Gating in RyR has been compared to VGSCs where there are low affinity activation sites and high affinity inactivation sites [21]. Inactivation of a channel implies refractoriness, there is a period in which the channel cannot be reactivated [137-140]. Reactivation of the channel would require the removal of Ca^{2+} from inactivation sites. A number of studies have produced evidence of refractoriness [81, 136, 137, 141] while others have shown that neighbouring sites can be activated [82]. Similarly, the mechanism of adaptation implies that after a peak $[Ca^{2+}]_i$ is reached, an even greater stimulus is needed to reactivate the channel [110, 138]. These mechanisms could be

physiological significant if they contribute to less spontaneous events and account for the decreased force-frequency relationship that is observed with activation-dependent inactivation [7].

Depletion of SR Ca^{2+} has been suggested as a mechanism for RyR closure simply because there would be no more Ca^{2+} to release. Also, recall that lumenal SR Ca^{2+} regulates the P_o RyR, so P_o RyR may be effectively decreased by Ca^{2+} depletion. Critics of this hypothesis point out that it doesn't account for repetitive Ca^{2+} sparks that occur in the same area [23] or Ca^{2+} sparks that last up to 200 ms [11], situations where Ca^{2+} stores are not exhausted.

Stochastic attrition, the "inherent random closing of an individual channel" is perhaps the least probable mechanism of RyR closure [18]. It would require the simultaneous closing of RyRs and DHPRs in the junctional cleft thereby preventing amplification of the Ca^{2+} signal, the positive feedback mechanism of CICR [18, 137, 142]. This hypothesis is not likely because a sufficient number of channels in a junction would have to close at once. Unless the channels are gated together this may not be a favourable theory [143].

SARCOPLASMIC / ENDOPLASMIC RETICULUM CALCIUM ADENOSINE TRIPHOSPATASE

Structure and Function

The characteristics of SERCA have been extensively studied over the past 50 years (for review see Carafoli [2]). In 1952 Marsh observed that fractions of skeletal muscle induced relaxation. Later in the 1960's Ebashi would show that relaxation was ATP-driven. Hasselbach and Makinose then deduced that Ca^{2+} was sequestered into the SR during

relaxation (1961). By 1970 MacLennan was the first to identify SERCA as a 100 kDa protein in skeletal muscle that comprised from 70% to 90% of SR membrane protein [144].

SERCAs belong to the P-type ATPase family, which are characterised by phosphorylation of an aspartate residue. Two subfamilies of Ca^{2+}-ATPases exist: plasma membrane Ca^{2+}-ATPases (PMCAs) and SERCAs. These pumps couple Ca^{2+} transport to ATP hydrolysis. The SERCA pump is constitutively active in SR membranes [35]. It creates a thousand-fold Ca^{2+} concentration gradient across the SR membrane that is non-electrogenic due to 2 H^+ release and anion exchangers [35].

At least 3 SERCA genes have been identified that share some homology. SERCA1 is mostly found in fast twitch muscle [145]. Its splice variants SERCA1a and SERCA1b differ slightly at the C-terminus [146] and are found respectively in adult and neonatal muscle. SERCA2 is also alternatively spliced into variants that differ at the carboxy terminus [147-149]. A restricted pattern of SERCA2a expression occurs in the heart while SERCA2b is elsewhere ubiquitously expressed in smooth and non-muscle tissue [147, 150, 151]. Less is known of SERCA 3 which is expressed in kidney [152], platelets [153], and endothelial cells [154].

The crystal structures of SERCA in its Ca^{2+} bound and free forms have recently been determined [155, 156]. The pump consists of 10 TM a-helices and three large cytoplasmic domains[157]. The 3 cytoplasmic domains are referred to as the A-, P-, and N- domains. The activation-domain or A-domain is a 125-residue loop connected to TM segments M2 and M3 [155]. The P-domain or phosphorylation domain is 410 residues, interacts with the M4 and M5 TM segments and contains Asp351 that is phosphorylated by ATP [158]. The N-domain or nucleotide binding domain is encased by the P-domain [158]. Approximately 5.7 Å apart are 2 Ca^{2+} binding sites located between M4, M5, M6 and M8 [158]. Site 1

consists of oxygen ligands from TM segments M5, M6 and M8 while site 2 is mostly found on M4 [158].

The rate of Ca^{2+} translocation by SERCA is much slower than an ion channel because conformational changes must occur for Ca^{2+} to reach high affinity binding sites from cytosol to TM, and then from the TM to the lumen [158]. A number of models exist that try to predict the conformational changes that SERCA undergoes during Ca^{2+} transport. The E1-E2 model is adapted from the Post-Elbers model for (Na^+-K^+)-ATPase (described in [158]). It predicts that SERCA oscillates between 2 conformations, E1 and E2. During E1 2 Ca^{2+} molecules from the cytosol can bind SERCA. Phosphorylation of Ca^{2+}-bound SERCA by ATP creates an intermediate, $E2PCa_2$ that aligns the low affinity Ca^{2+} binding site with the lumen. Bound Ca^{2+} is then deposited into the SR during E2 and SERCA is dephosphorylated. The E1 conformation is then recycled.

The four-site model builds on the E1-E2 model by incorporating the low affinity Ca^{2+} binding sites found on SERCA's lumenal side [159]. It predicts that Ca^{2+} molecules are transferred from the high affinity sites to the low affinity sites before release. Alternatively, the modified alternating four-site model agrees that Ca^{2+} is released from cytoplasmic sites, but Ca^{2+} can only bind to lumenal side in E1 or E2 and not the intermediary state [160].

Lipid Interactions

The lipid bilayer immediately surrounding SERCAs is called the annulus (for Review see [158]) . Annular lipid is immobile due to its interaction with the SERCA protein and altered lipid packing in that region [161]. It has been predicted that 30 lipid molecules reside in the annulus [158]. Most SERCA residues are hydrophobic and reside in the similarly charged lipid bilayer [158]. The few charged residues on SERCA maintain neutrality with its

surrounding either by forming ionic bonds or presenting themselves at the lumenal or cytosolic faces of the SR membrane [158]. Relative tilting of a-helices during pump cycles is influenced by TM domain thickness, lipid chain length and composition of annular regions [158, 162]. As constituents of the SR lipid bilayer, phospholipids, fatty acids, and cholesterol could all be potential modulators of SERCA function.

Phospholipids

Phopholipids (PLs) are rapidly exchanged from annular to bulk lipid as the Ca^{2+} pump changes conformation [161]. Both phosphatidylcholine (PC) and phosphatidylethanolamine (PE) impact SERCA function. Among PLs, PC binds preferentially to SERCA over PE regardless of acyl chain length [163]. PC content is 1.7 times higher in fast-twitch versus slow-twitch muscle resulting in increased membrane fluidity due to decreased sphingomyelin content [164]. This may account in part for increased pump kinetics in fast-twitch muscles. At the same time that PC is necessary for SERCA activity, PE increases SR coupling [165]. Unsaturated PE is closely associated with SERCA. The purpose of it may be to maintain membrane fluidity in the annulus and allow for easy transitions between pump conformations [166, 167]. While anionic PLs are thought to increase the coupling ratio of SERCA [168], methylation and glycosylation of PLs have been shown to decrease its activity [165].

Fatty Acids

Charged headgroups of fatty acids also bind SERCA in the lipid annulus [169, 170]. Binding in the annular layer is enhanced when SR lipid is in the liquid crystalline phase as opposed to gel phase lipid [163]. The fatty acid profile of SR membranes differs between longitudinal and terminal SR. The majority of unsaturated fatty acids are found in the

terminal cisternae [171]. Unsaturated fatty acids are necessary for SERCA activity [172]. Membrane thickness and fluidity as determined by FA acyl chain length vary directly with SERCA activity [173, 174]. SR FAs may correlate to muscle function, since fast-twitch muscle contains much higher amounts of docosahexaenoic acid than slow-twitch muscle [175].

Cholesterol

Studies of cholesterol effects upon SERCA function have been controversial. Much of the debate has centred around whether or not cholesterol is excluded from the slowly exchanging annular lipid region [176-179]. Under normally simulated Ca^{2+} transport conditions, cholesterol has no effect on SERCA activity [176, 177]. However, when SR membranes are made permeable, an inhibitory effect of cholesterol on SERCA activity is revealed [178, 179]. Another point to consider is that cholesterol could act at other non-annular SERCA sites, as exemplified by the binding of thapsigargin [158, 180]. Also, the cholesterol content of SR lipids also varies between fast- and slow-twitch muscle types, which suggests that cholesterol does influence SERCA activity [164]. This data indicates that cholesterol may interact with the protein or affect its surrounding fluidity [178].

Phospholamban

First identified by Tada and colleagues in 1975, phospholamban (PLB), an endogenous inhibitor of SERCA function, is expressed in smooth muscle cells, slow-twitch and cardiac muscle [2, 181]. It is a 22-27 kDa hydrophobic protein [44, 181]. PLB spans the SR membrane with two alpha helical regions extended into the transmembrane region and cytosolic space on either side of a ß-turn (Lamberth and Carafoli 2000). PLB is regulated by PKA and CaMK phosphorylation at serine 16 and threonine 17 [2, 44].

Dephosphorylated PLB binds near the P-site on SERCA [182]. It suppresses SERCA activity by interaction of a 30 residue cytoplasmic region with the pump's M6 transmembrane region lying immediately beneath its cytoplasmic N-domain [44, 183, 184]. This then inhibits SERCA by decreasing its Ca^{2+} affinity [2].

INVOLVEMENT OF THE SARCOPLASMIC RETICULUM IN ARRHYTHMOGENESIS

"When SR Ca^{2+} load is elevated, it may be the rise in $[Ca^{2+}]_{SR}$ which triggers the so-called spontaneous SR Ca^{2+} release during Ca^{2+} overload that can be directly arrhythmogenic." [7]

Inappropriate calcium mobilization is the cause of much cellular pathology including necrosis, apoptosis, and cardiac arrhythmias. Normally, an influx of Ca^{2+} from the interstitial fluid during cardiac excitation triggers the release of Ca^{2+} from the sarcoplasmic reticulum. This free cytosolic Ca^{2+} activates the contraction of the myofilaments. One type of cardiac arrhythmia, termed delayed after depolarisations (DADs), stems from improper Ca^{2+} handling. This additional excitation may appear near the very end of repolarisation or just after repolarisation in the excitatory potential of the myocardium. Such afterdepolarisations may themselves trigger propagated impulses. DADs are likely to occur when the basic cycle length of the initiating beats is short and when the cardiac cells are overloaded with Ca^{2+}.

Conditions that foster Ca^{2+} overload present themselves during heart failure. Free cytosolic Ca^{2+} has been shown to increase over several minutes during ischemia [185-187]. Ca^{2+} is initially increased by release of Ca^{2+} from the SR during ischaemia then the extracellular space during reperfusion [188]. A number of factors contribute to this rise in Ca^{2+} including reduced forward activity of Ca^{2+}-ATPases, outward NCX activity, and increased SR Ca^{2+} leak. Decreased ATP production during ischaemia prevents both

SERCAs and PMCAs from effectively removing Ca^{2+} from the cytosol [189]. NCX activity increases in the outward direction due to a rise in $[Na]_i$ thereby increasing Ca^{2+} influx [190, 191]. Cytosolic Ca^{2+} content may also be increased by PKA-induced phosphorylation of RyR, which would dissociate FKBP and promote RyR-mediated Ca^{2+} leaks [111]. Elevated cytosolic Ca^{2+} will also be perpetuated by increasing RyR P_o [192-195]. The net effect is a depletion of SR Ca^{2+} store and a rise intracellular Ca^{2+}.

There is potential for regulation of ischaemia-induced arrhythmias from Ca^{2+} overload by affectors of the SR. Cyclopiazonic acid and thapsigargin, SERCA antagonists, decrease the incidence of ventricular fibrillation arrhythmias in rat hearts after ischaemia and reperfusion [196]. Ryanodine prevents ouabain-induced arrhythmias in rabbit and dog hearts[197, 198] although it had no affect on ischaemia and electically induced arrhthymias in dog hearts [198]. The RyR antagonist dantrolene may delay Ca^{2+} overload development during myocardial ischaemia [188]. Both SERCA and RyR are potential targets for anti-arrhythmic agents.

EVIDENCE FOR CARDIOPROTECTION BY OMEGA-3 POLYUNSATURATED FATTY ACIDS

The omega-3 polyunsaturated fatty acids (n-3 PUFAs) have received a great deal of attention due to their many potential health benefits. Among these benefits are improved visual and neural development, immune response, anti-inflammatory and anticancer actions, protection against renal failure and improved lipoprotein levels. Of importance to this study are the cardiovascular effects of the n-3 PUFAs. Epidemiological, dietary, and cellular studies have lent to our understanding of how these lipids may function. To begin we will examine their structure, metabolism, and potential dietary sources.

Essential fatty acids (EFA) are termed so because they cannot be synthesised by the human body, and therefore must be acquired in the diet. The two precursors to the EFA families are linoleic acid (C18: 2 n-6) and linolenic acid (C18: 3 n-3). Both the n-3 and n-6 fatty acids belong to the family of polyunsaturated fatty acids. PUFAs are characterised by more than one double bond in the carbon chain that constitutes a fatty acid. Linolenic acid (ALA) is metabolised by a process of elongation and desaturation into the predominant n-3 PUFAs, eicosapentaenoic acid (EPA, C20: 5) and docosahexaenoic acid (DHA, C22: 6).

n-6 PUFAs are distinguished from n-3 PUFAs by the location of their double bond from the methyl terminus. n-6 fatty acids have a double bond six carbons in from the methyl-terminus, while n-3 PUFAs have a double bond at the third carbon from the methyl-terminus. A major n-6 fatty acid found in the body after conversion of linoleic acid is arachidonic acid (AA, C20: 4, n-6).

Metabolism is a collective term for the synthesis and degradation of a substance. PUFA metabolism is segregated into different locations of the cell. PUFA synthesis occurs in the cytoplasm, while PUFA degradation occurs in the mitochondria and peroxisomes. PUFA synthesis can arise from a variety of pathways in which acetyl CoA plays a central role. The catabolism of fatty acids is usually through the pathway of ß-oxidation.

The process by which PUFAs are metabolised is through a sequence of elongation and desaturation. Both n-3 and n-6 PUFA metabolism is catalysed by the ? 4-, ? 5-, ? 6-desaturases and elongase. Since n-3 and n-6 polyunsaturated fatty acids utilise the same enzymes in their metabolism, they compete with each other for these enzymes. To maintain homeostasis, an optimal balance must be achieved between the relative amounts of these two PUFA groups in the diet. This equilibrium is often described by a ratio of n-6 to n-3 PUFAs. The recommended ratio is 4:1 [199].

A general consensus in regards to fat intake and improving one's health has been to reduce total fat, saturated fat, and cholesterol intake [200]. Health and Welfare Canada recommends that of total caloric intake, 30 % consist of dietary fat. Of that 30 %, there should be an equal distribution of saturated-, monounsaturated-, and polyunsaturated- fatty acids. Potential sources of n-3 PUFA intake include fatty fish and flaxseed. The Adequate Intake (AI) values for n-3 are 1.6 g/d and 1.1 g/d for men and women respectively [201]. On the upper end, the Nutrition Committee of the American Heart Association deems an n-3 fatty acid intake of 15 % of total calories to be excessive while 10 % of calories to be acceptable [200]. Epidemiological and controlled randomised trials indicate that doses of 0.5-1.8 g/d EPA and DHA or 1.5 – 3 g/d ALA are preventive against CV mortality [202].

EPIDEMIOLOGY AND CONTROLLED TRIALS

Several studies have shown that the consumption of n-3 PUFAs in foods like fish and flaxseed decrease CV mortality. The premier studies in this field by Bang *et al.* [203] observed decreased cardiovascular mortality in Greenland's Inuit which they attributed to their n-3 PUFA enriched diet. This work set the stage for further analyses to establish the inverse relationship between populations consuming relatively high n-3 PUFA diets and their mortality and propensity for CV events [204-206]. Proposed mechanisms for this beneficial action initially focused on the prevention of coronary atherosclerosis [207]; however, direct actions on the myocardium are now proposed.

Some of the frequently cited studies on n-3 PUFAs and CV disease include the Chicago Western Electric Study, which examined nearly 2 000 men from the ages of 40-55 years. Over a 30 year follow-up fish consumption of more than 35 g/d was inversely correlated with death from CVD, especially from MI [208]. A study on women in the

Nurses Health Study also correlated n-3 PUFAs from fish with decreased incidence of coronary heart disease and death [209]. The Diet and Reinfarction Trial (DART) advised over 2 000 survivors of MI on diet over two years and demonstrated that fish eaters, who consumed 200-400 g fatty fish/week or 500-800 mg/day n-3 PUFAs, had a 29% decrease in mortality [210]. Burr *et. al* reasoned this was due to decreased fatal MIs, ventricular fibrillation and not changes in serum lipids [211]. The GISSI-Prevention Study also demonstrated a 20 % decrease in all-cause moratality and 45% decrease in sudden death from n-3 PUFA supplementation [212]. Other CV related effects establish that DHA and not EPA lowers blood pressure and heart rate significantly in humans [213]. Fish meals reduce primary cardiac arrest [214] and decrease infarct size [215, 216].

Specific data gathered on ALA consumption associates it with lower risk for MI and fatal ischaemic heart disease [202]. An inversely proportional relationship between 1.4 to 1.5 g/day ALA and risk of fatal ischaemic heart disease in women [217] and men [218] was established in addition to a similar relation to coronary artery disease in the National Heart, Lung, and Blood Institute Family Heart Study [219]. Also groups administered ALA-containing mustard oil and DHA- and EPA-containing fish oil both had decreased incidences of MI [220]. Using ALA supplements, the Lyon Study exhibited 70% lower CV mortality at 5 years compared to control without changing serum lipids [221].

While there is a great deal of data that correlates an effect of n-3 PUFA feeding with positive CV outcomes, some studies suggest that there is no effect [205, 222-224]. Kris-Etherton et al. [202] propose that an inverse relation between fish consumption and CV mortality is established only when a sufficiently large non-fish eating population is used as a control.

Evidence lends support that n-3 PUFAs may alter the myocardium. Both fish meals and fish oil supplements increase heart rate variability which is associated with decreased arrhythmia [225, 226]. Also, purified DHA and EPA alter the heart rate and increase diastolic filling of the heart [227]. Some postulated mechanisms for the positive CV outcomes associated with n-3 PUFAs are decreased occurrence of ventricular arrhythmias and atheroma progression, hypotriglyceridemic, antithrombogenic and hypotensive properties and increased NO-induced relaxation [228].

DIETARY MECHANISMS

Dietary studies by Peter McLennan on rat hearts have shown that feeding a diet rich in n-3 PUFAs significantly reduces the incidence and severity of arrhythmia occurring in ischaemia [229, 230]. PUFAs, including n-3 and n-6, but not MUFAs or SATs prevent ventricular fibrillation in a rat model of ischaemia and reperfusion [229]. More recent studies by McLennan indicate that an n-3 PUFA diet in rats decrease ischaemic markers like acidosis, K^+, lactate, CK, and increase contractile recovery during reperfusion due to reduced myocardial oxygen consumption (MVO_2) [231]. Other physiological findings in the field confirm that n-3 PUFA containing fish oils reduce ischaemic damage [232] and arrhythmia [233, 234]. In particular, spontaneous tachycardias, ventricular fibrillation, ventricular tachycardia and the duration of arrhythmias are all positively reduced [233, 234]. However, similar observations were not described in models of acute recurrent ischaemia [235].

A handful of studies have looked at the effects of dietary manipulation of n-3 PUFA content in the SR. Parameters examined include Ca^{2+} uptake, SERCA activity, PL and fatty acid composition, lipid order, membrane fluidity, SR Ca^{2+} content, and Ca^{2+} sparks. The most comprehensive study to date by Taffet et al looked at CSR from rats fed diets

containing either menhaden oil or corn oil, n-3 or n-6 PUFA sources [236]. Analysis of cardiac SR (CSR) phospholipid membranes revealed an increased DHA/AA ratio and corresponding n-3/n-6 content. Both Ca^{2+} uptake and SERCA activity were reduced in the n-3 PUFA group, which could not be accounted for by changes in membrane permeability or active pump sites [236]. Taffet showed that n-3 PUFAs are incorporated into CSR PLs without affecting permeability but interacting with membrane proteins, specifically SERCA. They proposed that dietary alteration of CSR lipids affects SERCA function by way of changes in the structure or composition of PL membranes. CSR may function like skeletal SR which displaces membrane lipid during conformational changes [237]. Changes in n-3 PUFA content in the lipids immediately surrounding SERCA and in the bulk lipid membrane could affect this process by resisting displacement [236].

Another study in fish oil fed rats, examined Ca^{2+} sparks in atrial myocytes [238]. Incorporation of n-3 PUFAs into these cells produced Ca^{2+} sparks with shorter duration than those formed in myocytes from rats fed a saturated fat diet. The group attributed their observations to either decreased activatory Ca^{2+} (I_{Ca}) or faster RyR kinetics (decreased P_o) [238]. This study suggests a direct effect of n-3 PUFAs on SR protein function.

Analysis of CSR from mice fed menhaden oil also exhibit incorporation of n-3 PUFAs, DHA and EPA, and a decreased n-6/n-3 ratio [239]. Both the initial rate and maximal rate of Ca^{2+} uptake were depressed in the n-3 PUFA group [239]. As in Taffet's study, this slowing of Ca^{2+} uptake was accounted for by SERCA activity that was decreased to $1/6^{th}$ of control. They proposed that since PC content and composition affects SERCA, a change in PC with n-3 PUFA content may alter SERCA activity and render SR less susceptible to "large rapid" fluxes in Ca^{2+} that can occur during reperfusion and ischaemia [239].

A second study on mice fed either ALA, EPA, or DHA for 14 days showed that CSR underwent marked membrane lipid changes as compared to control diets [240]. Here, there was a compensatory increase in saturated fatty acids and a decrease in oleic acid. Metabolism of precursor n-3 PUFAs into DHA produced corresponding decreases in AA and LA. In response, Ca^{2+} uptake was decreased in all groups compared to an n-6 diet, but Ca^{2+} uptake was depressed to a lesser extent in the ALA-fed group. In contrast to the aforementioned studies by Swanson and Taffet, maximal SERCA activity and SERCA Ca^{2+} affinity were not altered. Croset *et. al* suggest that Ca^{2+} uptake was decreased in n-3 PUFA fed groups due to uncoupling of the membrane due to changes in membrane permeability [240].

Diabetic cardiomyopathies may also be altered by n-3 PUFA supplementation. In studies by Black et. al. coronary and aortic flow rates and diastolic function were improved with the n-3 PUFA preparation, Promega [241, 242]. Their work revealed improved SR Ca^{2+} transport activity which may explain the improved diastolic function in this group [241].

Fluorescence anisotropy was used to assess membrane lipid order in skeletal SR, which can serve as a model for less abundant and robust CSR, from fish oil fed rats [243]. "Barely" significant changes in lipid order were established [243]. The authors thought that compensatory incorporation of cholesterol may allow for continued ordering of the membrane [243] even though cholesterol normally only accounts for 5% of SR lipids [244]. They "suggest(ed) that in natural membranes the complex mixture of phospholipid molecular species can, by itself, act as a passive buffer to prevent the response from large changes in fatty acyl chain compositions and motional properties [243]." In contrast to the preceding CSR data, SERCA catalytic activity was not affected by n-3 PUFA feeding in this skeletal SR preparation.

To date, n-3 PUFA feeding studies indicate that both CSR and skeletal SR fatty acids are responsive to changes in diet [236, 239, 241-243]. Confounding results have been reported on Ca^{2+} transport and SERCA catalytic activity in these groups [236, 239, 241, 243]. It is also debateable that membrane permeability, fluidity and order are not altered by relatively modest changes in n-3 PUFA content [236, 243]. Whether or not incorporation of n-3 PUFAs into SR phospholipids contributes to their known antiarrhythmic action requires further study. In addition, we have relatively little information on the effects that chronic dietary interventions may have that would be expected to alter ALA levels.

FREE FATTY ACID MECHANISM OF ACTION

The mechanism whereby unesterified n-3 PUFAs exert their anti-arrhythmic action has been actively researched over the past decade. The major findings have indicated that n-3 PUFAs act by enhancing the electrical stability of heart cells. Alexander Leaf and his associates have conducted many of the fundamental studies done in this area. In studies by Billman and Leaf, infusion of n-3 PUFA emulsions prevented the acute occurrence of ischaemia-induced ventricular fibrillation in exercising dogs [245-247]. Fish oil, non-esterified -ALA, -EPA and -DHA were each shown to prevent fibrillation when given as an infusion prior to an exercise-ischaemia test [245-247].

Leaf and his group have also focused on the cellular mechanism of the acute addition of unesterified ? 3 PUFAs (see Kang and Leaf for review [248]). Initial studies in neonatal rat cardiomyocytes illustrated that EPA prevented asynchronous contractility induced by high $[Ca^{2+}]$ or ouabain [249]. Voltage-clamp experiments revealed that n-3 PUFAs reduce the conductance of VGSCs (IC_{50} 4.8 μM EPA, DHA), L-type Ca^{2+} channels (IC_{50} 0.8 μM DHA) and initial outward K^+ current (I_{to}), and I_K delayed rectifier (I_K) [248, 250-253]. Based

on their observations they hypothesise that n-3 PUFAs electrically stabilize cardiac myocytes by increasing the electrical stimulus required to elicit an action potential by hyperpolarizing the membrane and raising the threshold of VGSC activation [248]. They are also thought to prolong the refractory period of cardiomyocytes [254].

Other studies have shown that alternative pathways of arrhythmogenesis may be inhibited by n-3 PUFAs. Na^+-H^+ exchanger inhibition by EPA and DHA could prevent the unfavourable activation of the NCX during ischaemia [255]. Studies have also examined the acute addition of n-3 PUFAs to ventricular myocytes to observe their effect on the SR [256-258]. n-3 PUFAs appear to decrease the frequency of both Ca^{2+} sparks and spontaneous Ca^{2+} release resulting in decreased amplitude of contraction. They concluded that PUFAs may exert their antiarrhythmic effects at the level of the SR in addition to the SL membrane.

Rodrigo's studies of EPA on permeabilised ventricular myocytes revealed an eventual decrease in contraction in both guinea pig and rat myocytes [256]. They proposed that the decreased amplitude of contraction is due to either decreased Ca^{2+} uptake because of L-type Ca^{2+} channel inhibition or increased threshold for Ca^{2+} release from the SR. They correlated a reduction in the frequency of spontaneous contractions with decreased P_o RyR, which is regulated by both SR and cytosolic Ca^{2+} [256]. It is suggested that EPA may decrease P_o RyR by acting on SR K^+ channels which maintain electroneutrality, since K^+ inhibition reduces Ca^{2+} uptake, Ca^{2+} content and ultimately affects spontaneous RyR activity [259].

Negretti *et al.* propose that EPA, an n-3 fatty acid, is a negative inotrope [257]. That is, it decreases the velocity and force of contraction of the heart. These two parameters are functions of Ca^{2+} concentration; as such the suspected mechanism by which EPA works is through Ca^{2+} handling. The effects of the two n-3 PUFAs, EPA, and DHA, on spontaneous

and electrically stimulated contractions in single, isolated ventricular myocytes from rat hearts were examined. The study concluded that EPA and DHA inhibit spontaneous waves of propagating Ca^{2+} release, frequency, amplitude, and resting cell length [257]. Another paper by the same authors concluded that EPA and DHA act through the inhibition of Ca^{2+} release and reduced availability of Ca^{2+} to the SR [258]. These effects were thought to be similar to tetracaine, a known antagonist of the RyR. Tetracaine decreases the open probability of the RyR and increases SR Ca^{2+} content [260]. Overend and colleagues describe this phenomenon as a compensatory mechanism where a decreased frequency of spontaneous release from the SR in the presence of tetracaine is made up for by an increase in release of Ca^{2+} when spontaneous release does occur.

A review of the literature indicates that n-3 PUFAs are anti-arrhythmic agents. The anti-arrhythmic action may occur after dietary administration, when we would expect n-3 PUFAs to be esterified to hyrdrophobic membrane constituents, as indicated by the studies of Black where SR Ca^{2+} uptake was enhanced in rats with diabetic cardiomyopathy [241]. Likewise, administration of n-3 PUFAs in their free or unesterified forms has been shown to alter ion channel conductance in studies by Leaf [248]. Our studies attempted to examine both of these aspects in relation to the SR. Isolated SR vesicles were assayed for function after the exogenous addition of n-3 PUFAs and after chronic dietary interventions. These studies on SR function formed part of a larger study where n-3 PUFA supplementation in rabbits was assessed in terms of arrhythmia and atherosclerosis in cholesterol-fed rabbits. Since arrhythmias contribute considerably to mortality from myocardial infarctions, n-3 PUFAs may act as positive modulators of cardiovascular health.

II. EXPERIMENTAL PROCEDURES

MATERIALS

Linoleic acid (LA, C18:2 n-6), alpha-linolenic acid (ALA, C18:3 n-3), eicosapenataenoic acid (EPA, C20:5 n-3), and docosahexaenoic acid (DHA, C22:6 n-3) were purchased from Doosan Serdary (Toronto, ON). Standard rabbit chow was from CO-OP Complete Rabbit Ration, Federated Co-operatives Limited (Sakatoon, SK) and Promega Flax was from Polar Foods Inc. (Fisher Branch, MB). Roche Molecular Biochemicals (Laval, PQ) supplied: adenosine triphosphate (ATP), creatine kinase (CK) creatine phosphokinase (CPK), dithiothreitol (DTT), leupeptin (Leu), nicotinamide adenine dinucleotide (NADH), phenylmethylsulfonyl fluoride (PMSF), and pyruvate kinase (PK). Calbiochem (San Diego, CA provided lactate dehydrogenase (LDH), ryanodine (Ry), and ionomycin (Io), while phosphoenol (PEP) was supplied by Fluka (St. Louis, MO). Calcium Green-2, Mag Fura-2, and Fura-2 were made available by Molecular Probes (Eugene, OR). Electrophoresis reagents and DC protein assay reagents were purchased from Bio-Rad Laboratories (Hercules, CA). All of the gas chromatography standards were obtained from Nu-chek Prep (Elysian, MN). NEN Life Sciences (Woodbridge, ON) supplied [^{3}H]-ryanodine. Millipore filters for the binding assay were obtained from Fisher Scientific (Napean, ON). All other reagents were of analytical grade and from Sigma (St. Louis, MO).

METHODS

ACUTE STUDY

SKELETAL MUSCLE SARCOPLASMIC RETICULUM ISOLATION

Isolation of skeletal HSR from White New Zealand male rabbits followed the method of Gilchrist *et al.* [261]. Briefly, Frozen rabbit skeletal muscle (160 g) was ground

under liquid nitrogen with a mortar and pestle. Ground muscle was then suspended in 800 mL of homogenization buffer containing 300mM sucrose, 20mM imidazole, 1mM PMSF, 1mM DTT, 500 μM ATP-TRIS, 500 μM EGTA, 2uM Leupeptin, pH 7.4. The suspension was then processed in a small Waring blender on low for 40 seconds twice with a 20 second interval. The processed muscle was subsequently centrifuged at 10 000 rpm (15 300 x g) for 15 minutes in a JA-14 Beckman Rotor. The supernatant was filtered through cheesecloth and re-centrifuged in a Beckman JA-20 rotor at 19 000 rpm (43 700 x g) for 1 hour. Pellets were then re-suspended in homogenization buffer containing 2 mM PMSF, 2 mM DTT, and 4 μM Leupeptin. Re-suspended pellets were gently homogenised under a glass mortar and pestle and volume was built up to 18 mL. The suspension was gently layered on previously thawed 25-45% linear sucrose gradients. The gradients were spun at 23 000 rpm on a Beckman SW-28 swinging bucket rotor for twenty-four hours. Following centrifugation, the light SR (LSR) and heavy SR (HSR) bands were harvested from the gradients. The two bands were then washed at 4 °C for one hour in 300mM sucrose and 200mM KCl. The two salt mixtures were centrifuged for 1 hour in a 50.2 Ti rotor at 35 000 rpm. The pellets were re-suspended in transport buffer containing 300mM sucrose, 50mM Dipotassium PIPES, pH 7.0 and stored at -70 °C.

SKELETAL SR PROTEIN DETERMINATION

HSR protein concentrations were determined from a bovine serum albumin standard according to Harrington's modified Lowry method [262].

STOPPED-FLOW RAPID KINETIC FLUORIMETRIC ASSAY OF EXTRALUMINAL Ca^{2+} TRANSPORT and SERCA1 CATALYTIC ACTIVITY IN SKELETAL SR

Freshly thawed HSR vesicles (0.25 mg/mL) were pre-incubated with 0.8 μM Calcium Green-2 (CG-2) in transport buffer (300 mM sucrose/50 mM dipotassium pipes, pH 7.0, 25 °C) containing 20 units/ml PK, 14 units/ml LDH, 200 μM NADH, 0 μM or 20 μM Ca^{2+}, and n-3 PUFAs at varying concentrations. Ca^{2+} transport was initiated with the combined addition of 2.5 mM PEP and 1 mM Mg·ATP in a SFA-20 Stopped Flow Accessory from Hi-tech Scientific (Salisbury, U.K.) with pneumatic drive set at 4 bars pressure. Fluorimetric measurements of Ca^{2+} transport and SERCA catalytic activity were made in a Photon Technology International Quantimaster™ steady state spectrofluorometer with a Delta Ram upgrade. Data was acquired with Windows©-based Felix-software. Extraluminal Ca^{2+} transport was monitored with CG-2 (Ex_{506}/Em_{531}) while NADH fluorescence (Ex_{377}/Em_{451}) was used as an indicator of SERCA1 hydrolysis of ATP through a coupled PEP/PYR enzyme pathway and ATP regenerating system.

Where skeletal SR was assayed solely for SERCA activity, HSR membranes (0.1 mg/mL) were permeabilized with 10 μM ionomycin, 20 units/ml PK, 14 units/ml LDH, 200 μM NADH, 5 μM Ca^{2+} free (256 μM Ca^{2+}/250 μM EGTA) prior to transport and pre-treated with varying concentrations of n-3 PUFAs in transport buffer (300 mM sucrose/50 mM dipotassium pipes, pH 7.0, 37 °C). Ca^{2+} transport was initiated with the combined addition of 2.5 mM PEP and 1 mM Mg·ATP in a SFA-20 Stopped Flow Accessory. NADH fluorescence (Ex_{377}/Em_{451}) was used as an indicator of SERCA1 hydrolysis of ATP through a coupled PEP/PYR enzyme pathway and ATP regenerating system.

FLUORIMETRIC ASSAY OF CA^{2+} PULSE-LOADING IN SKELETAL SR

Fluorimetric measurements of Ca^{2+} pulse-loading in skeletal SR were made in a Photon Technology International Quantimaster™ steady state spectrofluorometer with a Delta Ram upgrade. Data was acquired with Windows©-based Felix-software. The reaction mixture consisted of 0.8 μM CG-2, 400 μM NADH, 14 U/mL LDH, 20 U/mL PK, DHA, and transport buffer (300 mM sucrose/50 mM dipotassium pipes, pH 7.0, 25 °C) in a 3 mL cuvette with magnetic flea. It was equilibrated for approximately 80 seconds, after which the assay was initiated by the simultaneous addition of 1mM Mg·ATP and 2.5 mM PEP. When a steady baseline was obtained, incremental boluses of 10 μM Ca^{2+} were administered with a "repeator" pipette, returning to baseline before the next pulse was given.

SKELETAL ^{3}[H] – RYANODINE BINDING

Briefly, HSR (0.25 mg/mL) were incubated overnight in a reaction media consisting of transport buffer (300 mM sucrose/50 mM dipotassium pipes, pH 7.0, 25 °C), 1.03 mM $CaCl_2$ and 250 μM EGTA (10 μM Ca^{2+} free), 1 mM DTT, 4 μM leupeptin, 4 nM [^{3}H]-ryanodine, 10 mM creatine phosphate, 4 units/mL creatine phosphokinase, and 1 mM Mg·ATP. Aliquots (3 x 100 μl) were then vacuum filtered across a 0.45 μm HAWP 13 mm diameter filter. Each filter was washed twice with 500 μl transport buffer. Filters were then dissolved overnight in 2 ml CytoScint and 200 μl ethylene glycol. Radioactivity of specific [^{3}H]-ryanodine was then determined by liquid scintillation counting from total and non-specific counts in the presence of 100 μM cold ryanodine.

FATTY ACID PREPARATION

Stock solutions (1 mM) of individual n-3 PUFAs were prepared in silanized glass tubes. Pipetted volumes of n-3 PUFA and H_20 were combined in a glass test tube, sonicated and vortexed at 4 °C in a Branson 1200 bath sonicator (Markham, ON) until the emulsion resolved into a uniform cloudy suspension [255]. Where the n-3 PUFA, LA, was suspended in hexane, the solvent was dried-off with nitrogen gas before re-suspension in H_20.

SDS-PAGE OF SR PROTEINS TREATED WITH n-3 PUFAS

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of HSR membranes was carried out according to Gilchrist *et al.* [261]. Briefly, HSR (1 mg/mL) in 0.1 % CHAPS, 0.1% saponin, or 400 μM PUFA were vortexed for 1 hour at room temperature. Samples were then spun down for 30 minutes at 75 000 rpm in a Beckman TLA 100 rotor (150 000 x g). The supernatant was precipitated with trichloroacetic acid / deoxycholic acid on ice and spun at 13 500 rpm in an IEC MicroMax® desktop centifuge. The resulting pellet was resuspended in 25 μL of 1 x sample buffer for analysis. SR proteins were stained with Coomassie brilliant blue R-250.

FEEDING STUDY

FEEDING PROTOCOL

Male NZW rabbits were randomly fed one of four diets for 8 weeks: regular chow, or regular chow supplemented with 10% flaxseed (wt/wt), 0.5% cholesterol (wt/wt), or 10% flaxseed and 0.5% cholesterol. Briefly, diets were weighed, ground, reconstituted into pellets with H_20, dried, and stored in the dark at 4 °C. Rabbits that did not undergo an ischaemia-reperfusion protocol were fed *ad libitum* while those that were subjected to ischaemia-reperfusion were fed 125g/ day.

PLASMA SAMPLING AND TISSUE HARVESTING

Blood samples were taken from rabbit left marginal ear vein every 2 weeks beginning at week zero and collected in Vacutainer tubes. Samples were spun at 5 000 rpm (4 500 x g) for 10 minutes at room temperature in an Eppendorf Centrifuge 5804R with A-4-44 rotor. The upper plasma layer was pipetted into eppendorf tubes and frozen at -80 °C for later analysis. Cholesterol and triglyceride measurements were made with the VetTest 8008 blood chemistry analyser (IDEXX Laboratories Inc., Westbrook, ME, USA). Samples were ready for testing after they were brought to room temperature and re-spun at 8 000 rpm (6 800 x g) in an Eppendorf Centrifuge 5804R with F45-30-11 rotor.

At 8 weeks rabbits were anaesthetised with isoflurane. Hearts were rapidly excised, rinsed in 0.09% NaCl, and frozen in liquid nitrogen. Alternatively, some hearts underwent ischaemia-reperfusion on a modified Langendorff apparatus before quick freeze. These hearts were rapidly excised and placed into Tyrode's solution of pH 7.4 containing (mM) NaCl 115.0, $NaHCO_3$ 28.0, NaH_2PO_4 0.5, glucose 20.0, KCl 4.0, $CaCl_2$ 2.0, and $MgCl_2$ 0.7. The aorta was tied onto the cannula of the perfusion apparatus. Retrograde perfusion of the hearts was initiated within 60 s of excision. Hearts were perfused at a flow rate of 20 ml/min with Tyrode's solution maintained at 37.0 $\pm$ 0.5°C and bubbled with 95% O_2/5% CO_2. Hearts were equilibrated for 50 min prior to a 30 min test ischaemia. Global ischaemia was initiated by bypassing the flow to the heart and returning it to the buffer reservoir. The solution in the acrylic bath was bubbled with 95% N_2/5% CO_2 during ischaemia. Hearts were then reperfused for 45 min by re-establishment of flow with oxygenated Tyrode's and the immersion bath was again bubbled with 95% O_2/5% CO_2.

CARDIAC SARCOPLASMIC RETICULUM ISOLATION

CSR was isolated according to the method of Feher [263] with some modifications. Four rabbit hearts from different feeding groups were prepared at a time. Hearts were ground to a fine powder under liquid nitrogen and put in 5x weight volume ice-cold homogenization buffer (1M KCl, 10 mM Imidazole containing 2 μM leupeptin, 1 mM DTT, and 1 mM PMSF). The suspension was then homogenized at 6 000 rpm using a PT3100 Polytron by Kinematica. The slurry was pulsed for 20 seconds two times and then spun down in a Beckman JA-20 rotor at 9 000 rpm for 20 minutes (10 000 x gmax). The pellet, P1, was rehomogenized 2 x 20 seconds at 6000 rpm with the polytron in the original volume and centrifuged in a Beckman JA-20 rotor at 7 000 rpm for 20 minutes (6 000 x g max). The resulting supernatant, S2, was then centrifuged for 25 minutes at 14 000 rpm (24 000 x gmax). Supernatant, S3, was layered on a gradient of 3 mls of 35 % sucrose in homogenization buffer and 5 mls 25 % sucrose in homogenization buffer. The layered gradient was centrifuged at 18 500 rpm for 2 hours (41 000 x gmax). Pellet, P4, was built up with an equal volume of homogenization buffer and centrifuged again (41 000 x gmax) for 1 hour. The final pellet, P5, is resuspended in transport buffer (300 mM sucrose, 50 mM dipotassium pipes, pH 7.0) containing 2 μM leupeptin and 1 mM DTT. Aliquots of CSR were quick frozen with liquid nitrogen and stored at –70 °C.

CARDIAC SARCOPLASMIC RETICULUM PROTEIN DETERMINATION

CSR protein content was determined with the Detergent-Compatible (DC) Protein Assay supplied by Bio-Rad using BSA as a standard. Both standards (0.2-1.0 mg/mL BSA) and samples (20 μL) were built to 100 μL in transport buffer (300mM sucrose, 50mM dipotassium pipes, pH 7.0) containing 2 μM leupeptin, 1 mM DTT, and 0.1% SDS. Dilutions were then mixed with reagents according to the Bio-Rad DC Protein Assay

protocol and were read at 750 nm in a MRX® Revelation™ with Temperature Control Microplate Spectrophotometer by Dynex Technologies.

KINETIC MEASUREMENT OF EXTRALUMINAL Ca^{2+} TRANSPORT BY CARDIAC SARCOPLASMIC RETICULUM

Freshly thawed CSR vesicles (0.125 mg / mL) were mixed with 1 μM Fura-2 in transport buffer containing 20 U / mL CK, and 5 μM Ca^{2+} and pipetted into separate wells in a single column of a 96-well black flat bottom Microfluor® 1 microplate. The samples were then pre-incubated at 37 °C for 10 minutes in a SPECTRAmax® Gemini XS Dual-Scanning Microplate Fluorometer by Molecular Devices Coorporation. At time 0 seconds, Ca^{2+} transport was initiated by the combined addition of 10 mM CP, 1 mM Mg^{2+}ATP and 5 mM K^{+}-oxalate from a multichannel pipette to the microplate column. The reaction volume was then thoroughly mixed. At time 30 seconds, data collection of extraluminal Ca^{2+} transport began. Fura-2 fluorescence ($Ex_{340, 380}$ / Em_{510}) data was acquired in the microplate fluorometer with SOFTmax™ PRO software. A kinetic assay was run over 15 minutes with readings every 10 seconds. Each reading was an average of 6 reads/well. Dual-excitation of the dye occurred at both 340 nm and 380 nm with emission at 510 nm. The plate was automixed before and between readings for 1 second. The photo-multiplier tube was set to medium. Fura-2 was calibrated with both 5 mM Ca^{2+} and 100 mM EGTA in the reaction media. Ratios of the 340nm / 380nm signals were computed in Microsoft® Excel2000. Free calcium conversions and rates were then executed in Graphpad Prism™ software.

KINETIC ASSAY OF SERCA2a CATALYTIC ACTIVITY

At 37 °C CSR membranes (0.010 mg / mL) were incubated with transport buffer containing 20 U / mL PK, 14 U / mL LDH, 200 μM NADH and 10 μM 4-Bromo-A23187

in a 96-well black flat bottom Microfluor® 1 microplate. The SERCA antagonist, 10 μM Thapsigargin, or an equivalent amount of vehicle was added to determine specific SERCA activity. ATP hydrolysis was initiated with the combined addition of 2.5 mM PEP, 1mM Mg^{2+}ATP, 101 μM Ca^{2+} and 100 μM EGTA from a multichannel pipette to the microplate. NADH fluorescence (Ex_{377} / Em_{451}) was used as an indicator of SERCA2a hydrolysis of ATP through a coupled PEP / PYR enzyme pathway and ATP regenerating system. The reaction was monitored 30 seconds after initiation with SPECTRAmax® Gemini XS Dual-Scanning Microplate Fluorometer by Molecular Devices Coorporation using SOFTmax™ PRO software. A kinetic assay was run over 2 minutes. Readings were taken every 10 seconds as an average of 6 reads/well. The microplate was automixed for 1 second between reads. Data analysis was done in Graphpad Prism™ software.

SPECTROPHOTOMETRIC DETERMINATION OF CHOLESTEROL COMPOSTITION OF CARDIAC SARCOPLASMIC RETICULUM

Previously frozen CSR was double-extracted in 2:1 $CHCl_3$: MeOH and resuspended in 95% EtOH. CSR, 125 μg, was pipetted into a borosilicated glass tube. The volume of CSR was built to 60 μL with dd H_20. Then 1.2 mL of 2:1 $CHCl_3$: MeOH was added to the tube follwed by 0.25 mL of 0.73% NaCl. The tube was sealed with a Teflon-lined cap and vortexed every 15 minutes for 1 hour. Samples were spun for 5 minutes at 5000 rpm (4 500 x g) in an Eppendorf Centrifuge 5804R with Rotor A-4-44 at 22°C. The resulting bottom layer of $CHCl_3$ was transferred to a cuvette. The remaining aqueous solution was re-extracted with an additional 1.2 mL of 2:1 $CHCl_3$: MeOH and 0.25 mL of 0.73% NaCl. The tube was vortexed and spun as outlined above and the $CHCl_3$ layer was added to the

previous extract. The solvent was removed with N_2 (g). The remaining lipids were then dissolved in 100 μL 95% EtOH and stored at -20°C until needed.

A reaction media consisting of 0.5% Triton-X, 3 mM sodium cholate, 0.1 M Tris, pH 6.6 with 0.25 U / mL cholesterol oxidase, 0.1 mg / mL o-Dianisidine and peroxidase was added to CSR extracts in EtOH. The mixture was pipetted in triplicate into a 96-well 3595 microplate by costar® and incubated at 37°C for 20 minutes in a SPECTRAmax® PLUS^{384} Microplate Spectrophotometer by Molecular Devices Coorporation. To determine free cholesterol absorbance of the samples was read at 450 nm with the microplate spectrophotometer using SOFTmax™ PRO software. To obtain total cholesterol measurements aliquots were incubated with 1 U / mL cholesterol esterase at 37°C for 30 minutes and read again at 450 nm. Esterified cholesterol was then calculated from the total and free cholesterol values.

FATTY ACID ANALYSIS BY GAS CHROMATOGRAPHY WITH FLAME IONISATION DETECTOR

CARDIAC SARCOPLASMIC RETICULUM AND PLASMA ANALYSIS

Samples were prepared for analysis according to the methods of Lepage et al. [264] with adaptations by Ms. Andrea Edel. CSR and plasma samples were thawed and vortexed. Using a serological pipette, 2 mL of methanol-benzene 4:1 (v/v) (containing the internal standard) was added to a borosilicate glass tube. With a Drummond pipetter 100 μL plasma or 50 μL of SR was added to the methanol benzene solution and vortexed well. Acetyl chloride, 200 μL, was added to the mixture over several minutes during continuous agitation. The tubes were sealed with Teflon-lined caps and vortexed again. Methanolysis of the sample occurred over 60 minutes on a 90-95°C heating block. Samples were vortexed every 15 minutes while being heated. Tubes were then cooled at room temperature. To neutralize

the solution 5 mL of 6% K_2CO_3 was added to the tubes using a serological pipette. Each tube was vortexed 5 times and centrifuged for 5 minutes at 4500 g at 22°C in an Eppendorf Centrifuge 5804R with Rotor A-4-44. An aliquot of the upper benzene layer was then transferred with a Pasteur pipette to a low volume insert of an autosampler vial.

Samples were analysed in duplicate on a Varian CP-3800 GC equipped with 1177 injectors, CP-8400 Autosampler, Flame Ionization Detector (FID), 10 μL injection syringe, and CP-Sil 88 Column (50m x 0.25mm ID). Helium was used as the carrier gas and nitrogen as the make-up gas. Column flow was 1.5 mL/min. The split ratio used for injection is shown in the table below

Time (minutes)	Split State	Split Ratio
Initial	ON	5
0.01	ON	50
1.00	ON	5

The injection port temperature was 250°C and the FID temperature was set at 270°C. The column oven temperature followed the parameters outlined below.

Temp (°C)	Rate (°C/min)	Hold (min.)	Total Min.
80	-	1.00	1.00
140	30	0.00	3.00
200	5.0	0.00	15.00
225	5.0	10.00	30.00

The instrument was calibrated using a standard mixture of FAMES and an internal standard was used to check recoveries.

BRAIN, HEART, LIVER, AND KIDNEY ANALYSIS

Previously extracted samples of brain, heart, liver and kidney stored in $CHCl_3$ at -80 °C were removed from the freezer and vortexed. When samples reached 0 °C they were pipetted into pre-weighed tubes at varying volumes depending on the tissue extract (see

Table insert) using a Drummond Micropipette. Each sample was then purged with N_2 to remove the solvent. Once the tube was reequilibrated (approximately 5 minutes later), the tube was reweighed to obtain lipid weight. Next, a Calibra Macropipette was used to add 1 mL of 3:2 MeOH: Benzene solution containing an internal standard to each tube. Samples were vortexed and stored at -20 °C until the remaining samples were processed. Then 1 mL of 5:100 (v/v) acetyl chloride:MeOH was added to the tubes, tubes were vortexed and weighed. Tubes were heated between 95-100 °C for 1 hour and vortexed every 15 minutes to catalyse methanolysis of fatty acids. Samples were returned to room temperature and reweighed to determine if any sample was lost. The reaction in each tube was neutralized with 5 mL of 6% K_2CO_3. Finally, tubes were vortexed twice and centrifuged for 5 minutes at 4500 g, 22°C. An aliquot of the resolved benzene layer was removed with a Pasteur pipette and transferred into a low volume insert in an autosampler vial. Samples were injected into the gas chromatograph as outlined above.

Tissue	Stored in X mL $CHCl^3$	Volume of Tissue Extract Used for Derivatization	Grams of Tissue Used
Brain	3 mL	100 μL	1 g
Heart	2 mL	100 μL	0.25-0.50 g
Liver	3 mL	50 μL	1 g
Kidney	3 mL	100 μL	1 g

III. RESULTS

Recent studies suggest omega-3 polyunsaturated fatty acids (n-3 PUFA) reduce the arrhythmogenic potential of the myocardium. Since disturbances in intracellular Ca^{2+} regulation are arrhythmyogenic, we examined effects of exogenously added unesterified n-3 PUFAs on skeletal SR vesicles (HSR) Ca^{2+} transport. HSR membranes isolated in abundance from fast-twitch skeletal muscle were chosen because they allow for study of both SERCA pump function and RyR mediated Ca^{2+} leak within the same membrane preparation. HSR vesicles are enriched in both SERCA pumps and RyR channels, and they respond dynamically in Ca^{2+} transport assays [261].

The effects of esterified n-3 PUFAs on Ca^{2+} handling by the SR were also studied in a dietary model of n-3 PUFA enriched feed. Since our interest was to elucidate the anti-arrhythmogenic action of n-3 PUFAs in the heart, Ca^{2+} transport by the SR was assessed in isolated cardiac SR vesicles (CSR). In contrast to HSR vesicles, Ca^{2+} transport by CSR vesicles is less robust and its yield is less abundant. Despite these shortcomings Ca^{2+} transport assays were developed to capture Ca^{2+} handling. In addition, our studies on CSR were part of a larger study that assessed the electrophysiological and atherosclerotic effects of this dietary model.

EFFECTS OF EXOGENOUSLY APPLIED PUFAs ON ISOLATED SKELETAL HSR VESICLES

To quantify the response of HSR vesicles to PUFAs we employed recently described fluorimetric methods [261] adapted for stopped-flow rapid kinetic analysis (See Methods). Extraluminal Ca^{2+} transport by isolated SR vesicles was monitored with the fluorophore

Calcium Green-2 (CG-2, Ex_{506} / Em_{531}). NADH oxidation (Ex_{377} / Em_{451}) was used as an indicator of SERCA hydrolysis of ATP through a coupled PEP / PYR enzyme pathway and ATP regenerating system. Here, Ca^{2+} transport and SERCA activity were synchronously monitored and concentration-dependent effects of exogenously added PUFAs upon functional coupling were studied (Figure 1). We tested the n-3 and n-6 PUFAs: ALA, EPA, DHA, and LA. HSR vesicles were pre-treated with varying concentrations of PUFAs and rapidly mixed with ATP to activate Ca^{2+} uptake.

The rate of Ca^{2+} accumulation by HSR was measured by the decrease of CG-2 fluorescence. Thus relative to control traces, a leftward shift in the time-dependent Ca^{2+} uptake curve indicated an improvement in the rate of Ca^{2+} uptake, and a rightward shift indicated a delay in the rate of Ca^{2+} uptake. The rate of Ca^{2+} sequestration by HSR vesicles was not uniform in response to the PUFAs. PUFAs increased the rate of Ca^{2+} accumulation by HSR vesicles below a certain concentration threshold of PUFAs, but decreased the rate Ca^{2+} uptake above that limit (Figures 2-4, representative traces are shown). For example, Figure 4 demonstrates that relative to control, 10 μM DHA increased the rate of Ca^{2+} uptake by HSR, while 75 μM DHA decreased Ca^{2+} uptake. PUFA concentrations that delayed HSR Ca^{2+} sequestration exceeded 50 μM with n-3 PUFAs and approached 50 μM with the n-6 PUFA, LA (Figure 5). This data may indicate that Ca^{2+} transport is enhanced by PUFAs (<50 μM).

The SERCA catalytic activity that corresponded with the Ca^{2+} sequestration data was measured from the rate of NADH oxidation. A rapid decrease in the concentration of NADH was indicative of high SERCA activity, similarly a slow decline in NADH concentration represented low SERCA activity. Rates of ATP hydrolysis at PUFA concentrations that improved the rate of Ca^{2+}sequestration were unaffected when compared

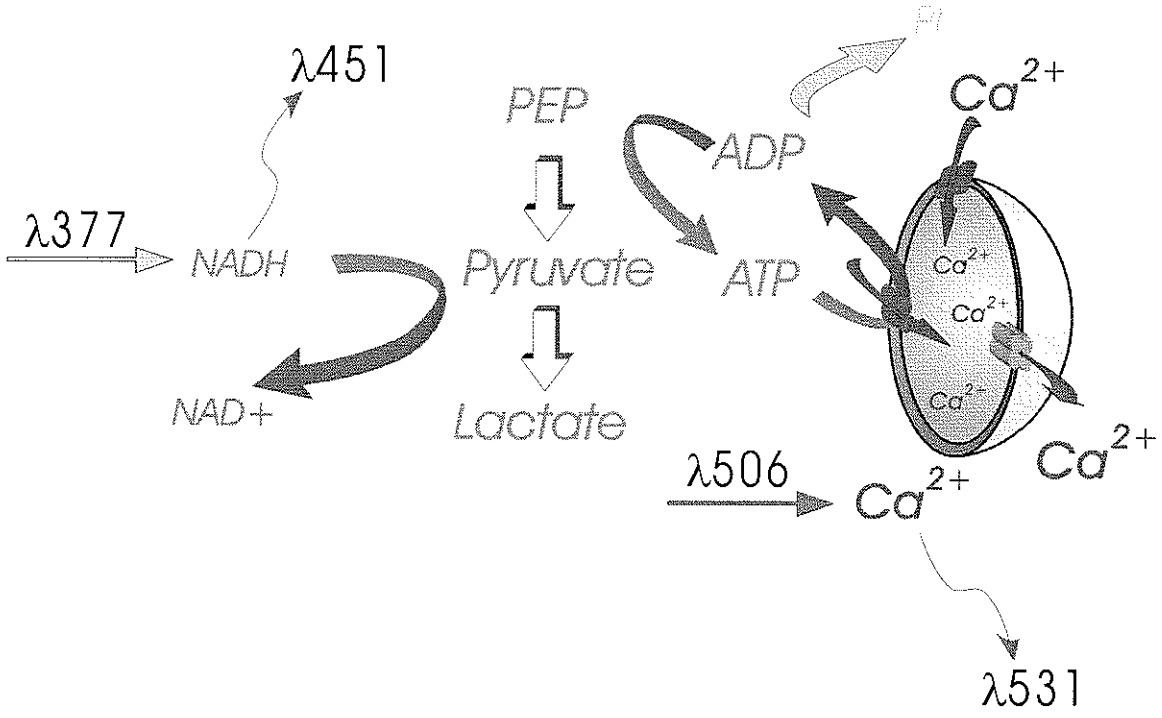


Figure 1. Synchronous Fluorimetry of SR Ca^{2+} transport activity and ATP hydrolysis

Extraluminal Ca^{2+} transport by isolated SR vesicles was monitored with the fluorophore CG-2 (Ex_{506} / Em_{531}). NADH oxidation (Ex_{377} / Em_{451}) was used as an indicator of SERCA hydrolysis of ATP through a coupled PEP / PYR enzyme pathway and ATP regenerating system.

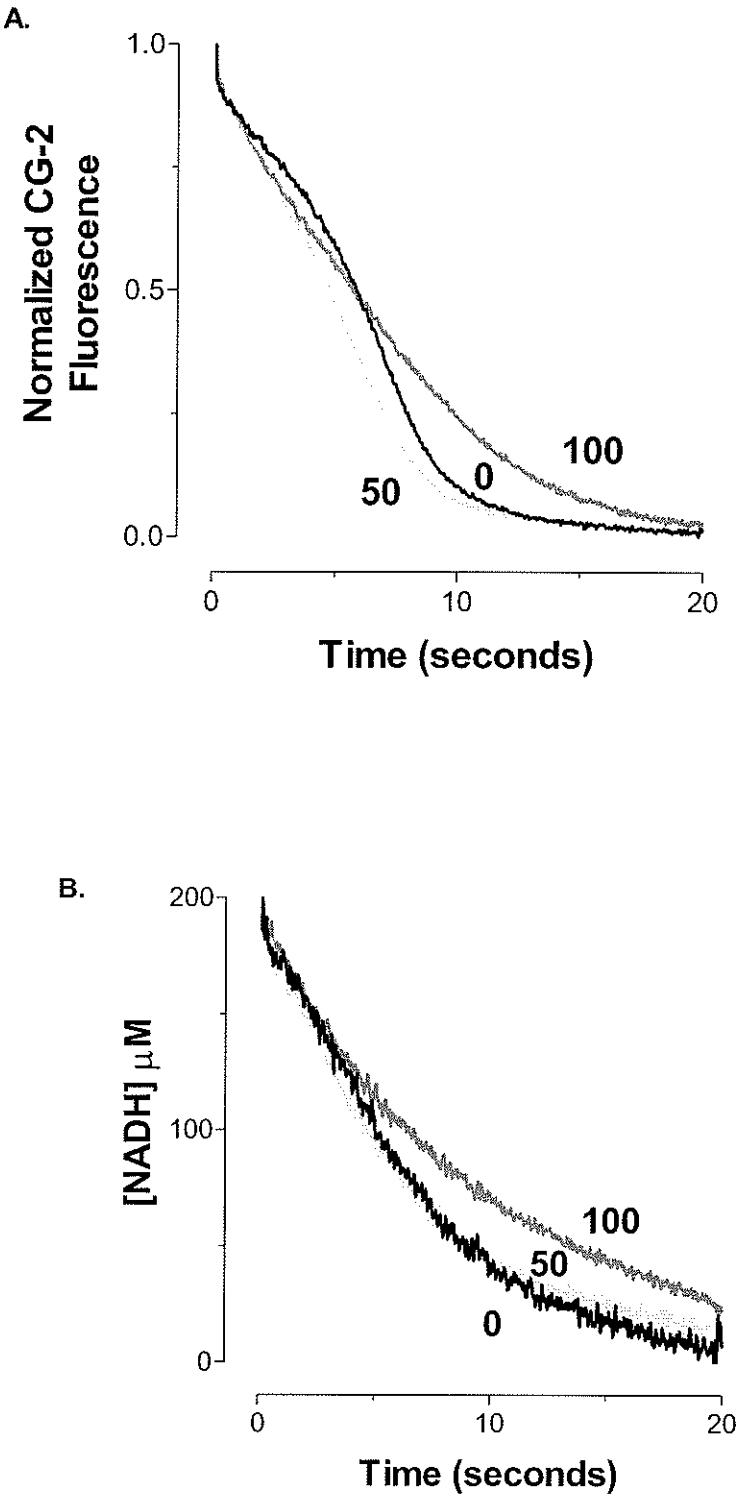


Figure 2. The Effects of Alpha-Linolenic Acid on A. Extraluminal Ca^{2+} Transport and B. SERCA ATP Hydrolysis by HSR Membranes

HSR membranes (0.25 mg/mL) were pre-treated with varying concentrations of alpha-linolenic acid (μM, C18:3 n-3). Ca^{2+} transport (Calcium Green-2 traces) and corresponding SERCA1 catalytic activity (NADH traces) were synchronously monitored as described in "Methods". Ca^{2+} transport was initiated by the combined addition of Mg·ATP and PEP.

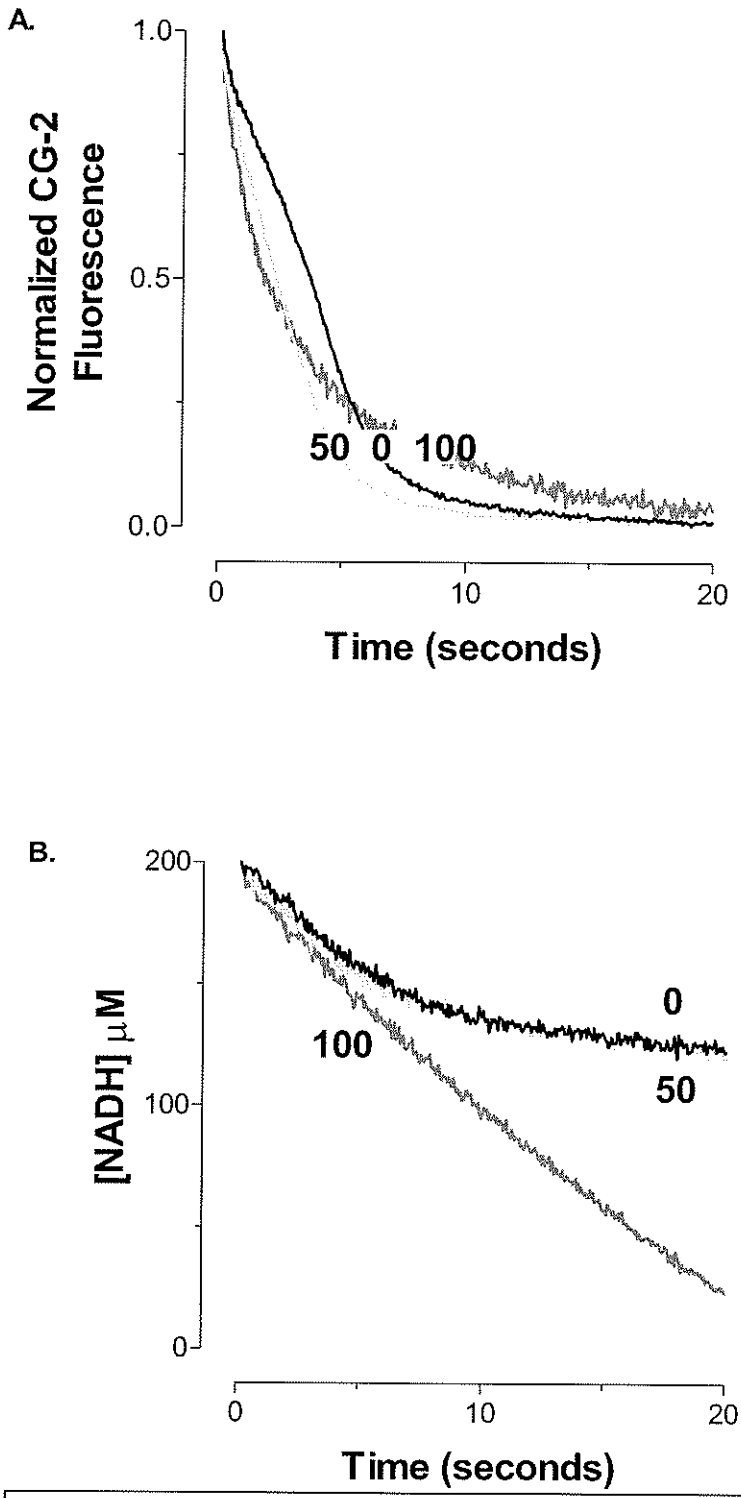


Figure 3. The Effects of Eicosapentaenoic Acid on A. Extraluminal Ca^{2+} Transport and B. SERCA ATP Hydrolysis by HSR Membranes

HSR membranes (0.25 mg/mL) were pre-treated with varying concentrations of eicospentaenoic acid (μM, C20:5 n-3). Ca^{2+} transport (Calcium Green-2 traces) and corresponding SERCA1 catalytic activity (NADH traces) were synchronously monitored as described in "Methods". Ca^{2+} transport was initiated by the combined addition of Mg·ATP and PEP.

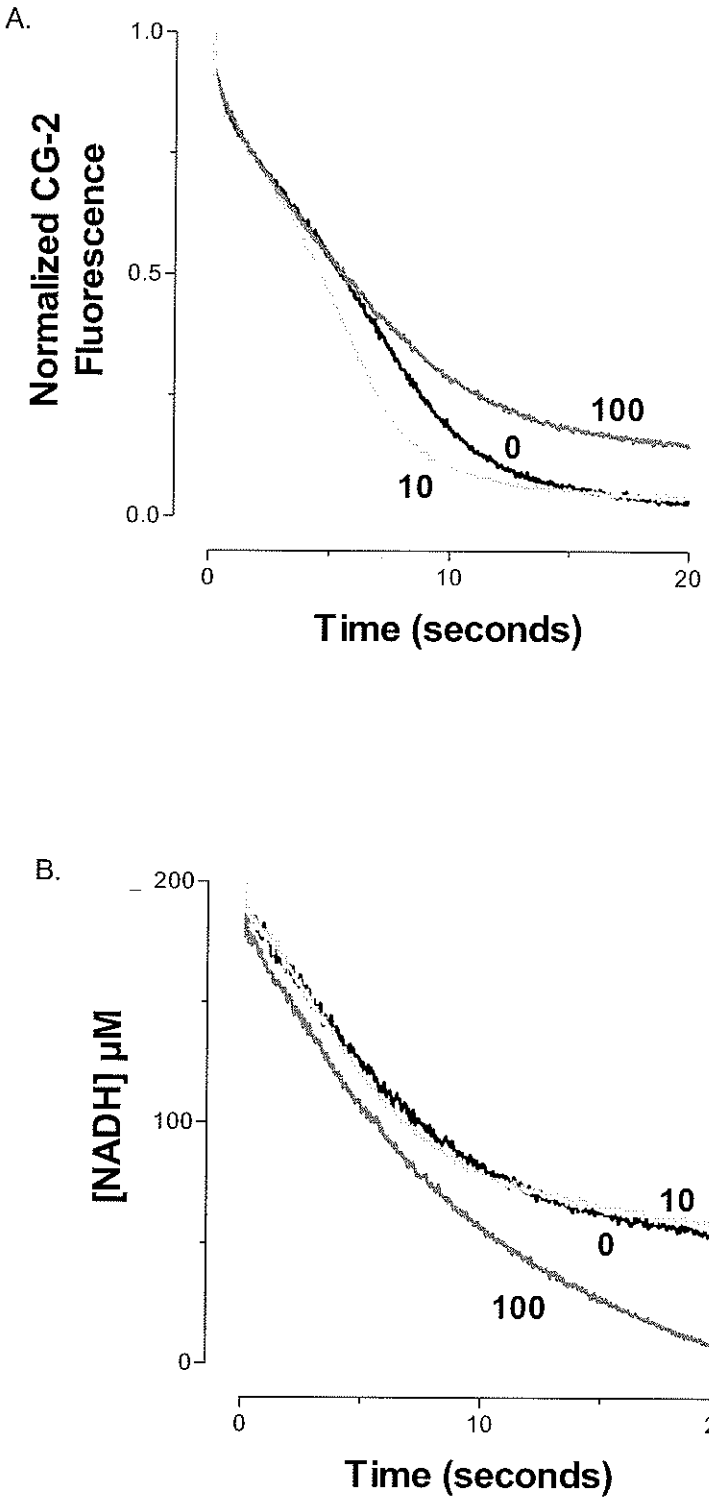


Figure 4. The Effects of Docosahexaenoic Acid on A. Extraluminal Ca^{2+} Transport and B. SERCA ATP Hydrolysis by HSR Membranes

HSR membranes (0.25 mg/mL) were pre-treated with varying concentrations of docosahexaenoic acid (µM, C22:6 n-3). Ca^{2+} transport (Calcium Green-2 traces) and corresponding SERCA1 catalytic activity (NADH traces) were synchronously monitored as described in "Methods". Ca^{2+} transport was initiated by the combined addition of Mg·ATP and PEP.

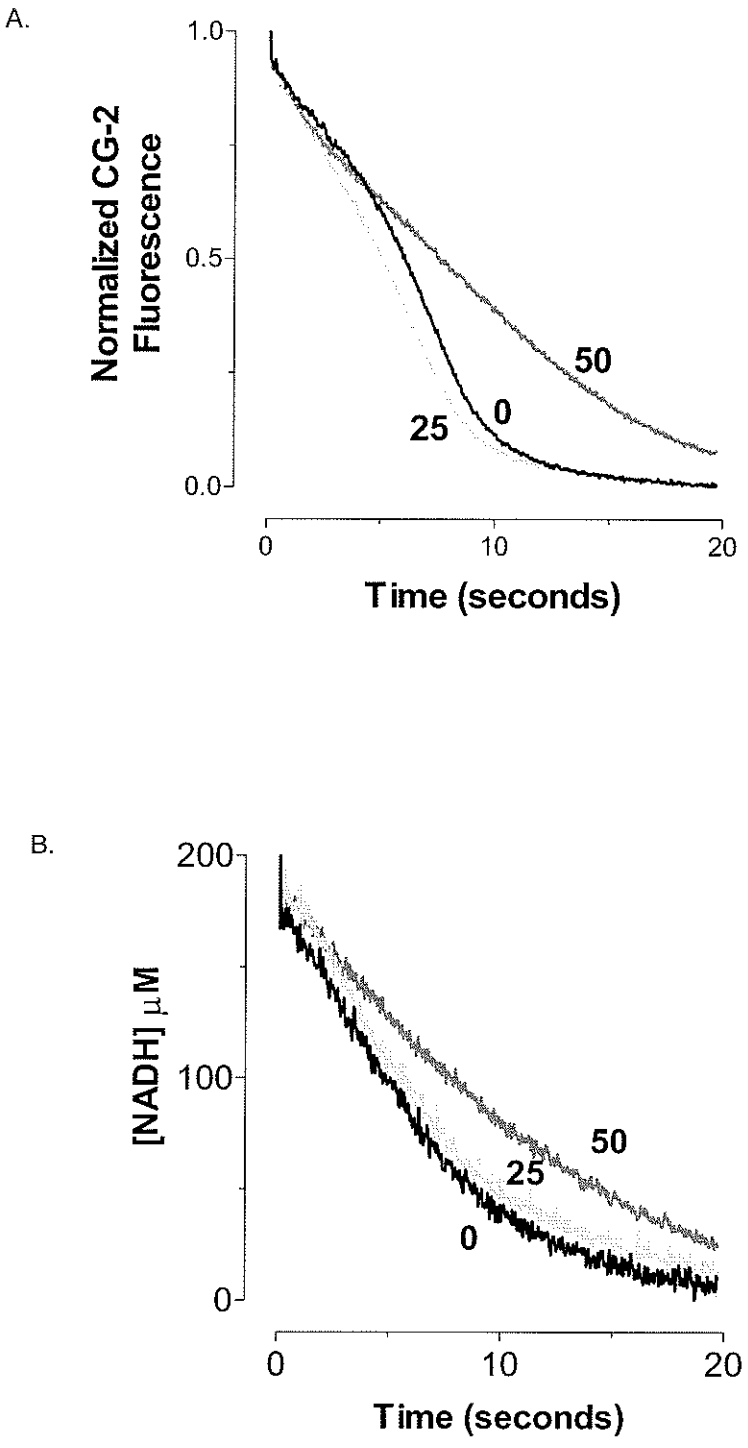
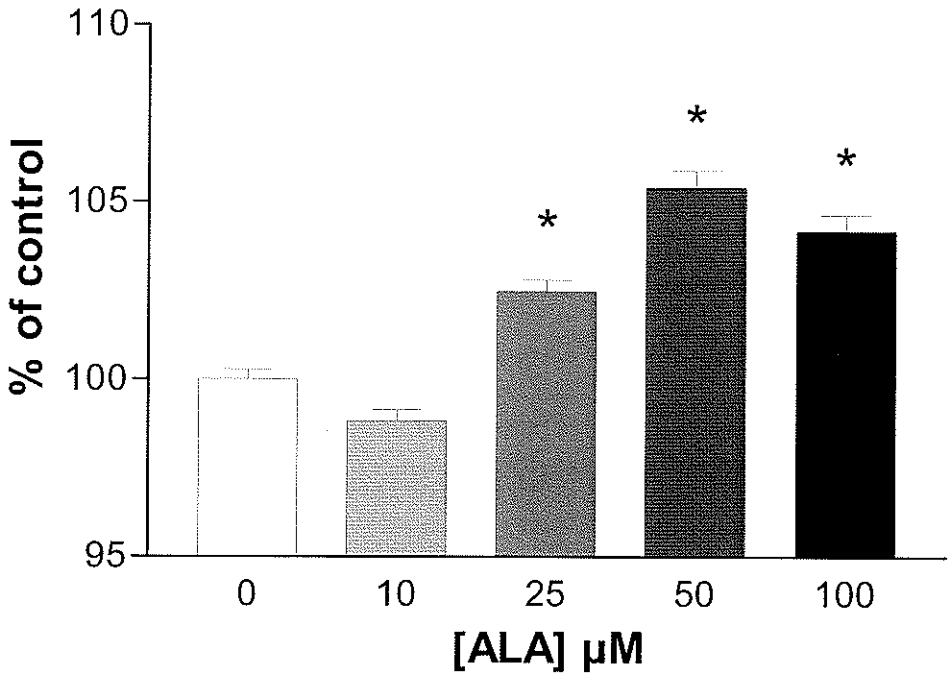


Figure 5. The Effects of Linoleic Acid on A. Extraluminal Ca^{2+} Transport and B. SERCA ATP Hydrolysis by HSR Membranes

HSR membranes (0.25 mg/mL) were pre-treated with varying concentrations of linoleic acid (μM, C18:2 n-6). Ca^{2+} transport (Calcium Green-2 traces) and corresponding SERCA1 catalytic activity (NADH traces) were synchronously monitored as described in "Methods". Ca^{2+} transport was initiated by the combined addition of Mg·ATP and PEP.

to control NADH consumption (Figures 2-5). For example, Figure 4 shows that the rate of NADH oxidation was unchanged from control despite its effect on Ca^{2+} transport. However, the related catalytic activity seen with PUFA concentrations that delayed Ca^{2+} uptake was variable. When Ca^{2+} uptake was delayed, comparatively long chain n-3 PUFAs (EPA and DHA) hydrolysed ATP slower than control (Figures 3-4). Comparatively shorter chain PUFAs (ALA and LA) consumed ATP faster than control under the same conditions (Figures 2, 5). Despite this variability, a sustained activation of the terminal phase of the NADH trace (last 10 seconds) is a common feature of all of the rates of ATP hydrolysis when Ca^{2+} uptake is prolonged by PUFAs. This may indicate that SERCA is sequestering Ca^{2+} into the SR in the presence of an opposing Ca^{2+} leak created by PUFAs. These initial characterisations of both Ca^{2+} handling and SERCA activity suggest that relatively low concentrations of PUFAs improve coupling of Ca^{2+} transport whereas relatively higher PUFA concentrations impair coupling of Ca^{2+} transport.

To account for the observed enhanced sequestration of Ca^{2+} by HSR with activating concentrations of PUFAs, uncoupled Ca^{2+}-dependent SERCA1a activity in ionomycin-permeabilised HSR vesicles was assessed using a stopped-flow rapid kinetic assay of NADH fluorescence. This method allowed us to examine SERCA1a activity independently of RyR1 leak status. Maximum activation rates of SERCA1a catalytic activity were derived from linear regression analysis of the NADH oxidation rates using Graphpad Prism™ software (see Methods). Generally, a concentration-dependent increase in the rate of NADH oxidation by permeabilised HSR vesicles was observed in response to PUFAs (Figures 6-9). These small, albeit statistically significant, increases only accounted for a 5-10% increase in overall ATP hydrolysis. This suggests that PUFA effects on SERCA activity are limited; consequently they may act at a different site in the SR membrane.



* P < 0.001

Figure 6. Effect of Alpha-Linolenic Acid on SERCA1 ATP Hydrolysis

HSR membranes (0.10 mg/ml) were pre-treated with varying concentrations of alpha-linolenic acid (ALA; μM). SERCA1 activity as measured by NADH consumption in ionomycin-permeabilized HSR membranes is shown. * P < 0.001 vs. 0 μM ALA

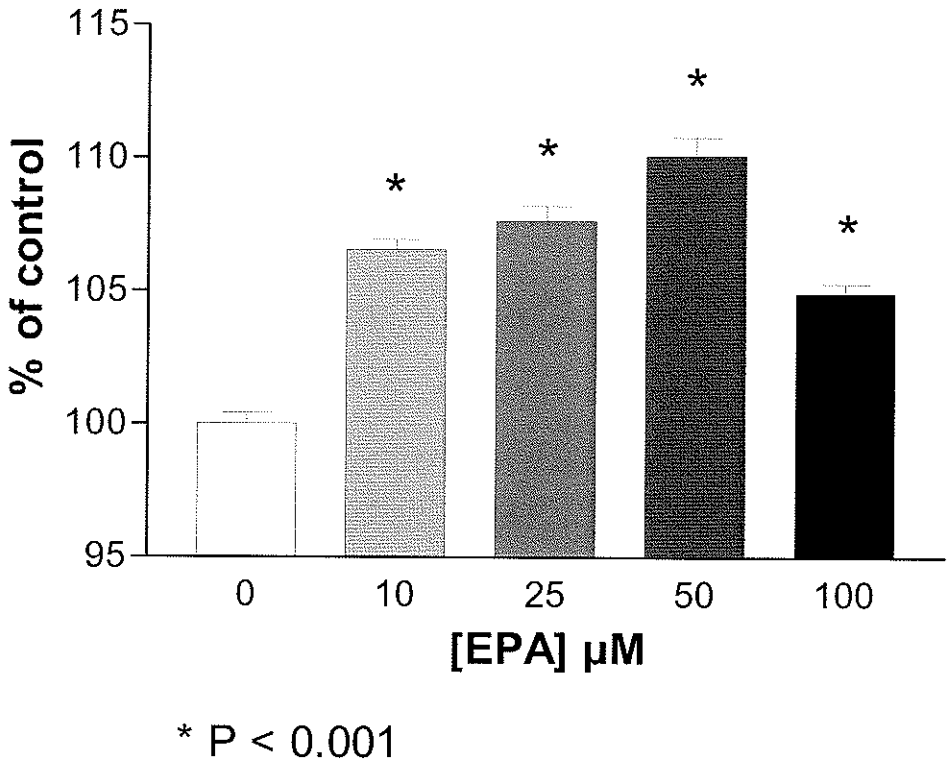


Figure 7. Effect of Eicosapentaenoic Acid on SERCA1 ATP Hydrolysis

HSR membranes (0.10 mg/ml) were pre-treated with varying concentrations of eicosapentaenoic acid (EPA, μM). SERCA1 activity as measured by NADH consumption in ionomycin-permeabilized HSR membranes is shown. * $P < 0.001$ vs. 0 μM EPA

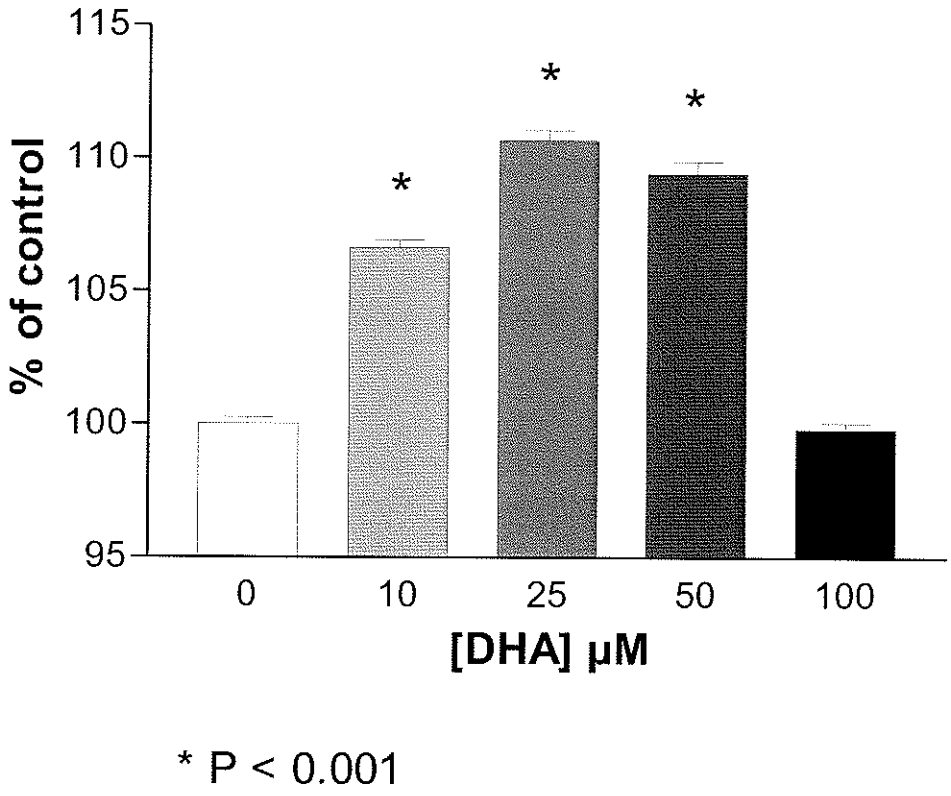


Figure 8. Effect of Docosahexaenoic Acid on SERCA1 ATP Hydrolysis

HSR membranes (0.10 mg/ml) were pre-treated with varying concentrations of docosahexaenoic acid (DHA, μM). SERCA1 activity as measured by NADH consumption in ionomycin-permeabilized HSR membranes is shown. * $P < 0.001$ vs. 0 μM DHA

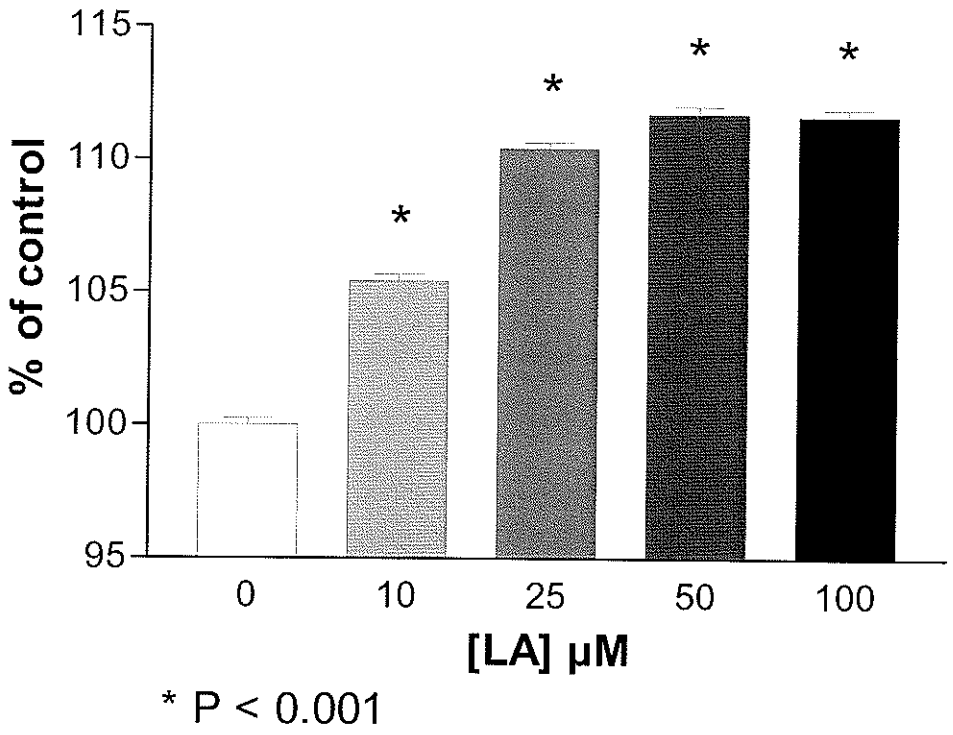


Figure 9. Effect of Linoleic Acid on SERCA1 ATP Hydrolysis

HSR membranes (0.10 mg/ml) were pre-treated with varying concentrations of linoleic acid (LA, μM). SERCA1 activity as measured by NADH consumption in ionomycin-permeabilized HSR membranes is shown. * $P < 0.001$ vs. 0 μM LA

Since PUFA-induced enhanced sequestration of Ca^{2+} in HSR could be due to decreased leak status of vesicles, rather than an increased rate of SERCA activity, we examined this alternate hypothesis. Ca^{2+} leak pathways in SR membranes are often mediated by RyR subconductance states, so a [^{3}H]-ryanodine binding assay was used to determine the effect of the selected PUFAs on RyR integrity. Specific effects of [^{3}H]-ryanodine binding were derived from total and non-specific binding data conducted under maximal binding Ca^{2+} buffered conditions (see Methods). In all cases, n-3 and n-6 PUFAs decreased [^{3}H]-ryanodine binding in a concentration-dependent manner (Figures 10-13). Interestingly, concentrations of PUFAs that delayed Ca^{2+} transport significantly decreased [^{3}H]-ryanodine binding suggesting that relatively high PUFA concentrations disrupt RyR functional status and in so doing potentiate Ca^{2+} leaks. Still, the improved coupling of Ca^{2+} transport and ATP hydrolysis seen with lower PUFA concentrations was not explained by this data.

To further examine the effect of PUFAs on Ca^{2+} release processes from HSR, we incrementally loaded vesicles with Ca^{2+} to activate CICR using a previously published pulse-loading protocol [261]. Usually 3-4 pulses of 10 μM Ca^{2+}, represented by rapid increases in CG-2 fluorescence, were needed to stimulate Ca^{2+} release depending on the preparation of HSR vesicles and residual Ca^{2+} found in the buffers (approximately 400 nM). CICR was characterized by an increase in CG-2 fluorescence and a slow return to baseline as compared to initial Ca^{2+} pulses. Figure 14, Panel A shows that under control conditions, with vehicle, a CICR-like phenomenon was observed after four pulses of 10 μM Ca^{2+} (equivalent to a loading level of 40.4 μM Ca with 0.25 mg/mL HSR). A progressive inhibition of CICR was observed with relatively low concentrations (μM) of DHA; addition of 2.5 μM DHA to the reaction media delayed the CICR response by one pulse, but 5 μM DHA inhibited CICR (Figure 14 Panel A). In a similar preparation of HSR, where a comparable control of CICR

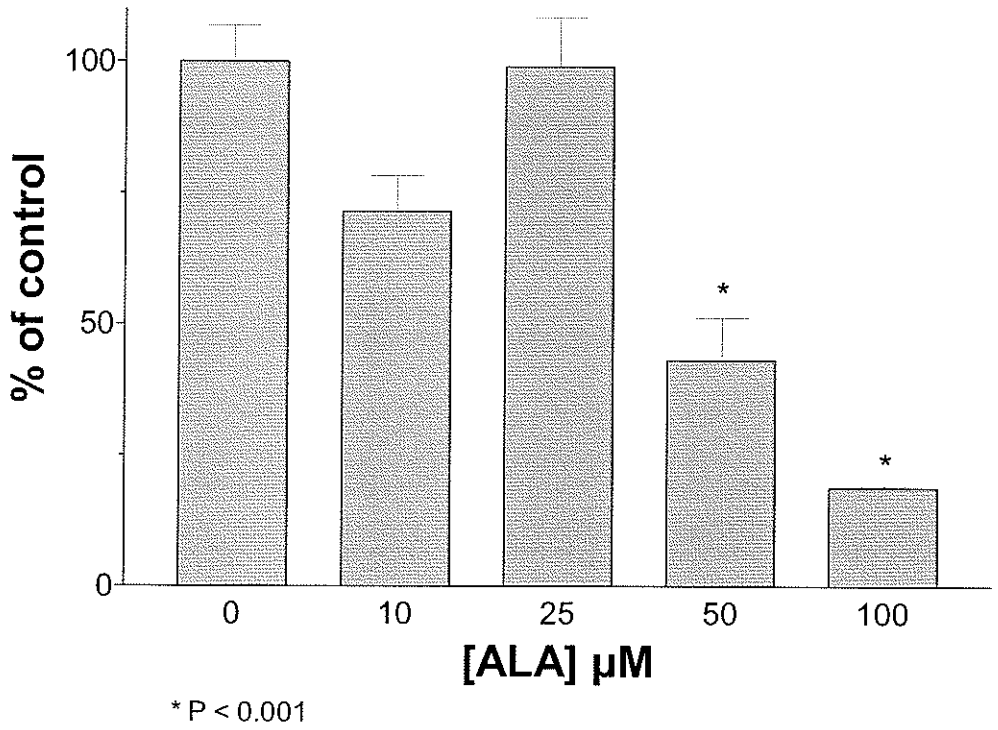


Figure 10. Effect of Alpha-Linolenic Acid [^{3}H]-ryanodine Binding

HSR membranes (0.25 mg/ml) were pre-treated with varying concentrations of alpha-linolenic acid (ALA, C18:3 n-3). Specific [^{3}H]-ryanodine binding was determined from non-specific binding, as determined with 100 μM ryanodine, subtracted from total binding (n = 3). * P < 0.001 vs. 0 μM ALA

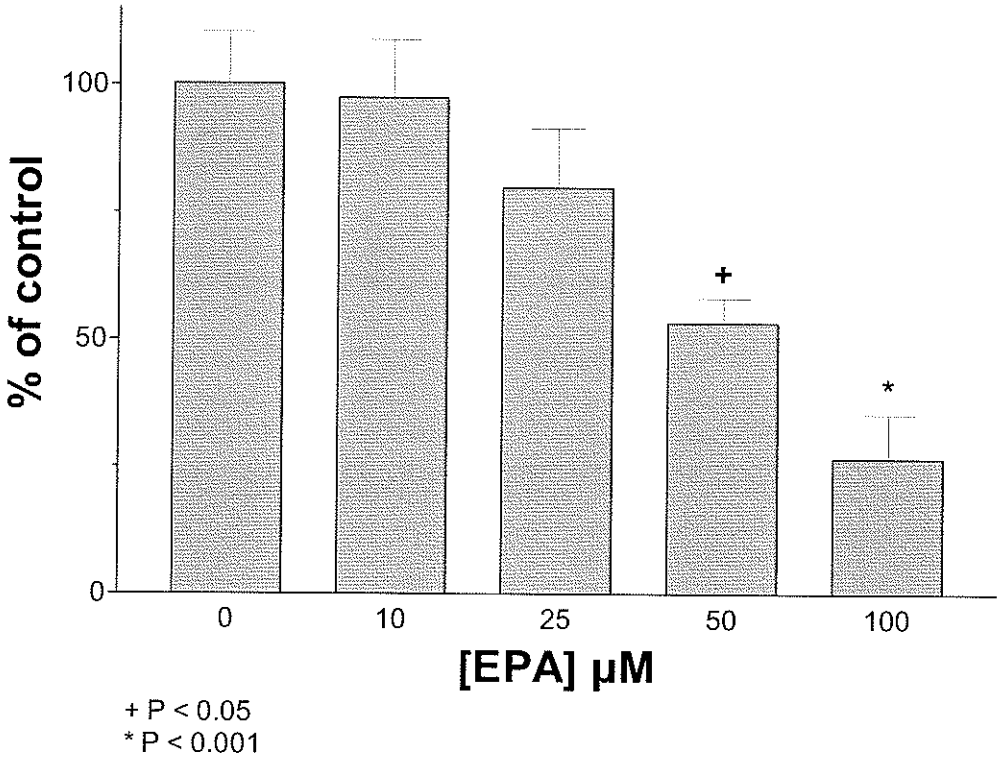


Figure 11. Effect of Eicosapentaenoic Acid on [^{3}H]-ryanodine Binding

HSR membranes (0.25 mg/ml) were pre-treated with varying concentrations of eicosapentaenoic acid (EPA, C20:5 n-3). Specific [^{3}H]-ryanodine binding was determined from non-specific binding, as determined with 100 μM ryanodine, subtracted from total binding (n = 3). + $P < 0.05$ vs. 0 μM EPA * $P < 0.001$ vs. 0 μM EPA

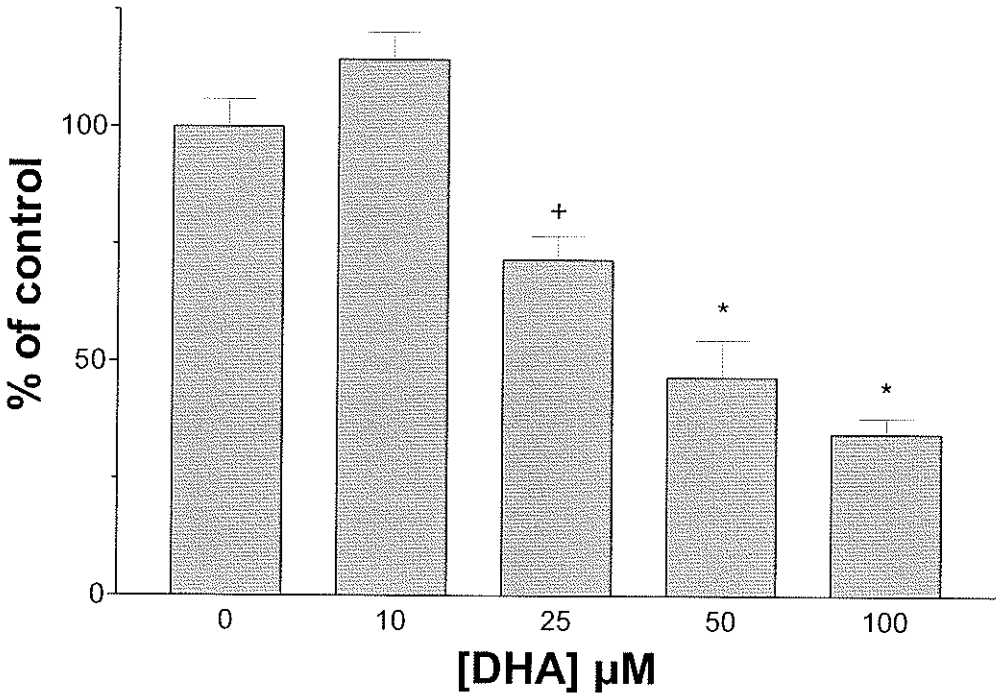


Figure 12. Effect of Docosahexaenoic Acid on [^{3}H]-ryanodine Binding

HSR membranes (0.25 mg/ml) were pre-treated with varying concentrations of docosahexaenoic acid (DHA, C22:6 n-3). Specific [^{3}H]-ryanodine binding was determined from non-specific binding, as determined with 100 μM ryanodine, subtracted from total binding (n = 3). + $P < 0.05$ vs. 0 μM DHA * $P < 0.001$ vs. 0 μM DHA

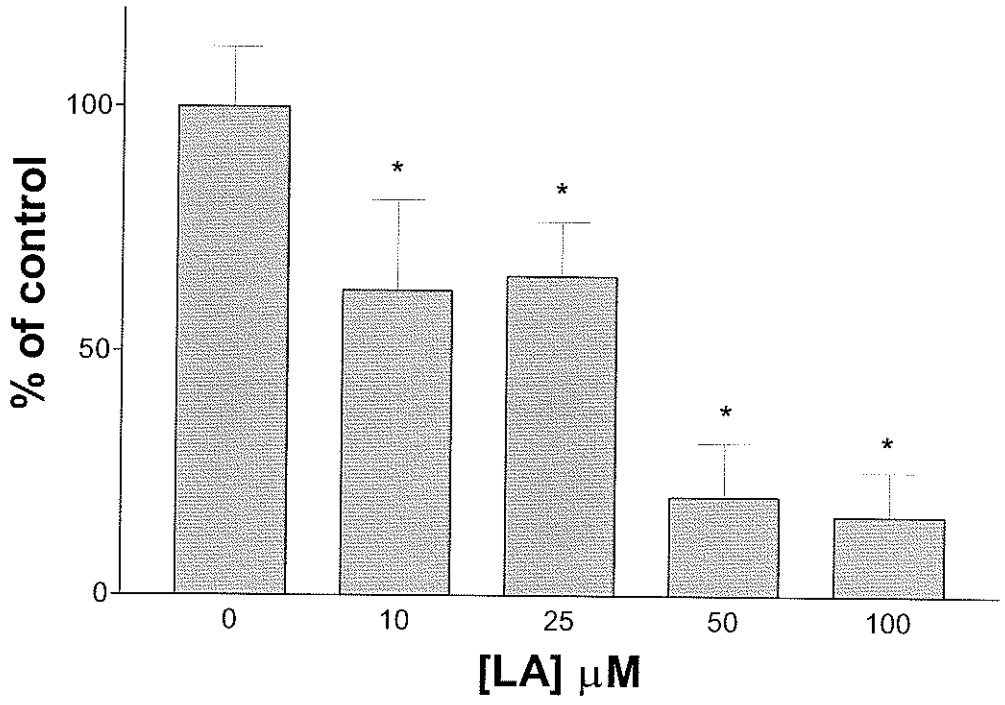
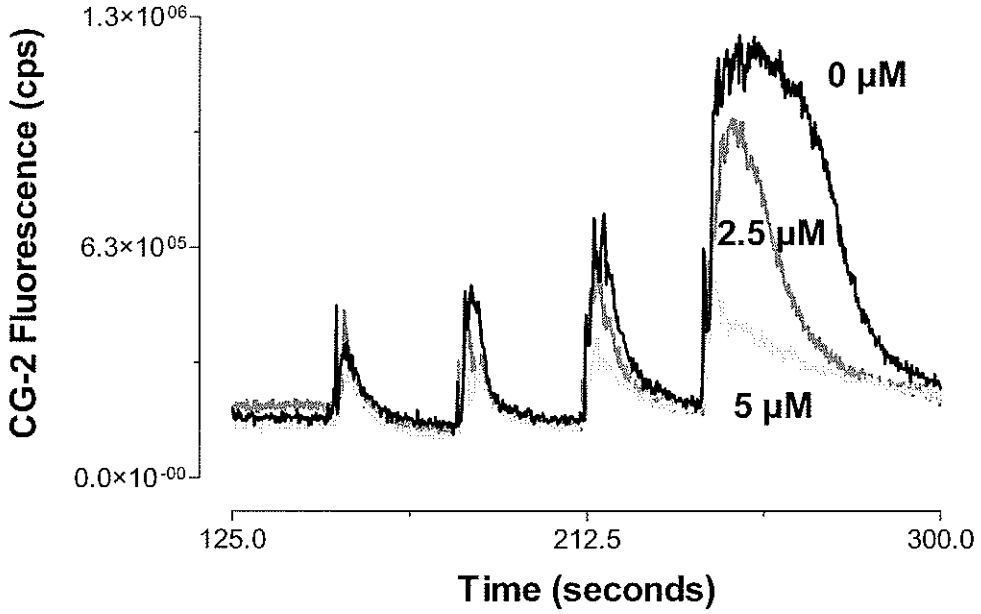


Figure 13. Effect of Linoleic Acid on [^{3}H]-ryanodine Binding

HSR membranes (0.25 mg/ml) were pre-treated with varying concentrations of linoleic acid (LA, C18:2 n-6). Specific [^{3}H]-ryanodine binding was determined from non-specific binding, as determined with 100 μM ryanodine, subtracted from total binding (n = 3). * $P < 0.001$ vs. 0 μM LA

A.



B.

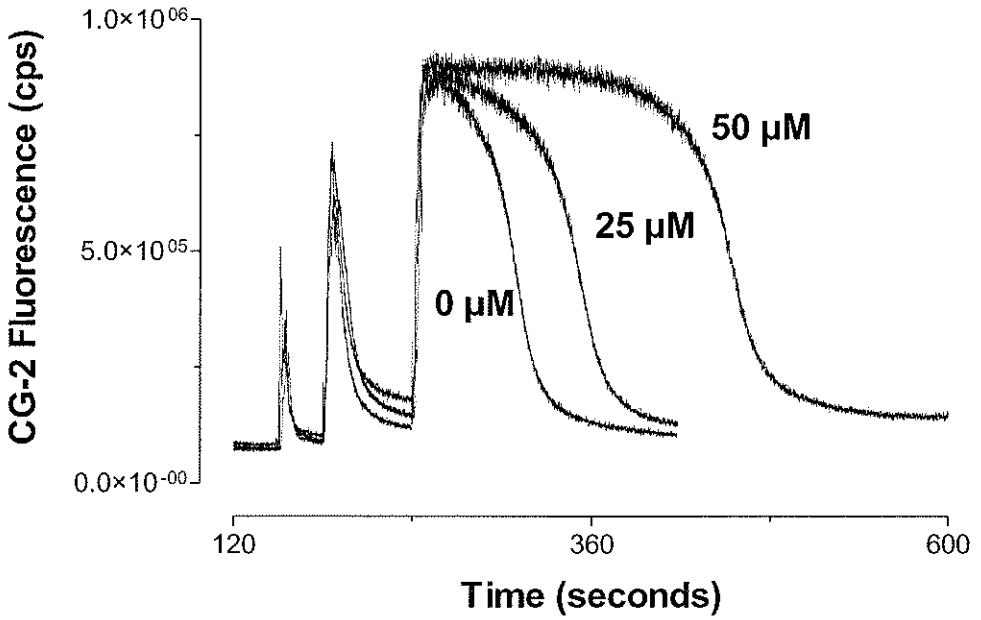


Figure 14. Effects of Docosahexaenoic Acid on Ca^{2+} Pulse-loading in HSR Membranes

HSR membranes (0.25 mg/mL) were pre-treated with varying concentrations of docosahexaenoic acid (C22:6 n-3). Ca^{2+} transport (CG-2 traces) was monitored as described in "Methods". Transport was initiated by the combined addition of Mg·ATP and PEP. Ca^{2+} additions were made with a repeater pipette as 10 μM pulses and are seen as rapid rises in CG-2 fluorescence. CICR is characterised by a rapid rise in CG-2 fluorescence and a time-dependent delay in a return to baseline fluorescence. *CICR was suppressed by low-end (A.) and potentiated by high-end (B.) concentrations of docosahexaenoic acid.*

occurred after three pulses of 10 μM Ca^{2+} (equivalent to a loading level of 30.4 μM Ca^{2+} with 0.25 mg/mL HSR) relatively high concentrations of DHA potentiated Ca^{2+} release in a concentration-dependent manner (Figure 14 Panel B). Results that exhibited a gradual increase in the Ca^{2+} threshold needed for CICR (Figure 14 Panel A) were remarkably similar to the known effects of ruthenium red, a RyR antagonist (Figure 15). Perhaps PUFAs specifically inhibit a RyR response at relatively low concentrations, but prevent normal kinetics of RyR activation and inactivation at higher concentrations.

We then speculated that PUFA-activated Ca^{2+}-leak conditions were mostly likely caused by a loss of SR membrane integrity in the bulk lipid rather than direct effects on RyR. To determine if the observed PUFA effects on HSR vesicles were due to a disruption of HSR membrane protein, HSR vesicles were incubated for an hour with various PUFAs, centrifuged to separate out protein, the supernatants were then precipitated and analysed. PUFAs appeared to have no effect on SR protein levels as compared to untreated HSR vesicles. Detergents known to solubilise HSR membranes, like CHAPS and saponin, were used as outside controls to show that solubilisation of SR membranes increased the recovery of SERCA proteins from supernatants of treated HSR on SDS-PAGE gels (Figure 16). Qualitative analysis of SERCA protein (110 kDa) and calsequestrin (44 kDa) in the preparations indicated that PUFAs do not increase the recovery of these proteins (Figure 16). Therefore PUFAs do not disrupt HSR membranes to such an extent that integral SR membrane proteins are removed.

EFFECTS OF CHOLESTEROL AND FLAXSEED FEEDING ON CARDIAC SR FUNCTION

The second half of this study attempted to modulate the lipid compostion of cardiac SR membranes with fatty acids and cholesterol feeding. Fatty acid content of the

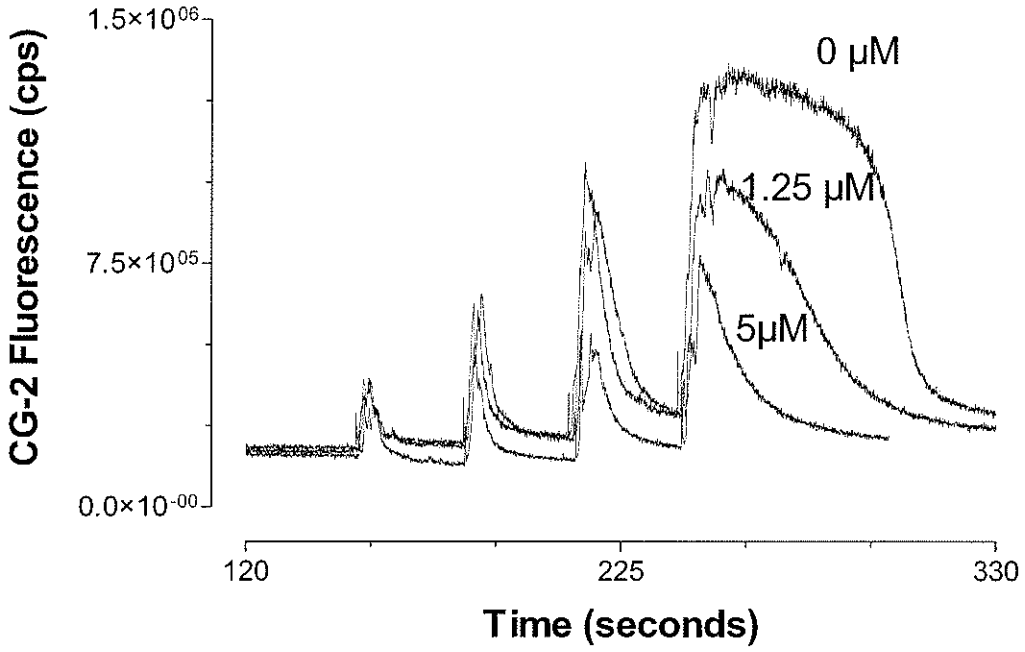


Figure 15. Effect of Ruthenium Red Ca^{2+} pulse-loading

HSR membranes (0.25 mg/mL) were pre-treated with varying concentrations of ruthenium red (μM). Ca^{2+} transport (CG-2 traces) was monitored as described in "Methods". Transport was initiated by the combined addition of Mg-ATP and PEP. Ca^{2+} additions were made with a repeater pipette as 10 μM pulses and are seen as rapid rises in CG-2 fluorescence. CICR is characterised by a rapid rise in CG-2 fluorescence and a time-dependent delay in a return to baseline fluorescence.

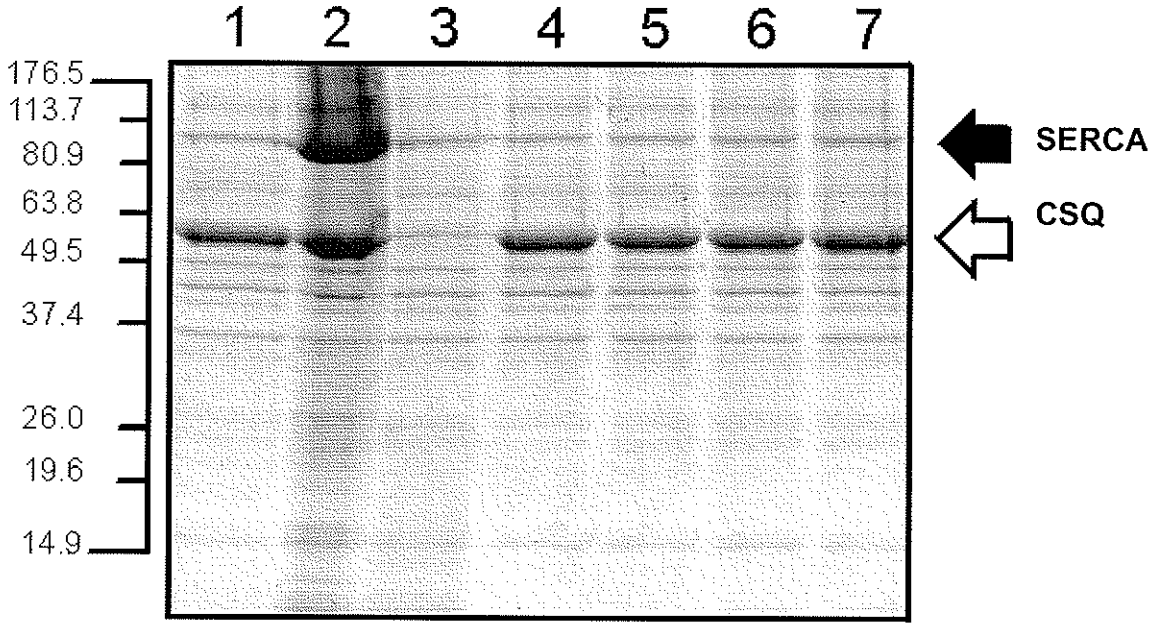


Figure 16. SDS-PAGE of SR response to PUFAs

Lanes 1-7 respectively contain untreated HSR, 0.1% CHAPS + HSR, 0.1 % Saponin + HSR, 400 μM ALA / mg HSR, 400 μM EPA / mg HSR, 400 μM DHA / mg HSR, and 400 μM LA / mg HSR Marker 1 indicates SERCA (110) kDa while marker 2 indicates CSQ (40 kDa).

diets was varied with flaxseed incorporation. In addition cholesterol chow was fed to alter both membrane rigidity and fatty acid content. Finally, a cholesterol/flaxseed diet was given to observe their combined effects. Plasma and tissue analyses were used as indicators of incorporation of dietary substituents. Isolated CSR vesicles were then characterised for fatty acid, cholesterol content, and corresponding Ca^{2+} transport and SERCA activities.

DIETARY ANALYSIS

NZW rabbits were fed a regular chow, or a regular chow supplemented with 10% flaxseed, or 0.5% cholesterol, or 10% flaxseed and 5% cholesterol for 8 weeks. Promega flaxseed was chosen as a rich-source of n-3 PUFAs because it was fortified in ALA (70% of total fatty acids compared to 55% in regularly grown seed). Based on the studies of Prasad [265] and our preliminary studies, which indicated that the texture and palate of flaxseed-augmented regular chow could be maintained, we chose to supplement rabbit diets with 10% flaxseed (equivalent to 12.5 g for a typical 125 g diet). This was less than the 7.5 g flaxseed/ kg body weight (equivalent to 18.75 g for a 2.5 kg rabbit) fed by Prasad[265]. However, we still expected significant levels of n-3 PUFAs to be reached in the rabbits since the flaxseed was ground to aid in its digestion and the rabbits did not experience the gastrointestinal distress he reportedly observed. In the interest of observing changes that were clinically relevant, diets were also reconstituted with 0.5 % cholesterol, which we knew promoted atherosclerotic plaque formation in NZW rabbits over a time-course of 8 weeks [266]. In addition, the cholesterol may be incorporated into membranes like CSR [267] and alter its function and physical characteristics.

Analysis of the fatty acid composition of the diets confirmed ALA-enrichment in the flaxseed diets. ALA content increased approximately ten-fold in diets containing flaxseed.

The flaxseed and cholesterol/flaxseed diets respectively contained 20.077± 0.841 mg ALA/ g and 22.535± 0.679 mg ALA/ g diet in comparison to the regular and cholesterol diets that contained 1.993± 0.120 mg ALA/ g and 2.119± 0.036 mg ALA/ g respectively (Table 1). Significant increases in palmitic acid (C16:0), and oleic acid (C18: 1) were also observed in the flaxseed diets (Table 1).

The amount of diet received by the animals was not controlled in the study where hearts did not undergo ischaemia-reperfusion, since the resulting weight of the animals at 8 weeks did not affect our ability to normalise the yield of isolated CSR vesicles. However, during the ischaemia-reperfusion study rabbits were rationed 125 g feed /day to allow for the comparison of electrophysiological data between like groups (This data will not be discussed here). After 8 weeks of feeding, rabbit weights were normal between rabbits limited to 125 g of diet/day (Figure 17). Caloric intake for 125 g of each of the prepared diets did not differ statistically. Regular chow, 10% flaxseed, 0.5% cholesterol, and 10% flaxseed / 5% cholesterol contained 423, 445, 421, 450 calories respectively.

CHARACTERISATION OF RABBITS

Over the course of the study, rabbits were monitored for plasma fatty acid and cholesterol composition as an indication of the effectiveness of the feeding protocol. A steady time-dependent increase in n-3 PUFAs from flax-fed rabbits was noted during the feeding period. Gas chromatography revealed that cholesterol/flax-fed rabbits exhibited significant increases in plasma ALA as compared to all other fed rabbits (Table 2). Plasma from cholesterol-fed groups was easily distinguished from others by its cloudy colour. Indicators of plasma cholesterol, as measured by the VetTest 8008, confirmed that the study design promoted cholesterol absorption in fed animals. Total plasma fatty acids, cholesterol

FAME	Regular Chow	10 % Flaxseed	0.5 % Cholesterol	10 % Flaxseed and 0.5 % Cholesterol
C14:0	0.293 ± 0.014	0.371 ± 0.013	0.267 ± 0.002	0.337 ± 0.016
C14:1	0.131 ± 0.001	0.0000 ± 0.0000	0.033 ± 0.033	0.0000 ± 0.0000
C16:0	6.502 ± 0.247	9.567 ± 0.349	6.338 ± 0.044	9.364 ± 0.232
C16:1	0.403 ± 0.024	0.540 ± 0.031	0.354 ± 0.001	0.442 ± 0.004
C18:0	2.200 ± 0.147	3.942 ± 0.157	2.048 ± 0.006	3.643 ± 0.186
C18:1 (n-9) Oleic	10.693 ± 0.325	17.055 ± 0.862	10.292 ± 0.010	16.361 ± 0.840
C18:1 Vaccenic	1.719 ± 0.148	2.775 ± 0.002	1.644 ± 0.064	2.611 ± 0.067
C18:2 (n-6) LA	11.191 ± 0.343	11.781 ± 0.513	12.328 ± 0.170	13.713 ± 0.490
C20:0	0.113 ± 0.011	0.165 ± 0.009	0.117 ± 0.001	0.159 ± 0.025
C18:3 (n-6) GLA	0.000 ± 0.000	0.115 ± 0.002	0.000 ± 0.000	0.125 ± 0.004
C20:1 (n-9)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
C18:3 (n-3) ALA	1.993 ± 0.120	20.077 ± 0.841	2.119 ± 0.036	22.535 ± 0.679
C20:2 (n-6)	0.092 ± 0.012	0.125 ± 0.009	0.087 ± 0.002	0.106 ± 0.007
C22:0	0.160 ± 0.012	0.196 ± 0.002	0.166 ± 0.006	0.199 ± 0.036
C22:1	0.112 ± 0.032	0.292 ± 0.009	0.095 ± 0.009	0.094 ± 0.042
C20:3	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.193 ± 0.003
C22:6 (n-3) DHA	0.000 ± 0.000	0.214 ± 0.004	0.105 ± 0.105	0.115 ± 0.115

Table 1. Fatty Acid Composition of Rabbit Diets

Fatty acid composition of rabbit diets was measured as mg of fatty acid methyl esters (FAME) per gram of diet ± SEM. Abbreviations: LA- linoleic acid, GLA- gamma linoleic acid, ALA- alpha linolenic acid, DHA- docosahexaenoic acid.

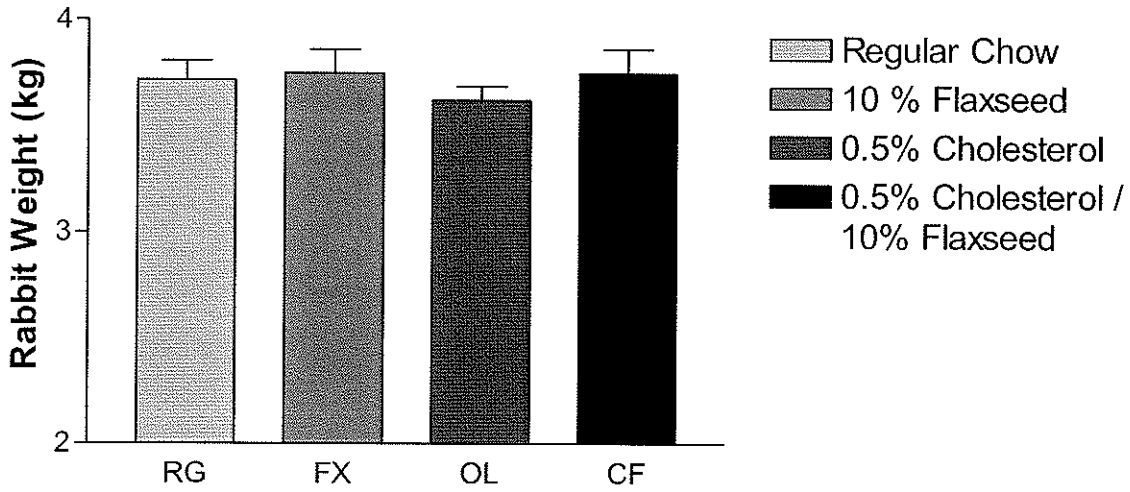


Figure 17. Mean Rabbit Weights of Feeding Groups at 8 Weeks

Data shown represents weight in kg of rabbits fed 125 g/day for 8 weeks. There were no significant changes in rabbit weights after 8 weeks of feeding.

FAME	Regular Chow	10 % Flaxseed	0.5 % Cholesterol	10 % Flaxseed and 0.5 % Cholesterol
C8:0	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
C10:0	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
C12:0	0.049 ± 0.002	0.034 ± 0.002	0.036 ± 0.002	0.000 ± 0.000
C14:0	0.007 ± 0.002	0.002 ± 0.001	0.019 ± 0.002	0.017 ± 0.002
C14:1	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
C16:0	0.150 ± 0.015	0.096 ± 0.011	0.798 ± 0.072 a, b	0.795 ± 0.062 a, d
C16:1	0.019 ± 0.003	0.008 ± 0.001	0.194 ± 0.019 a, b	0.137 ± 0.014 a, d
C17:0	0.002 ± 0.001	0.000 ± 0.000	0.042 ± 0.005 a, b	0.040 ± 0.003 a, d
C17:1	0.000 ± 0.000	0.000 ± 0.000	0.003 ± 0.002	0.000 ± 0.000
C18:0	0.149 ± 0.010	0.109 ± 0.008	0.284 ± 0.024 a, b	0.343 ± 0.029 a, d
C18:1 (n-9) Oleic	0.130 ± 0.030	0.089 ± 0.009	0.004 ± 0.000	0.751 ± 0.229 a, c, d
C18:1 Vaccenic	0.029 ± 0.015	0.009 ± 0.001	0.516 ± 0.152 a, b	0.484 ± 0.228 a, d
C18:2 (n-6) LA	0.126 ± 0.022	0.088 ± 0.012	0.774 ± 0.087 a, b	0.821 ± 0.073 a, b
C20:0	0.000 ± 0.000	0.000 ± 0.000	0.009 ± 0.001	0.010 ± 0.001
C18:3 (n-6)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
C20:1	0.000 ± 0.000	0.000 ± 0.000	0.002 ± 0.001	0.000 ± 0.000
C18:3 (n-3) ALA	0.005 ± 0.003	0.061 ± 0.010	0.167 ± 0.019	0.994 ± 0.195 a, c, d
C20:2	0.000 ± 0.000	0.000 ± 0.000	0.013 ± 0.002	0.011 ± 0.003
C22:0	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
C20:3 gamma	0.000 ± 0.000	0.000 ± 0.000	0.422 ± 0.045 a, b	0.300 ± 0.047 a, d
C22:1	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
C20:3 11-14-17	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.008 ± 0.003
C20:4	0.020 ± 0.003	0.013 ± 0.000	0.063 ± 0.005 a, b	0.053 ± 0.003 a, d
C24:0	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
C20:5 (n-3)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.003 ± 0.002
C24:1	0.000 ± 0.000	0.000 ± 0.000	0.060 ± 0.007 a, b	0.068 ± 0.005 a, d
C22:6 (n-3) DHA	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000

Table 2. Effects of Feeding on Plasma Fatty Acid Profiles at 8 Weeks

Fatty acid composition of rabbit plasma at 8 weeks was measured as mg of fatty acid methyl esters (FAME) per mL of plasma ± SEM. Abbreviations: LA- linoleic acid, ALA- alpha linolenic acid, DHA- docosahexaenoic acid. Statistical Significance: RG vs. FX, OL, CF = a; FX vs. OL = b; OL vs. CF = c; FX vs. CF = d.

and triglycerides in cholesterol-fed animals were significantly higher than non-cholesterol fed animals (Figure 18, 19). Substantially higher levels of fatty acids were present in both cholesterol-fed and cholesterol/flax-fed animals because cholesterol diets contained additional fatty acids esterified to cholesterol molecules. These results provided evidence that components of both flaxseed and cholesterol were digested and relatively enriched in these subjects.

The fatty acid profile of the harvested tissues from both flax-fed rabbit groups showed an incorporation of ALA and downstream metabolites. Brains from flax-fed rabbits demonstrated significant levels of ALA, EPA, and docosapentaenoic acid (C22:5, n-3) (Table 3). Kidneys from the same flax-fed animals also demonstrated an increase in the n-3 PUFAs ALA, EPA, and DHA, and a concomitant decrease in AA (arachidonic acid C20:4, n-6) (Table 4). This increase in n-3/n-6 ratio is consistent with our understanding that n-3 and n-6 PUFAs are in competition for the same enzymes. Livers also had significant levels of ALA and EPA in both flax groups (Table 5). Both livers and kidneys had a striking increase in the amount of oleic acid in cholesterol-fed animals from these tissues (Tables 4, 5). This could be a compensatory reaction to cholesterol feeding since the cholesterol diet does not include significant amounts of oleic acid as compared to the other diets (Table 1). Both non-ischaemic/perfused hearts and ischaemic-perfused hearts successfully included ALA into their lipid fractions. Non-ischaemic hearts were significantly enhanced with ALA and had a small decrease in AA (Table 6). Ischaemic-reperfused hearts also showed an increase in ALA in the flax-fed group, but a similar increase was not observed in the cholesterol/flax group (Table 7). Overall, the most significant incorporations of ALA (mg/ per g tissue) were seen in liver; which was not surprising since it is the major site for lipid metabolism in animals. These data established that cholesterol and flaxseed feeding in rabbits changed the

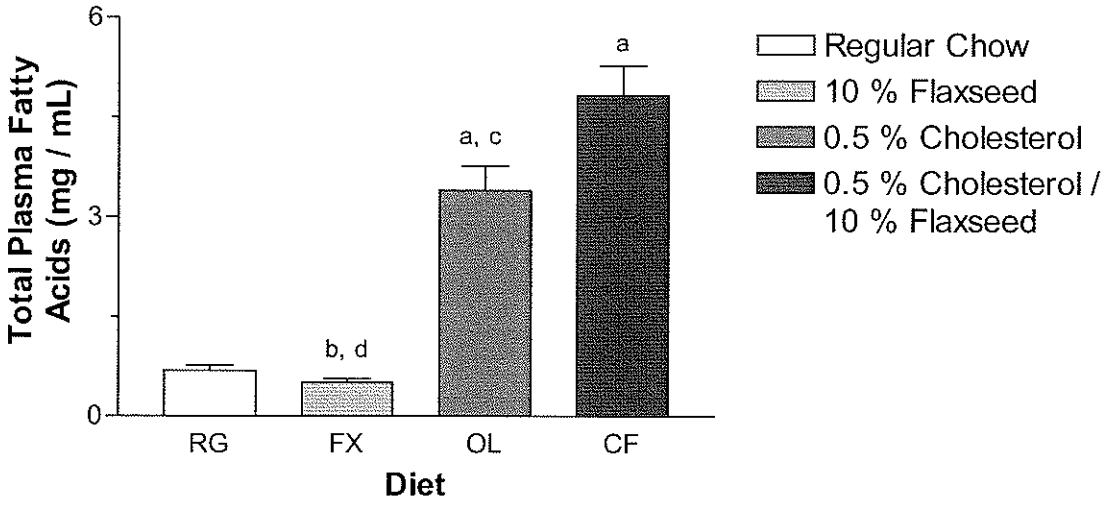


Figure 18. Total Plasma Fatty Acid Concentrations After 8 Weeks of Feeding

Total fatty acid concentration of rabbit plasma at 8 weeks was measured as mg of fatty acid methyl esters (FAME) per mL of plasma ± SEM. Statistical Significance: RG vs. FX, OL, CF = a; FX vs. OL = b; OL vs. CF = c; FX vs. CF = d.

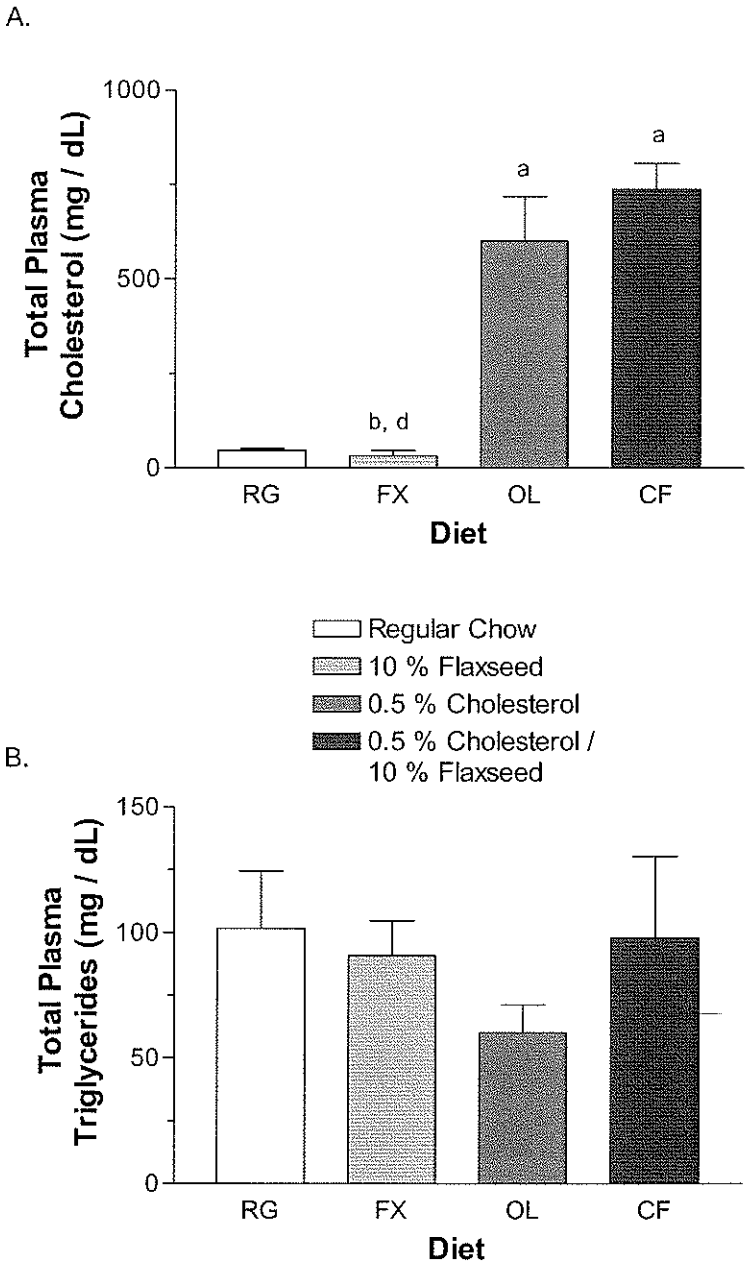


Figure 19. Total Plasma Cholesterol and Triglyceride Concentrations after 8 Weeks Feeding

Total plasma cholesterol (A.) and triglyceride (B.) concentrations of rabbit plasma at 8 weeks were measured in mg / dL per mL ± SEM. Statistical Significance: RG vs. FX, OL, CF = a; FX vs. OL = b; OL vs. CF = c; FX vs. CF = d.

FAME	Regular Chow (RG)	10 % Flaxseed (FX)	0.5 % Cholesterol (OL)	10 % Flaxseed and 0.5 % Cholesterol (CF)
C14:0	0.0576 ± 0.0019	0.0672 ± 0.0045	0.0638 ± 0.0028	0.0596 ± 0.0038
C14:1	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C16:0	4.9853 ± 0.0983	4.9430 ± 0.1016	4.8583 ± 0.1328	4.5909 ± 0.1486
C16:1	0.0961 ± 0.0046	0.1261 ± 0.0128	0.1267 ± 0.0057	0.1101 ± 0.0067
C18:0	5.6523 ± 0.1319	5.3414 ± 0.1417	5.5393 ± 0.2105	5.0987 ± 0.1666
C18:1 (n-9) Ol	6.0108 ± 0.4207	5.9380 ± 0.3647	6.3931 ± 0.2013	5.7161 ± 0.2245
C18:1 Vac	1.1697 ± 0.0492	1.0937 ± 0.0493	1.2392 ± 0.0361 [c]	0.9930 ± 0.0244 [a]
C18:2 (n-6) LA	0.4227 ± 0.0112	0.6021 ± 0.0536 [a]	0.5502 ± 0.0238 [a]	0.5873 ± 0.0135 [a]
C20:0	0.1104 ± 0.0149	0.1050 ± 0.0130	0.1265 ± 0.0113	0.1009 ± 0.0095
C18:3 (n-6) GLA		0.0000 ± 0.0000	0.0000 ± 0.0000	0.0083 ± 0.0083
C20:1 (n-9)	0.4630 ± 0.0690	0.4464 ± 0.0568	0.5523 ± 0.0410	0.4163 ± 0.0395
C18:3 (n-3) ALA	0.0000 ± 0.0000	0.1682 ± 0.0489 [a,b]	0.0068 ± 0.0046 [b,c]	0.1091 ± 0.0167 [a,c]
C20:2 (n-6)	0.0890 ± 0.0107	0.1025 ± 0.0127	0.1217 ± 0.0115	0.0992 ± 0.0084
C22:0	0.3607 ± 0.0203	0.2741 ± 0.0198 [a]	0.3230 ± 0.0244 [c]	0.2415 ± 0.0148 [ac]
C20:3 (n-6) 8-11-14	0.1448 ± 0.0079	0.1637 ± 0.0112	0.1608 ± 0.0106	0.1834 ± 0.0093
C20:3 (n-3) 11-14-17	0.0000 ± 0.0000	0.0132 ± 0.0056	0.0067 ± 0.0067	0.0025 ± 0.0025
C20:4 (n-6)	2.9023 ± 0.1059	2.4139 ± 0.1066 [a]	2.6983 ± 0.1467	2.3300 ± 0.1220 [a]
C24:0	0.2586 ± 0.0311	0.2346 ± 0.0366	0.2987 ± 0.0363	0.2048 ± 0.0243
C20:5 (n-3) EPA	0.0000 ± 0.0000	0.0280 ± 0.0057 [a,b]	0.0077 ± 0.0077 [b]	0.0239 ± 0.0056 [a]
C24:1 (n-9)	0.4328 ± 0.0712	0.3734 ± 0.0631	0.4902 ± 0.0488	0.3578 ± 0.0446
C22:6 (n-3) DHA	2.2416 ± 0.0961	2.3914 ± 0.1403	2.3382 ± 0.0867	2.3769 ± 0.1369

Table 3. Effect of Feeding on Brain Fatty Acid Profile at 8 Weeks

Fatty acid composition of rabbit brains were measured as mg of fatty acid methyl esters (FAME) per gram of tissue ± SEM. Abbreviations: LA- linoleic acid, GLA- gamma linoleic acid, ALA- alpha linolenic acid, EPA- eicosapentaenoic acid, DHA- docosahexaenoic acid. Statistical Significance: RG vs. FX, OL, CF = a; FX vs. OL = b; OL vs. CF = c; FX vs. CF = d.

FAME	Regular Chow (RG)	10 % Flaxseed (FX)	0.5 % Cholesterol (OL)	10 % Flaxseed and 0.5 % Cholesterol (CF)
C14:0	0.0617 ± 0.0096	0.0596 ± 0.0098	0.1423 ± 0.0377	0.0924 ± 0.0120
C14:1	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0092 ± 0.0062	0.0000 ± 0.0000
C16:0	3.5842 ± 0.2915	3.2856 ± 0.2554	4.5931 ± 0.4040 b	3.9677 ± 0.2517
C16:1	0.2173 ± 0.0328	0.1736 ± 0.0228	0.4949 ± 0.0954 a,b,c	0.2853 ± 0.0299
C18:0	3.2784 ± 0.0847	3.1656 ± 0.0857	3.0982 ± 0.0910	3.7325 ± 0.2093 a,c,d
C18:1 (n-9) Ol	3.5885 ± 0.1656	3.2317 ± 0.1460	5.1458 ± 0.4045 a,b	4.6528 ± 0.3120 a,d
C18:1 Vac	0.5317 ± 0.0410	0.4540 ± 0.0161	0.7819 ± 0.0558 a,b,c	0.5722 ± 0.0372
C18:2 (n-6) LA	5.7473 ± 0.2484	5.5979 ± 0.2042	6.1090 ± 0.2477	6.7557 ± 0.4430
C20:0	0.0813 ± 0.0032	0.0824 ± 0.0042	0.0974 ± 0.0040	0.1050 ± 0.0082 a,d
C18:3 (n-6) GLA	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0154 ± 0.0042 a,b,c	0.0000 ± 0.0000
C20:1 (n-9)	0.0711 ± 0.0058	0.0577 ± 0.0026	0.1344 ± 0.0143 a,b	0.1279 ± 0.0114 a,d
C18:3 (n-3) ALA	0.2128 ± 0.0148	0.8693 ± 0.0354 a,b	0.3264 ± 0.0483	1.6329 ± 0.1037 a,c,d
C20:2 (n-6)	0.1010 ± 0.0034	0.0817 ± 0.0063	0.1431 ± 0.0124 a,b	0.1293 ± 0.0087 a,d
C22:0	0.1538 ± 0.0073	0.1396 ± 0.0083	0.1510 ± 0.0048	0.1504 ± 0.0104
C20:3 (n-6) 8-11-14	0.2227 ± 0.0061	0.1393 ± 0.0095 a	0.3299 ± 0.0171 a,b,c	0.2794 ± 0.0127 a,d
C20:3 (n-3) 11-14-17	0.0173 ± 0.0047	0.1004 ± 0.0095 a,b	0.0149 ± 0.0061	0.1646 ± 0.0106 a,c,d
C20:4 (n-6)	3.6501 ± 0.1163	2.8567 ± 0.1216 a,b	3.5198 ± 0.0585	3.0396 ± 0.1117 a,c
C24:0	0.0889 ± 0.0071	0.0884 ± 0.0074	0.0747 ± 0.0040	0.0957 ± 0.0067
C20:5 (n-3) EPA	0.1188 ± 0.0071	0.3071 ± 0.0098 a,b	0.1055 ± 0.0034	0.5273 ± 0.0155 a,c,d
C24:1 (n-9)	0.1207 ± 0.0140	0.1074 ± 0.0088	0.1220 ± 0.0069	0.1178 ± 0.0095
C22:6 (n-3) DHA	0.1518 ± 0.0084	0.2143 ± 0.0140 a,b,d	0.1360 ± 0.0103	0.1486 ± 0.0077

Table 4. Effect of Feeding on Kidney Fatty Acid Profile at 8 Weeks

Fatty acid composition of rabbit kidneys were measured as mg of fatty acid methyl esters (FAME) per gram of tissue ± SEM. Abbreviations: LA- linoleic acid, GLA- gamma linoleic acid, ALA- alpha linolenic acid, EPA- eicosapentaenoic acid, DHA- docosahexaenoic acid. Statistical Significance: RG vs. FX, OL, CF = a; FX vs. OL = b; OL vs. CF = c; FX vs. CF = d

FAME	Regular Chow (RG)	10 % Flaxseed (FX)	0.5 % Cholesterol (OL)	10 % Flaxseed and 0.5 % Cholesterol (CF)
C14:0	0.5126 ± 0.0473	0.7067 ± 0.1548	0.6310 ± 0.0210	0.5684 ± 0.0330
C14:1	0.0703 ± 0.0469	0.0840 ± 0.0560	0.2116 ± 0.0576	0.0682 ± 0.0454
C16:0	8.1294 ± 1.1202	11.4887 ± 3.9729	10.8560 ± 0.7503	10.7096 ± 0.5404
C16:1	0.9066 ± 0.1838	1.0584 ± 0.3164	2.7018 ± 0.1769 a, b, c	1.9986 ± 0.1489 a, d
C18:0	7.1766 ± 0.4043	7.1323 ± 0.8955	4.7677 ± 0.2547 a, b	5.5854 ± 0.2602
C18:1 (n-9) Ol	7.7614 ± 1.0342	9.0362 ± 2.6771	17.2156 ± 2.1619 a, b	19.8219 ± 0.9423 a, d
C18:1 Vac	1.2253 ± 0.1610	1.3075 ± 0.2685	2.3949 ± 0.1165 a, b	2.1130 ± 0.0851 a, d
C18:2 (n-6) LA	10.3348 ± 0.8535	11.1191 ± 2.0993	10.7335 ± 0.5608	10.5330 ± 0.3000
C20:0	0.0000 ± 0.0000	0.0430 ± 0.0286	0.2143 ± 0.0245 a, b	0.2337 ± 0.0025 a, d
C18:3 (n-6) GLA	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C20:1 (n-9)	0.2354 ± 0.0642	0.0000 ± 0.0000 a	0.4007 ± 0.0089 a, b, c	0.0000 ± 0.0000 a
C18:3 (n-3) ALA	0.7809 ± 0.1142	4.8023 ± 1.5053 a, b	1.2094 ± 0.0861	8.2202 ± 0.4645 a, c, d
C20:2 (n-6)	0.4353 ± 0.0324	0.3844 ± 0.0154	0.4328 ± 0.0172	0.4139 ± 0.0137
C22:0	0.3885 ± 0.0068	0.3546 ± 0.0052	0.3342 ± 0.0379	0.3438 ± 0.0041
C20:3 (n-6) 8-11-14	N/m	N/m	N/m	N/m
C20:3 (n-3) 11-14-17	N/m	N/m	N/m	N/m
C20:4 (n-6)	2.6550 ± 0.1107	1.9899 ± 0.2397 a	1.7740 ± 0.0515 a	1.5160 ± 0.0699 a
C24:0	N/m	N/m	N/m	N/m
C20:5 (n-3) EPA	0.2256 ± 0.0379	0.4026 ± 0.0387 a, b	0.0820 ± 0.0418 a	0.4765 ± 0.0105 a, c
C24:1 (n-9)	0.1529 ± 0.0779	0.0504 ± 0.0504	0.1531 ± 0.0780	0.4678 ± 0.0522 a, c, d
C22:6 (n-3) DHA	0.5675 ± 0.0122	0.6582 ± 0.0545 b	0.4415 ± 0.0096	0.7555 ± 0.0960 c

Table 5. Effect of Feeding on Liver Fatty Acid Profile at 8 Weeks

Fatty acid composition of rabbit livers were measured as mg of fatty acid methyl esters (FAME) per gram of tissue ± SEM. Abbreviations: LA- linoleic acid, GLA- gamma linoleic acid, ALA- alpha linolenic acid, EPA- eicosapentaenoic acid, DHA- docosahexaenoic acid. Statistical Significance: RG vs. FX, OL, CF = a; FX vs. OL = b; OL vs. CF = c; FX vs. CF = d

FAME	Regular Chow (RG)	10 % Flaxseed (FX)	0.5 % Cholesterol (OL)	10 % Flaxseed and 0.5 % Cholesterol (CF)
C14:0	0.4658 ± 0.0495	1.1073 ± 0.2428	1.1052 ± 0.2344	1.0768 ± 0.2757
C14:1	0.0585 ± 0.0209	0.1537 ± 0.0552	0.2205 ± 0.0713	0.0707 ± 0.0477
C16:0	5.3704 ± 0.5801	10.0459 ± 2.4056	9.6224 ± 2.0754	10.4529 ± 2.5926
C16:1	0.7009 ± 0.0780	1.4567 ± 0.3301	1.8670 ± 0.4046	1.5590 ± 0.4546
C18:0	3.1909 ± 0.1362	3.6275 ± 0.3077	3.4253 ± 0.2994	3.7663 ± 0.3697
C18:1 (n-9) Ol	5.6605 ± 0.6167	9.9385 ± 2.2124	8.9732 ± 1.8087	9.8353 ± 2.1913
C18:1 Vac	1.0551 ± 0.0706	1.5121 ± 0.2174	1.6044 ± 0.1983	1.4345 ± 0.1700
C18:2 (n-6) LA	7.6513 ± 0.3685	9.2899 ± 1.1469	9.0073 ± 1.0986	9.6167 ± 0.9072
C20:0	0.0779 ± 0.0178	0.2209 ± 0.0319 [a]	0.1455 ± 0.0440	0.1742 ± 0.0104
C18:3 (n-6) GLA	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0412 ± 0.0278
C20:1 (n-9)	0.0000 ± 0.0000	0.0000 ± 0.0000	0.1469 ± 0.0448 [a, b, c]	0.0000 ± 0.0000
C18:3 (n-3) ALA	0.8243 ± 0.1270	4.8547 ± 1.1819 [a, b]	1.1365 ± 0.2648	5.0081 ± 0.6255 [a, c]
C20:2 (n-6)	0.1888 ± 0.0255	0.3550 ± 0.0178 [a]	0.3846 ± 0.0188 [a]	0.2547 ± 0.0096 [a, c, d]
C22:0	0.1920 ± 0.0345	0.3929 ± 0.0150 [a]	0.4107 ± 0.0166 [a]	0.2691 ± 0.0069 [a, c, d]
C20:3 (n-6) 8-11-14	N/m	N/m	N/m	N/m
C20:3 (n-3) 11-14-17	N/m	N/m	N/m	N/m
C20:4 (n-6)	3.5188 ± 0.0412	2.5688 ± 0.1080 [a, b]	3.0947 ± 0.0680 [a]	2.8636 ± 0.0806 [a, c, d]
C24:0	N/m	N/m	N/m	N/m
C20:5 (n-3) EPA	0.2430 ± 0.0363	0.6789 ± 0.0177 [a, b]	0.2981 ± 0.0651	0.6264 ± 0.0300 [a, c]
C24:1 (n-9)	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C22:6 (n-3) DHA	0.3861 ± 0.0473	0.5932 ± 0.0263 [a, d]	0.6258 ± 0.0148 [a, c]	0.4055 ± 0.0057

Table 6. Effects of Feeding on Fatty Acid Profile of Non-ischaemic Hearts at 8 Weeks

Fatty acid composition of non-ischaemic rabbit hearts were measured as mg of fatty acid methyl esters (FAME) per gram of tissue ± SEM. Abbreviations: LA- linoleic acid, GLA- gamma linoleic acid, ALA- alpha linolenic acid, EPA- eicosapentaenoic acid, DHA- docosahexaenoic acid. Statistical Significance: RG vs. FX, OL, CF = a; FX vs. OL = b; OL vs. CF = c; FX vs. CF = d

FAME	Regular Chow (RG)	10 % Flaxseed (FX)	0.5 % Cholesterol (OL)	10 % Flaxseed and 0.5 % Cholesterol (CF)
C14:0	1.2261 ± 0.2525	0.8945 ± 0.1521	1.5021 ± 0.2564 [c]	0.5988 ± 0.0713
C14:1	0.1839 ± 0.0446	0.1061 ± 0.0319 [a, b]	0.2921 ± 0.0405	0.0393 ± 0.0266 [a, c]
C16:0	12.5761 ± 2.7792	9.1968 ± 1.5056	14.6030 ± 2.5380 [c]	5.8137 ± 0.6835
C16:1	1.6192 ± 0.3011	1.1777 ± 0.1591	2.2835 ± 0.4641 [b, c]	0.8232 ± 0.0817
C18:0	4.0223 ± 0.4841	3.5663 ± 0.3053	4.1500 ± 0.4247	2.8378 ± 0.1771
C18:1 (n-9) Ol	13.8393 ± 3.0498	10.8639 ± 1.8124	16.5863 ± 2.9404 [c]	6.1201 ± 0.6705
C18:1 Vac	1.8090 ± 0.2759	1.4639 ± 0.1782	2.0564 ± 0.2829 [c]	0.9545 ± 0.0723 [a]
C18:2 (n-6) LA	12.0125 ± 2.0462	9.6363 ± 1.1693	13.8617 ± 2.0113 [c]	6.9836 ± 0.5712
C20:0	0.1694 ± 0.0416	0.1309 ± 0.0297	0.2424 ± 0.0302	0.1497 ± 0.0294
C18:3 (n-6) GLA	0.0418 ± 0.0284	0.0000 ± 0.0000	0.0441 ± 0.0299	0.0000 ± 0.0000
C20:1 (n-9)	0.1859 ± 0.0484	0.0000 ± 0.0000 [a, b]	0.3077 ± 0.0463 [a]	0.0000 ± 0.0000 [a, c]
C18:3 (n-3) ALA	2.0883 ± 0.4853	5.8914 ± 1.0354 [a, b, d]	2.6473 ± 0.5199	2.6830 ± 0.4789
C20:2 (n-6)	0.3668 ± 0.0228	0.2387 ± 0.0420 [a, b]	0.3933 ± 0.0313 [c]	0.2741 ± 0.0239
C22:0	0.2709 ± 0.0459	0.2607 ± 0.0278	0.3500 ± 0.0149	0.2938 ± 0.0249
C20:3 (n-6) 8-11-14	N/m	N/m	N/m	N/m
C20:3 (n-3) 11-14-17	N/m	N/m	N/m	N/m
C20:4 (n-6)	2.5435 ± 0.1499	2.1031 ± 0.0480	2.5482 ± 0.1035	2.1559 ± 0.1830
C24:0	N/m	N/m	N/m	N/m
C20:5 (n-3) EPA	0.3192 ± 0.0533	0.5511 ± 0.0217 [a, b]	0.3346 ± 0.0400	0.5586 ± 0.0494 [a, c]
C24:1 (n-9)	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C22:6 (n-3) DHA	0.5230 ± 0.0079	0.5001 ± 0.0268	0.4918 ± 0.0171	0.4577 ± 0.0381

Table 7. Effects of Feeding on Fatty Acid Profile of Ischaemic-Reperfused Hearts at 8 Weeks

Fatty acid composition of ischaemic-reperfused rabbit hearts were measured as mg of fatty acid methyl esters (FAME) per gram of tissue ± SEM. Abbreviations: LA- linoleic acid, GLA- gamma linoleic acid, ALA- alpha linolenic acid, EPA- eicosapentaenoic acid, DHA- docosahexaenoic acid. Statistical Significance: RG vs. FX, OL, CF = a; FX vs. OL = b; OL vs. CF = c; FX vs. CF = d

fatty acid compostion of major organs; especially hearts from which CSR were isolated.

CHARACTERISATION OF CSR

Before analysis of CSR isolated from the above study, control CSR vesicles were characterised for specific protein content, Ca^{2+} transport properties, and fatty acid composition. The SR proteins RyR, CSQ, and SERCA were sparse in CSR as compared to skeletal SR vesicles. CSR vesicles were compared to HSR vesicles in a SDS-PAGE gel stained with Coomassie brilliant blue R-250 (Figure 20). Qualitative observations revealed that the CSR preparation was relatively devoid of RyR monomer (550 kDa) and CSQ (44 kDa) opposed to HSR. SERCA protein bands (110 kDa) were visible in the CSR lane, but were less abundant than HSR.

CSR were also characterised for responses to known SR Ca^{2+} transport affectors. Pharmacological agents acting on SERCA, but not RyR nor IP_3R affected Ca^{2+} transport. A Ca^{2+} transport assay was refined in a microplate spectrofluorometer to allow for the simultaneous monitoring of up to 8 assays and to accommodate the small yield of CSR (see Methods). Potassium oxalate was used to facilitate conductance of Ca^{2+} into the SR lumen in a timely fashion, since CSR vesicles had very little CSQ. Figure 21 shows that CSR was unresponsive to the RyR inhibitor ruthenium red (Panel A). Moreover, RyR activating concentrations of ryanodine produced only a slight delay in Ca^{2+} uptake (Figure 21, Panel E). Ca^{2+} transport in isolated CSR vesicles was not responsive to changes in RyR conductance induced by RyR ligands, RR and ryanodine. IP_3R antagonists, xestospongin C and heparin had no effect on Ca^{2+} transport, which indicated that these CSR preparations were not enriched in IP_3Rs. (Figure 21, Panels C and D). As expected, thapsigargin, a SERCA antagonist inhibited Ca^{2+} uptake by these vesicles (Figure 21, Panel B). The Ca^{2+} transport

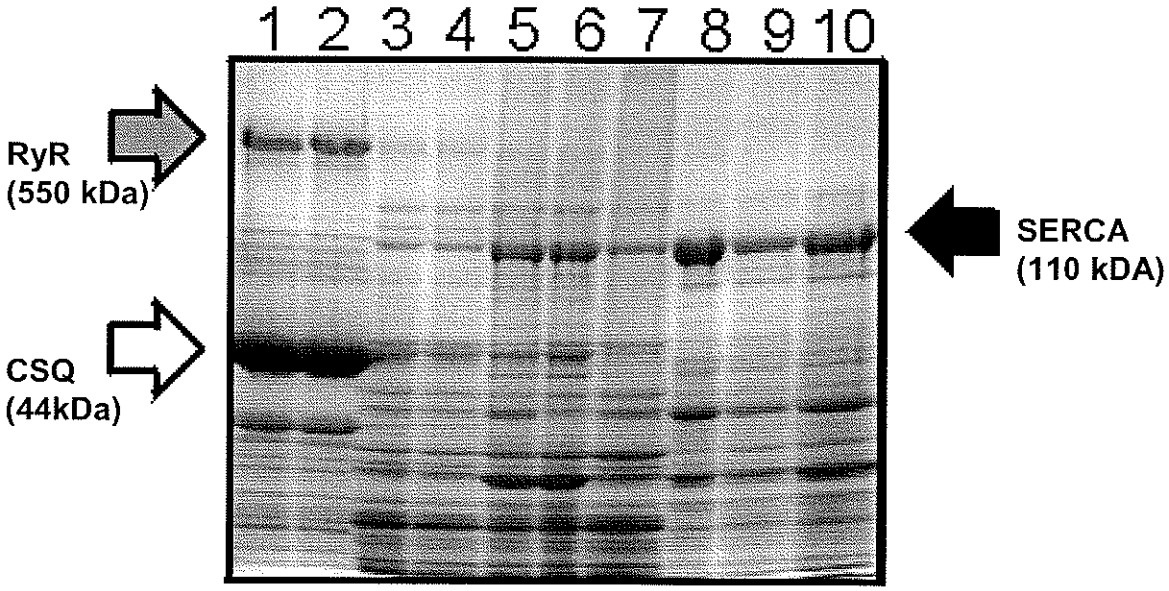


Figure 20. Comparison of CSR and HSR by SDS-PAGE Gel

Lane 1-10 respectively represent HSR, HSR, CSR, CSR, Pellet 1, Pellet 2, Pellet 3, Supernatant 1, Supernatant 2, and Supernatant 3 as sampled from CSR isolation.

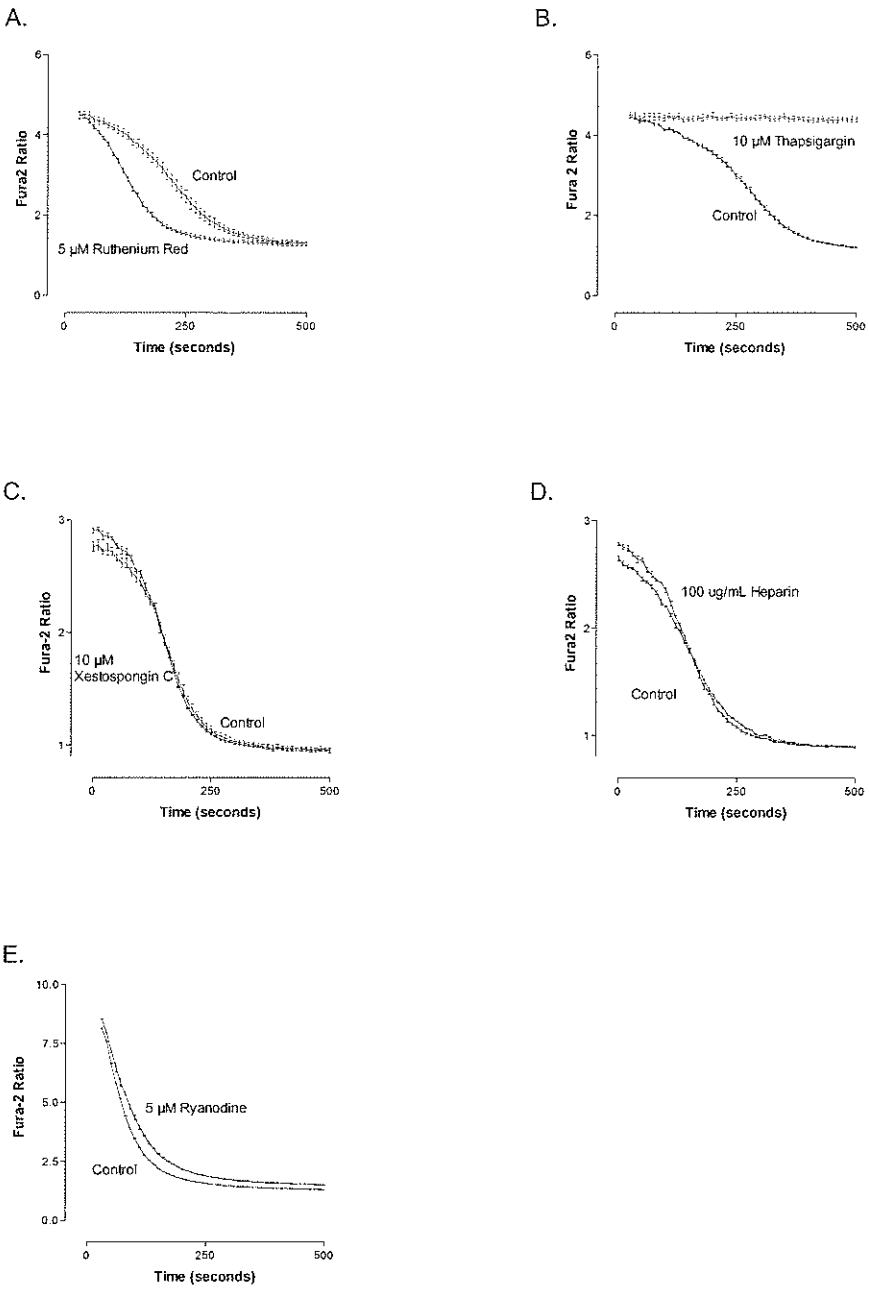


Figure 21. Characterisation of Extraluminal Ca^{2+} Transport by Isolated CSR Vesicles

HSR membranes (0.25 mg/mL) were pre-treated with varying concentrations of A. ruthenium red, B. thapsigargin, C. xestospongin, D. heparin, E. ryanodine . Ca^{2+} transport (CG-2 traces) was monitored as described in "Methods". Transport was initiated by the combined addition of Mg·ATP and PEP.

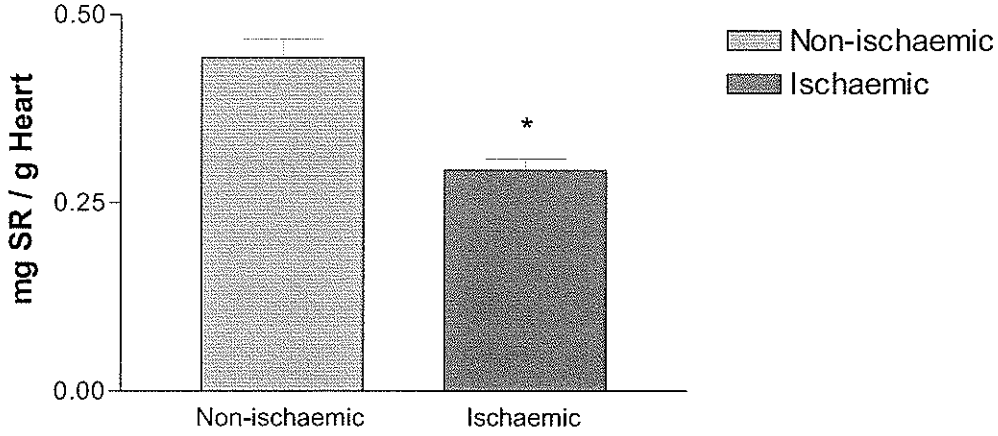
data suggests that CSR vesicles isolated in this manner and under these conditions (see Methods) are responsive to alterations in SERCA activity, but not channel conductance.

CSR from both non-ischaemic and ischaemic-reperfused rabbit hearts fed one of the four diets were characterised to see if dietary alterations in lipid profile could modify their function. CSR were isolated from 8-week rabbit hearts and assayed for protein content recovery as an indication of the comparability of the preparations. Diet did not affect the yield of CSR (mg CSR / g heart), but perfusion status did. Isolated CSR vesicles from non-ischaemic reperfused hearts had similar yields between all feeding groups (Figure 22, Panel B). Likewise, CSR yields were similar amongst ischaemic-reperfused hearts (Figure 22, Panel B). However, protein recovery of CSR from ischaemic-reperfused hearts was significantly reduced compared to CSR from non-ischaemic reperfused hearts ($P<0.05$) (Figure 22, Panel A).

To measure if dietary flaxseed changed the fatty acid composition of isolated CSR vesicles, gas chromatography analysis was performed on extracted lipids. Fatty acid analysis of CSR from non-ischaemic hearts exhibited a significant increase in the content of ALA and its metabolite EPA in CSR from both groups of flax-fed rabbits (Table 8). Fatty acid analysis of isolated CSR from ischaemic-reperfused hearts also indicated that ALA, EPA, and even DHA (in the 10% flax/0.5% cholesterol group) were incorporated into flax-fed animals (Table 9). Hence, dietary flaxseed supplementation successfully increased the n-3 PUFA content of isolated CSR vesicles from rabbit and was not altered by ischaemia-reperfusion.

We were also interested in the extent to which cholesterol was incorporated into SR membranes during feeding. For the most part there is a trend of increasing amounts of free, esterified, and total cholesterol in CSR vesicles from rabbits fed cholesterol, although these

A.



B.

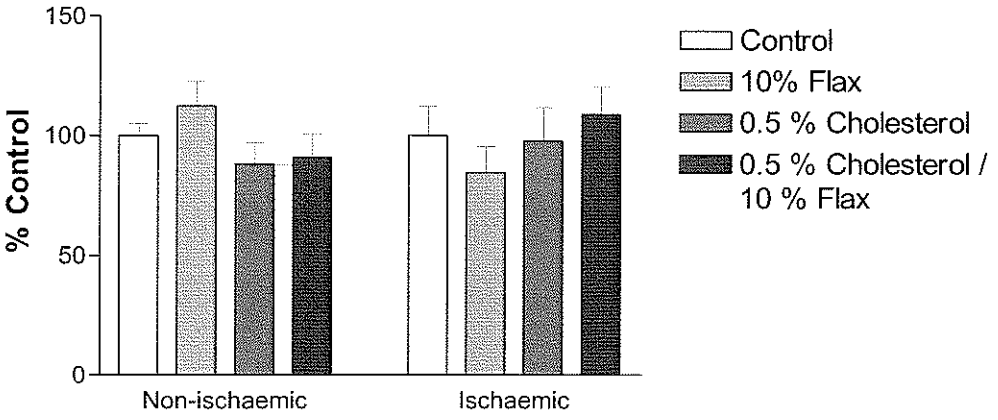


Figure 22. Yield of CSR from Feeding Groups

A. Comparison of CSR yield between CSR isolated from non-ischaemic hearts and ischaemia-reperfused hearts (mg SR / g heart). * $P < 0.05$ B. Relative comparison of CSR yield from non-ischaemic hearts and ischaemia-reperfused hearts from rabbits fed similar diets, measured as % control (regular chow diet). Not significantly different.

FAME	Regular Chow (RG)	10 % Flaxseed (FX)	0.5 % Cholesterol (OL)	10 % Flaxseed and 0.5 % Cholesterol (CF)
C14:0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C14:1	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C16:0	0.0629 ± 0.0024	0.0602 ± 0.0020	0.0610 ± 0.0018	0.0645 ± 0.0038
C16:1	0.0098 ± 0.0013	0.0112 ± 0.0022	0.0129 ± 0.0026	0.0042 ± 0.0041
C18:0	0.0731 ± 0.0020	0.0709 ± 0.0019	0.0694 ± 0.0013	0.0780 ± 0.0036
C18:1 (n-9) Ol	0.0531 ± 0.0020	0.0498 ± 0.0016	0.0456 ± 0.0020	0.0460 ± 0.0031
C18:1 Vac	0.0197 ± 0.0006	0.0171 ± 0.0005	0.0190 ± 0.0007	0.0183 ± 0.0011
C18:2 (n-6) LA	0.1486 ± 0.0038	0.1404 ± 0.0046	0.1388 ± 0.0030	0.1535 ± 0.0052
C20:0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C18:3 (n-6) GLA	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C20:1 (n-9)	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C18:3 (n-3) ALA	0.0056 ± 0.0001	0.0161 ± 0.0004 a, b	0.0018 ± 0.0008 a, c	0.0167 ± 0.0006 a
C20:2 (n-6)	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C22:0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0001 ± 0.0001
C20:4 (n-6)	0.1040 ± 0.0029	0.0780 ± 0.0022 a, b, d	0.0903 ± 0.0019 a	0.0892 ± 0.0047 a
C20:5 (n-3) EPA	0.0000 ± 0.0000	0.0102 ± 0.0003 a, b	0.0000 ± 0.0000	0.0108 ± 0.0005 a, c
C24:1 (n-9)	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C22:6 (n-3) DHA	0.0052 ± 0.0004	0.0047 ± 0.0001	0.0038 ± 0.0006	0.0048 ± 0.0006

Table 8. Effects of Feeding on Fatty Acid Profiles of CSR from Non-ischaemic Hearts at 8 Weeks

Fatty acid composition of CSR was measured as mg of fatty acid methyl esters (FAME) per gram of tissue ± SEM. *n-3 PUFA content of CSR lipids from flax-fed rabbits increased significantly, including ALA (C18:3) and its metabolite EPA (C20:5), possibly at the expense of AA (C20:4 n-6).* Abbreviations: LA- linoleic acid, GLA- gamma linoleic acid, ALA- alpha linolenic acid, EPA- eicosapentaenoic acid, DHA- docosahexaenoic acid. Statistical Significance: RG vs. FX, OL, CF = a; FX vs. OL = b; OL vs. CF = c; FX vs. CF = d.

FAME	Regular Chow (RG)	10 % Flaxseed (FX)	0.5 % Cholesterol (OL)	10 % Flaxseed and 0.5 % Cholesterol (CF)
C14:0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C14:1	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C16:0	0.0526 ± 0.0020	0.0641 ± 0.0063	0.0552 ± 0.0037	0.0534 ± 0.0027
C16:1	0.0000 ± 0.0024	0.0036 ± 0.0018 [a]	0.0000 ± 0.0053	0.0092 ± 0.0035 [a]
C18:0	0.0594 ± 0.0024	0.0776 ± 0.0065 [a]	0.0632 ± 0.0034	0.0707 ± 0.0030
C18:1 (n-9) Ol	0.0389 ± 0.0017	0.0486 ± 0.0038	0.039 ± 0.0024	0.0388 ± 0.0021
C18:1 Vac	0.0173 ± 0.0008	0.0215 ± 0.0027	0.0187 ± 0.0013	0.0157 ± 0.0001
C18:2 (n-6) LA	0.1121 ± 0.0042	0.1384 ± 0.0121	0.1193 ± 0.0056	0.1393 ± 0.0039
C20:0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C18:3 (n-6) GLA	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C20:1 (n-9)	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C18:3 (n-3) ALA	0.0002 ± 0.0002	0.0180 ± 0.0020 a, b, d	0.0000 ± 0.0000	0.0148 ± 0.0007 a, c
C20:2 (n-6)	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C22:0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C20:4 (n-6)	0.0740 ± 0.0024	0.0785 ± 0.0069	0.0779 ± 0.0040	0.0748 ± 0.0026
C20:5 (n-3) EPA	0.0000 ± 0.0000	0.0082 ± 0.0011 a, b	0.0000 ± 0.0000	0.00092 ± 0.0009 a, c
C24:1 (n-9)	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
C22:6 (n-3) DHA	0.0003 ± 0.0002	0.0009 ± 0.0004	0.0000 ± 0.0000	0.0027 ± 0.0004 a, c, d

Table 9. Effects of Feeding on Fatty Acid Profiles of CSR from Ischaemia-Reperfused Hearts at 8 Weeks

Fatty acid composition of CSR was measured as mg of fatty acid methyl esters (FAME) per gram of tissue ± SEM. *n-3 PUFA content of CSR lipids from flax-fed rabbits increased significantly, including ALA (C18:3) and its metabolites EPA (C20:5) and DHA (C22:6).* Abbreviations: LA- linoleic acid, GLA- gamma linoleic acid, ALA- alpha linolenic acid, EPA- eicosapentaenoic acid, DHA- docosahexaenoic acid. Statistical Significance: RG vs. FX, OL, CF = a; FX vs. OL = b; OL vs. CF = c; FX vs. CF = d.

results are not significant. A colorimetric assay was adapted to measure both free and total cholesterol, and consequently esterified cholesterol in a microplate reader (see Methods). Cholesterol content of CSR isolated from rabbits fed a flax, cholesterol, or cholesterol/ flax supplemented diets are shown in Figure 23. There were not any significant changes in cholesterol composition of CSR from non-ischaemic hearts (Figure 23, Panel A). Within the reperfused-ischaemic group, CSR from cholesterol / flax-fed rabbits had significantly higher amounts of cholesterol over control and flax fed rabbits (Figure 23, Panel B). CSR from reperfused-ischaemic hearts had significantly lower levels of total cholesterol than CSR from non-ischaemic hearts ($P= 0.0156$), so the groups were not comparable. There was a general increase in cholesterol content in all cholesterol-fed animals; however the trend was only significant in one case.

After determining that the CSR lipid composition was altered by changes in diet, we assessed their response to Ca^{2+} handling. Extraluminal Ca^{2+} uptake of CSR isolated from rabbits fed a flax, cholesterol, or cholesterol/flax supplemented diet from both non-ischaemic and ischaemic-reperfused hearts was monitored using a novel microplate assay (see Methods). The averaged values of Ca^{2+} uptake are shown as traces in Figure 24, Panel A. Ca^{2+} uptake by non-ischaemic CSR was not affected by diet, but Ca^{2+} uptake by reperfused-ischaemic CSR was changed. Figure 24, Panel B compares the rate constants of extraluminal Ca^{2+} uptake as determined by exponential decay in Graphpad Prism software. Analysis of these rates revealed that neither flaxseed nor cholesterol supplementationto the diet altered Ca^{2+} transport in CSR isolated from non-ischaemic hearts. However, a striking improvement in Ca^{2+} transport was shown in CSR from hearts of flaxseed-fed rabbits that were subjected to ischaemia reperfusion. This may suggest that flaxseed feeding may improve Ca^{2+} handling in CSR after ischaemia-reperfusion

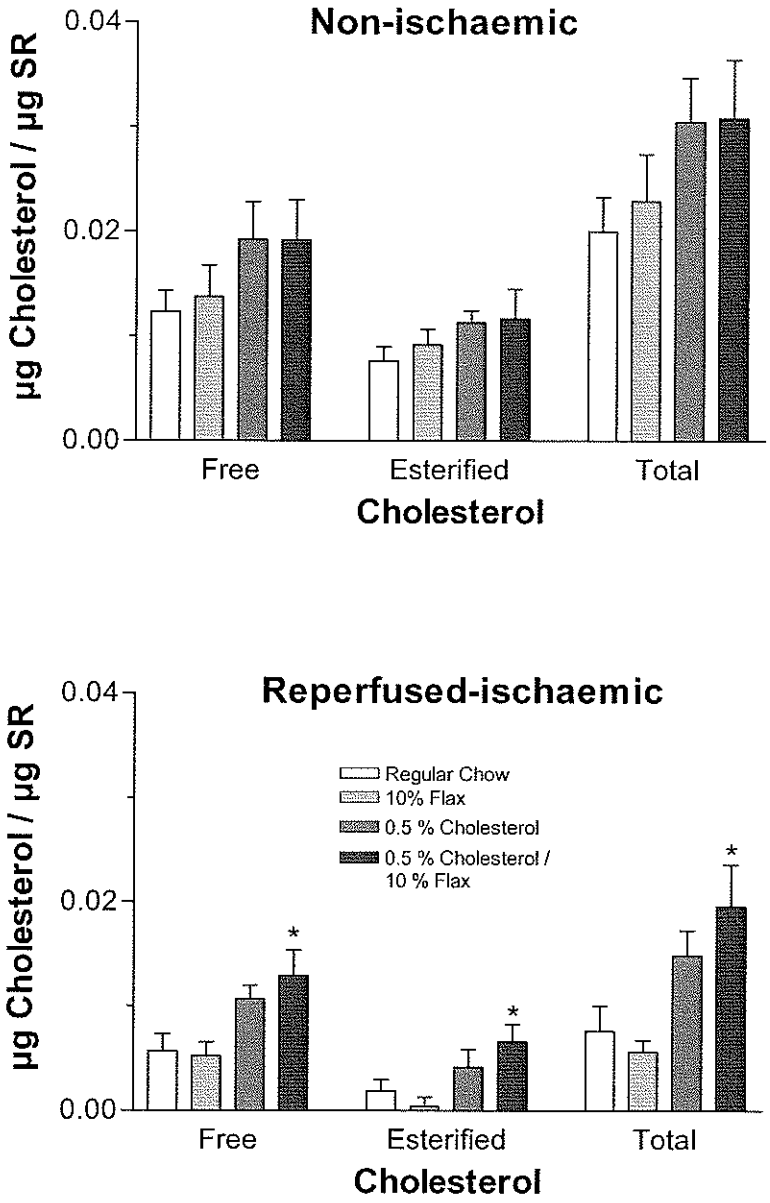


Figure 23. Cholesterol content of CSR isolated from rabbits fed a flax, cholesterol, or cholesterol / flax supplemented diet from both non-ischaemic and ischaemic reperfused hearts

CSR lipids were extracted in $CHCl_3$: MeOH and assayed for free and total cholesterol content as in 'Methods'. *No significant changes in cholesterol composition of CSR from non-ischaemic hearts were observed. CSR from reperfused-ischaemic hearts had significantly lower levels of total cholesterol than CSR from non-ischaemic hearts. Within the reperfused-ischaemic group, CSR from cholesterol / flax-fed rabbits had significantly higher amounts of cholesterol over control and flax fed rabbits.* * $P < 0.05$

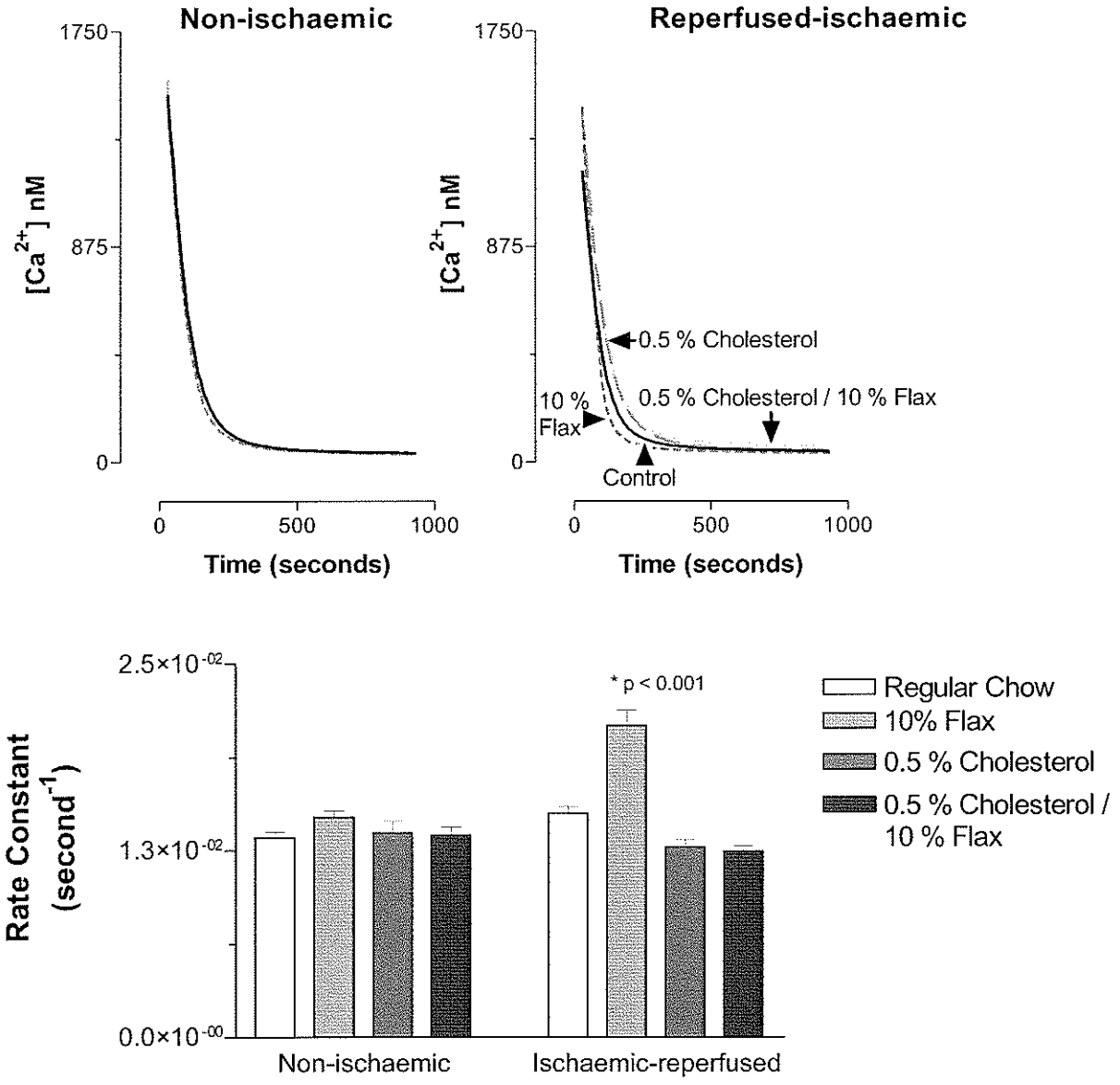


Figure 24. Panel A. Extraluminal Ca^{2+} uptake by CSR isolated from rabbits fed a flax, cholesterol, or cholesterol / flax supplemented diet from both non-ischaemic and ischaemic-reperfused hearts. Mean values of oxalate-dependent uptake of 5 μM Ca^{2+} by 0.125 mg / mL CSR at 37 °C are shown. As described in 'Methods' Ca^{2+} transport was initiated by the combined addition of MgATP, CP and K^{+}-oxalate. Y-axis represents free extraluminal Ca^{2+} as converted from Fura-2 ratio. **Panel B. Comparison of rates of extraluminal Ca^{2+}uptake by CSR isolated from rabbits fed a flax, cholesterol or cholesterol / flax supplemented diet from both non-ischaemic and ischaemic-reperfused hearts.** While there were no significant differences in the rates of Ca^{2+}uptake within the non-ischaemic group, flax-fed ischaemic-reperfused CSR sequestered Ca^{2+} at a significantly higher rate than all other groups ($p < 0.001$).

This may suggest that flaxseed feeding may improve Ca^{2+} handling in CSR after ischaemia-reperfusion.

SERCA activity in these preparations of CSR vesicles was also studied. The rate of SERCA activity as measured in nmol NADH/ minute·mg SR was similar in the non-ischaemic reperfused preparations (Figure 25). This was in agreement with the CSR data that showed no change in Ca^{2+} transport rates. Overall, the ischaemic reperfused-group had higher rates of ATP hydrolysis compared to the non-ischaemic reperfused groups. However, the rates of NADH oxidation were significantly slower in the two flax-fed groups. A discussion of these results follows.

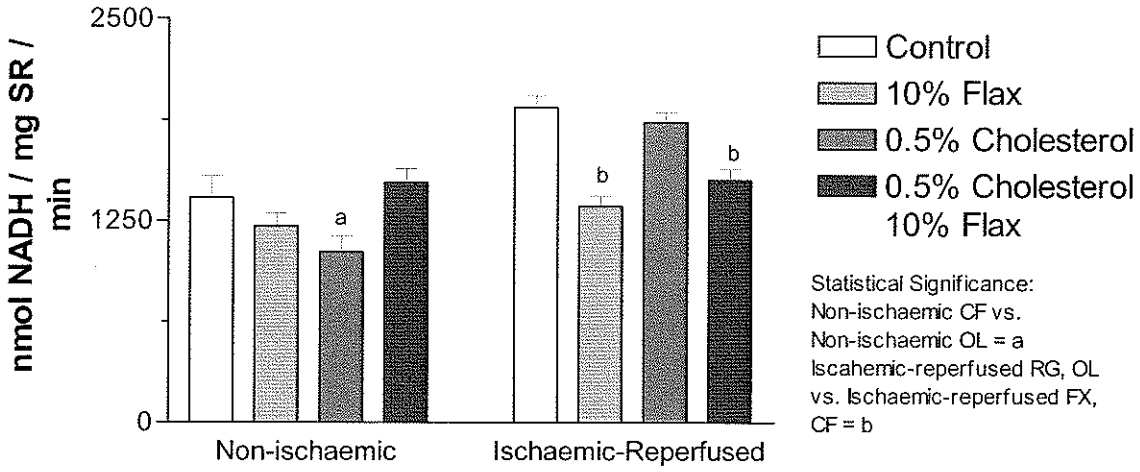


Figure 25. Comparison of SERCA catalytic activity between CSR isolated from rabbits fed a flax, cholesterol, or cholesterol / flax supplemented diet from both non-ischaemic and ischaemic-reperfused hearts. NADH fluorescence decay was used as a measure of SERCA catalytic activity by 0.010 mg / mL CSR at 37 oC, see 'Methods'. Specific SERCA2a activity was determined in the presence of the Ca^{2+} ionophore, 4-bromo-A23187 (10 μM) and SERCA inhibitor, Thapsigargin (10 μM). CSR from perfused-ischaemic hearts showed significantly faster catalytic activity ($p < 0.001$) than CSR from non-ischaemic hearts regardless of diet.

IV. DISCUSSION

We sought to elucidate the effects of dietary flaxseed, enriched in n-3 PUFAs, on SR function. Our experimental design examined both free fatty acid and esterified fatty acid effects; by exogenous addition of n-3 PUFAs to isolated SR preparations and isolation of SR from animals fed diets high in n-3 PUFAs, respectively. Both studies suggest that n-3 PUFAs improve SR function. Unesterified n-3 PUFAs enhanced Ca^{2+} sequestration of the SR at least in part by impairing Ca^{2+} efflux via RyRs. Esterified n-3 PUFAs also improved Ca^{2+} uptake in isolated SR vesicles, but only after ischaemia-reperfusion. The implication of how these fatty acids act pertains to their potential clinical relevance as anti-arrhythmic agents and their recommended route of administration as nutraceuticals or dietary supplements.

One theory reasons that n-3 PUFAs prevent arrhythmia in their free form. A number of studies suggest that FAs may simply partition into the lipid membrane to exert antiarrhythmic effects because these actions are rapidly reversed with the FA chelator BSA [245]. This could mean that these acting n-3 PUFAs are not covalently bound nor are they esterified to any membrane compontents [245]. A long-chain FFA mechanism may be physiologically relevant since they are liberated from phospholipids during ischaemia by phospholipase A_2 [245, 268-271]. Also, mitochondrial fatty acid oxidation leads to accumulation of C16 and C18 chains and carnitine derivatives in cytosol, which may explain pump failure in ischaemic heart [271-273]. In fact cytosolic FFA concentrations may reach up to 20 μM at which point we would expect to see direct effects on SR function [274].

Unesterified n-3 PUFA effects on SR function generally point towards an inhibiton of RyR Ca^{2+} release [258]. These SR effects are manifested in permeabilised ventricular

myocytes by decreased frequency, velocity, and amplitude of contraction [256, 257]. Our initial studies also predicted that n-3 PUFAs reduce the open probability (P_o) of RyRs in isolated HSR vesicles. When HSR vesicles were pre-treated with relatively low concentrations of n-3 PUFAs we observed an increase in the rate of Ca^{2+} uptake without an increase in the corresponding catalytic activity. This is consistent with improved coupling of Ca^{2+} transport and/or a decrease in the RyR P_o. More efficient cycling of Ca^{2+} into HSR vesicles per mole of ATP would increase the rate of Ca^{2+} sequestration without necessarily increasing the rate of ATP hydrolysis. Reagents known to improve coupling can act by inhibiting a tonic leak of Ca^{2+} out of the SR. For example, the known RyR antagonist ruthenium red (RR) produces a sharp increase in the rate at which the SR accumulates Ca^{2+} without increasing SERCA activity. It can be reasoned that RyR antagonists increase Ca^{2+} uptake by blocking the inherent leak of Ca^{2+} out of the SR. Similarly, improved coupling of Ca^{2+} transport may arise from a decrease in the mean open time of RyRs. Hence, under these conditions, n-3 PUFAs appear to inhibit RyR leaks in a similar manner to pharmacological RyR antagonists.

At relatively higher n-3 PUFA concentrations, we observed a decrease in Ca^{2+} uptake rates, while SERCA1 activity increased. This may be due to uncoupling of Ca^{2+} transport or an increase in RyR P_o may explain this effect. A leak of Ca^{2+} out of the SR would result in futile cycling as can be seen with Ca^{2+} ionophores like ionomycin. Likewise, a RyR-mediated leak would also decrease coupling of Ca^{2+} sequestration to ATP hydrolysis. The general effect of these n-3 PUFAs appears to be improved coupling below a threshold, and above which the ratio of Ca^{2+} transport to ATP hydrolysis decreases. This is consistent with the idea that ampiphiles stabilize membrane lipids at low concentrations and disrupt membrane lipids at high concentrations [275-277].

Oxidized forms of both LA and AA have been shown to decrease the rate of Ca^{2+} accumulation by SR vesicles independently of SERCA or detergent-like effects[278]. However, our n-3 PUFA preparations were purged with nitrogen gas to attenuate their oxidation. Alternatively, palmitic acid (C16:0), and not oleic acid (C18:1), enhanced Ca^{2+} uptake in isolated light skeletal SR fractions [271-273]. While these preparations would not be enriched in RyRs, they still provide insight into a possible mechanism of action for fatty acids. They too hypothesised that enhanced Ca^{2+} uptake could be due to an alteration in SERCA activity, leak status of the vesicles, or both factors [271].

To account for the increased rate of Ca^{2+} uptake in HSR vesicles in the presence of relatively low amounts of n-3 PUFAs, we further examined the possibility that intrinsic SERCA1 activity may have increased, thereby increasing the rate of Ca^{2+} uptake. Under completely uncoupled conditions of Ca^{2+} transport, n-3 PUFAs induced a slight 5-10% increase in the rates of NADH oxidation as a marker of SERCA1 activity ($p<0.001$). This indicated that n-3 PUFAs might influence SERCA1 kinetics by ligand-mediated actions on the pump directly. However we reasoned that a faster rate of ATP hydrolysis did not account for increased Ca^{2+} uptake, since others have shown that even though SERCA activity was stimulated by more than 75% with palmitic acid, less than 1% of palmitic acid was associated with the annular layer, so it was not reasonable to suggest that improved SERCA activity accounted for increased Ca^{2+} transport [279, 280]. Furthermore, palmitic acid only increased the rate of E2P decay and did not affect phosphoenzyme intermediate conformations of the pump [280].

An alternative hypothesis to our observations in Figures 2-5 was that n-3 PUFAs inhibited RyR-mediated leaks. To examine this hypothesis we used a [^{3}H]-ryanodine binding assay. The data indicated that n-3 PUFAs decreased [^{3}H]-ryanodine binding in a

concentration-dependent manner. The results of this experiment did not necessarily display competitive binding between [^{3}H]-ryanodine and n-3 PUFAs, but perhaps a change in the functional characteristics of RyR such that it could no longer bind [^{3}H]-ryanodine. A decrease in [^{3}H]-ryanodine binding which occurs in the presence of RyR channel inhibitors may indicate that n-3 PUFAs decrease RyR P_o [67].

Observed effects of n-3 PUFAs on RyR P_o are supported in the literature. Rodrigo postulated that an observed decrease in contraction in permeabilised ventricular myocytes in the presence of EPA was due to an increased threshold for Ca^{2+} release from the SR or blockade of SR membrane K^+ channels that maintain electrical homeostasis [256]. The second hypothesis may be discounted on the basis that valinomycin, a known K^+-selective ionophore, has no effects on the HSR Ca^{2+} transport [281]. Furthermore, both EPA and DHA have been shown to inhibit spontaneous Ca^{2+} release causing contraction of ventricular myocytes [257]. Subsequent studies by O'Neill indicate that these n-3 PUFAs inhibit the SR Ca^{2+} release mechanism [258].

To further examine the hypothesis that n-3 PUFAs inhibit RyR we investigated their effects upon CICR. We chose to examine DHA, since it has been shown to be the most potent of the n-3 PUFAs [282]. We observed a concentration-dependent inhibition of Ca^{2+} release. Like the Ca^{2+} transport traces, the response of HSR vesicles to Ca^{2+} pulse-loading with DHA was similar to the ruthenium red-response, which also increased the threshold for Ca^{2+} release (Figure 15). A contrary effect was observed when pulse-loading HSR with higher concentrations of DHA (Figure 14 Panel B). Ca^{2+} release was potentiated, but the rate of re-accumulation of Ca^{2+} was unchanged, as indicated by the slope of the Ca^{2+} transient. These results suggest that RyR kinetics, but not SERCA kinetics, were altered by n-3 PUFAs. This is consistent with results of a study by Leifert in which asynchronous

contractility of ventricular myocytes was decreased by feeding n-3 PUFAs. This study also showed that changes in the decay rate of the Ca^{2+} transient were independent of SERCA and SR Ca^{2+} content [283]. Additional data concurs that FAs may alter subconductance states of RyRs. AA (20-50 μM), but not 5 μM AA, has been shown to induce partially RR-sensitive Ca^{2+} release from CSR [284]. Also, oleic acid and not palmitic acid accelerated spontaneous Ca^{2+} release from SR vesicles [280].

The mechanism by which unesterified n-3 PUFAs exert their action on the SR is not well defined. Unesterified n-3 PUFAs most likely act by partitioning into the hydrophobic domains of SR lipid, particularly the discrete TM domain regions of ion channels, since its effects on SR function are reversed in the presence of BSA [257, 285]. Furthermore, n-3 PUFAs may act by increasing membrane fluidity [282, 286]. Electron microscopy indicates that FFAs that delay Ca^{2+} uptake may increase membrane permeability by solubilising SR membrane vesicles [279]. However, FFAs that enhanced Ca^{2+} transport had no affect on SR membrane morphology, which suggests that these lower concentrations do not alter membrane packing [279]. An increase in fluidity due to higher concentrations of n-3 PUFAs may disrupt normal membrane protein function or increase the permeability of the SR membrane to Ca^{2+}. Both pumps and channels may malfunction under such high concentrations. At lower concentrations, n-3 PUFAs may facilitate Ca^{2+} transport by acting as Ca^{2+}-precipitating anions or buffers in the SR [279, 280, 287]. FFAs that improve SR Ca^{2+} transport form aggregates in response to high Ca^{2+} concentrations and create a poorly exchangeable Ca^{2+} pool [280, 287]. It is reasonable then to suggest that n-3 PUFAs may uncouple SR Ca^{2+} transport by increasing membrane fluidity and permeability to the point of dysfunction in the range of 25-100 μM. However, a more specific effect may occur at low-

end n-3 PUFA concentrations that decrease RyR P_o possibly by decreasing the free pool of SR Ca^{2+}.

The physiological extension of these findings could find its place in the attenuation of arrhythmogenesis. When incorporated into phospholipids n-3 PUFAs, activated by PLA_2 may mediate enhanced sequestration of Ca^{2+} during ischaemia [288, 289]. In the cardiomyocyte this would lower resting cytosolic Ca^{2+} and thereby decrease the probability of spontaneous Ca^{2+} release from the SR. Additionally, decreased RyR P_o would increase the threshold for Ca^{2+} release from the SR and possibly prevent unwanted Ca^{2+} release, since ischaemic SR is less effective at sequestering Ca^{2+} due to Ca^{2+} efflux occurring at RyRs [290].

Our interests in the potential for creating a store of n-3 PUFAs available to the SR lead us to pursue a flaxseed-supplemented dietary model from which we could assess the function of isolated SR vesicles. Flaxseed enriched with the n-3 PUFA ALA was chosen over sources of EPA and DHA because it has a better taste, odor and is more resistant to auto-oxidation [245]. However, ALA may not be stored as well as the longer-chain n-3 PUFAs, since it is metabolised more readily [291, 292]. n-6 PUFAs have also been shown to be less potent antiarrhythmic agents because they can form pro-arrythmic metabolites [282, 293]. Analysis of rabbit tissues confirmed that changes in n-3 PUFA content in the diet were reflected in major organs and especially CSR vesicles. Likewise, other studies that have attempted to alter SR lipid compostion with dietary feeding have had similar success in decreasing the n-6/n-3 ratio in SR membranes [236, 240-242]. Fatty acids can change the physical structure of PL membranes and affect transmembrane ion channels [285, 294].

Cholesterol feeding was included in this study to create both fatty streaks in animals that could potentially create ischaemic conditions *in vivo* and to alter membrane fluidity and function. We were not successful in achieving significant changes in cholesterol compostion

of SR membranes with a 0.5% cholesterol diet over 8 weeks. Previous studies in Yorkshire swine have shown that the effects of a 1% cholesterol diet on SR function are controversial. After 7-11 months of feeding a cholesterol-enriched diet, SR Ca^{2+} uptake was increased [295]. Another study used a cholesterol-supplemented diet for up to 1 year and reported a decrease in Ca^{2+} uptake and a corresponding decrease in SERCA activity [267]. Our lack of significant changes in cholesterol content and SR function in cholesterol-fed groups may indicate that the given dose of cholesterol was too low, the myocardium was capable of resisting changes to cholesterol content, or most likely the duration of feeding was too short to induce increases in membrane cholesterol content so SR activity could not be altered. However, a combined cholesterol/flaxseed diet masked the flaxseed effects on Ca^{2+} transport in CSR from ischaemia-reperfused hearts.

To assess SR function we used the rate of net Ca^{2+} uptake in isolated SR vesicles, which represents the equilibrium between Ca^{2+} influx and efflux. These studies on isolated SR vesicles reveal a limited amount of information about SR function since they are removed from a physiological setting. However, they do allow for dynamic analysis of the interacting pumps and channels of the SR. The use of oxalate in our Ca^{2+} transport conditions is advantageous because it augments a relatively slow process. An obvious limitation to our study is the lack of negative control, SR from reperfused hearts, for the ischaemia-reperfusion group. Nevertheless, we were able to observe some significant trends relative to the positive control, SR from ischaemia-reperfused hearts. Feeding rabbits with ALA-enriched flaxseed protected SR function from ischaemia-reperfusion-induced injury as measured by Ca^{2+} transport studies. This agrees with our FFA study on isolated SR vesicles that showed that n-3 PUFAs, including ALA, enhance Ca^{2+} uptake. However, in our model of dietary intervention, Ca^{2+} uptake was improved relative to control by flaxseed feeding

only in the post-ischaemic period. It could be that CSR is altered by ischaemia-reperfusion in such a way that newly incorporated n-3 PUFAs can protect against impaired Ca^{2+} handling. More likely, the changes in FA content in the SR membrane did not alter normal basal SR function. However, when stressed, as in I/R challenge, the FA in the membrane induced significant protective effects on SR function.

The response of SR Ca^{2+} transport to ischaemia has been extensively studied, but is not well defined. It is generally agreed upon that oxalate-dependent Ca^{2+} transport is depressed after ischaemia due to a combination of reduced SERCA activity and concurrent efflux of Ca^{2+} via RyRs [290, 296-301]. However, some studies indicate that Ca^{2+} transport is unchanged after bouts of ischaemia lasting 60 minutes or less [300, 302-304]. Ischaemia may not affect SERCA activity after 60 minutes either [296, 300]. These data are in agreement with the present results.

Some potential limitations of our data should be examined. Since each assay was equilibrated in the same buffers before Ca^{2+} uptake was initiated, we can rule out the possibility that confounding concentrations of ions like Ca^{2+}, H^{+}, Mg^{2+} were present in our ischaemic-reperfused SR preparations [298]. We can also exclude the possibility that our isolating process may select for "good" SR, since this procedure has been shown to reveal damaged SR when it is assayed [296]. There is evidence that variability exists between SR isolated from different areas of the myocardium [305]. Our SR vesicles were not isolated from a single area of the heart,but rather a whole heart was used for the isolation of SR. Thus our results could not detect any variability in SR function within specific regions of the heart. Another limitation to consider in the present study is that changes in the efflux pathway cannot be observed in this preparation, because it is unresponsive to effectors of RyR.

There are a number of mechanisms that could be occurring in our isolated CSR vesicles that would account for the beneficial effects of dietary flaxseed on Ca^{2+} transport after ischaemia-reperfusion. For example, alterations in phosphorylation status could be a cause for altered Ca^{2+} handling. CaMKII has been implicated in the maintenance of SR function after ischaemic preconditioning by phosphorylation of both phospholamban and SERCA [306]. Since the SERCA pump was not maximally activated in the Ca^{2+} transport experiments, we would expect to see differences in rates of uptake as affected by phosphorylation status [298]. Mechanistic data also points towards a reactive oxygen species (ROS)–induced damage of SR that is mediated by CaMK [307]. It has been suggested that "reoxygenation upon reperfusion of the myocardium may then result in promoting inorganic as well as organic-free radical chain reactions ultimately affecting unsaturated fatty acids and membrane bilayer functions" [300]. It follows then that newly incorporated PUFAs and/or cholesterol may be oxidized during reperfusion and thereby alter Ca^{2+} uptake properties.

As discussed earlier, membrane fluidity as determined by cholesterol and fatty acid compostition may or may not affect SERCA activity [174, 283, 286, 308]. According to Almeida, cholesterol decreased Ca^{2+} uptake and SERCA activity is only slightly decreased most likely due to a loss of efficiency in the ionophoretic channel of the pump [308]. n-3 PUFA-induced changes in membrane fluidity did not account for the termination of asynchronous contractility in cardiomyocytes. Rather, mechanisms independent of SERCA affected Ca^{2+} handling [283]. SERCA activity is also modulated by membrane thickness as determined by esterified fatty acids and alkanes [173]. Ultimately, homoviscous adaptation, the maintenance of membrane integrity, may allow for continued function of SERCAs despite dietary modification [174, 283, 309].

Direct effects of flaxseed feeding on SERCA function were determined by a unique assay. To exclude contaiminating ATPase activity from our analysis, we used the SERCA antagonist thapsigargin to find specific SERCA activity as a function of NADH oxidation over time. Other studies in the literature have attempted to assay SERCA activity as a measure of inorganic phosphate liberation in isolated SR preparations, but the source of this activity is not clear [296, 310]. Our data indicates that SERCA activity after ischaemia-reperfusion in control and cholesterol-fed rabbits is significantly higher than in SR from non-ischaemic hearts. As discussed above, this contradicts studies by Singal that establish that both Ca^{2+} transport and SERCA activity are unchanged after ischaemia reperfusion within our given time-frame [300]. This data did not give any indication as to why CSR from ischaemia-reperfused hearts from flaxseed-supplemented rabbits sequestered Ca^{2+} at a higher rate. It is likely that the Ca^{2+}transport-related SERCA effects may have been masked because the SERCA pump was maximally stimulated under our assay conditions [298]. In fact, SERCA activity was significantly slower in CSR from ischaemia-reperfused flax-fed animals than control ischaemia-reperfused CSR. We can hypothesise based on our study of the literature that oxidation of n-3 PUFAs after reperfusion may affect SERCA function. Future studies may account for differences in Ca^{2+} transport by assaying SERCA activity under similar conditions.

Improved SR Ca^{2+} handling after ischaemia-reperfusion may have clinical applications, since impaired SR Ca^{2+} handling may result in increased diastolic Ca^{2+}, which may cause spontaneous Ca^{2+} release, arrhythmias, and a weaker amplitude of contraction. In fact, the corresponding electrophysiological data from ischaemia-reperfused hearts indicates that flax-fed rabbits showed delayed contracture during the onset of ischaemia and were not susceptible to ventricular fibrillation during the ischaemia phase as were controls [311].

Cholesterol-flax fed rabbits were also resistant to ventricular fibrillation during reperfusion [311]. A number of SR-mediated mechanisms may contribute to these beneficial effects including alterations in coupling, RyR efflux, lumenal free Ca^{2+}, phosphorylation of SERCA, ROS and membrane fluidity. Overall ,these data suggest that n-3 PUFAs in both unesterified and esterified forms may attenuate changes in SR Ca^{2+} handling associated with ischaemia.

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