Dietary and Free n-3 Polyunsaturated Fatty Acids Modify Calcium Handling Mechanisms in the Heart

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Submitted for the Doctor of Philosophy
March 2003

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ABSTRACT

It is well recognized that the consumption of fish correlates with a reduction in mortality due to cardiovascular disease. Whole heart studies have identified that dietary fish oil confers protection from cardiac arrhythmias. Many studies have shown that the acute application of the polyunsaturated fatty acids present in fish oil, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) to cardiac myocytes significantly reduce the amplitude of the various sarcolemmal ion currents responsible for the cardiac action potential. It is believed that this reduction in electrical excitability is the mechanism by which fish oil confers protection from cardiac arrhythmias. However not all arrhythmias arise from disturbances in membrane electrical excitability, a class of arrhythmias arise from calcium mishandling.

The aim of this thesis was to determine whether the antiarrhythmic actions of polyunsaturated fatty acids present in fish oil are due to modulation of calcium handling. Using fast line scan confocal microscopy, calcium sparks, transients and waves were studied.

These studies show that in rats supplemented with 10% dietary fish oil, the width and duration of spontaneous calcium sparks was significantly reduced in both atrial and ventricular myocytes compared to myocytes from lard supplemented rats. This was also found in rat ventricular myocytes incubated with 15 μM EPA compared to the controls. Calcium transients in ventricular myocytes from rats supplemented with fish oil displayed a reduced doubling time of the rising phase of the transient without altering the

decaying phase compared to myocytes from lard supplemented rats. This was also found in ventricular myocytes incubated with 15 μM EPA compared to the controls.

Dietary fish oil supplementation was not found to alter the properties of calcium waves in rat ventricular myocytes.

When added to isolated ryanodine receptors in artificial bilayers, 50 μM EPA was found to significantly reduce the open probability of the channel compared to the controls.

The reduction in spark size and ryanodine receptor open probability may indicate that the polyunsaturated fatty acids may provide protection against arrhythmias by reducing spontaneous calcium release thereby reducing spontaneous membrane depolarization resulting from calcium extrusion from the cytosol by the Na^{+}-Ca^{2+} exchanger.

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Bonny Honen

ACKNOWLEDGEMENTS

Thank you to Assoc. Prof. Ted Mc Murchie and Mrs Sharon Burnard, (CSIRO Heath Sciences and Nutrition) for performing the membrane fatty analysis on both the atrial and ventricular tissue.

Thank you to Wayne Leifert and Anisa Jahangiri (CSIRO Heath Sciences and Nutrition) for their assistance with animal feeding for the dietary components of the experiments.

Thank you to Assoc. Prof. Derek Laver (School of Biomedical Science, University of Newcastle) for his assistance and the use of his Laboratory for the isolated Ryanodine Receptor experiments.

Thank you to Prof. Angela Dulhunty, Suzy Pace and Joan Stivala (Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, Australian National University) for supplying the SR vesicles used for the isolated Ryanodine Receptor experiments.

Thank you to Dr David Saint (Department of Physiology, University of Adelaide) for all his help and support throughout my PhD.

1. INTRODUCTION

1.1 General Introduction

1.1.1 Epidemiological Studies

Cardiovascular disease is the leading cause of death in Australia accounting for 40% of the total deaths in 1998. Of these deaths, 22% were due to coronary heart disease (mainly heart attacks) (Heart Foundation statistics 2001). It is well recognized that the regular consumption of fish correlates with a reduction in the mortality due to cardiovascular disease (Kromhout *et al.* 1985, Burr *et al.* 1989 Siscovick *et al.* 1995, Oomen *et al.* 2000, Yuan *et.al.* 2001).

In a population of middle age men it was shown that the mortality due to cardiovascular disease was reduced by fifty percent over a twenty-year period in those who consumed fish daily in comparison to those who did not eat fish at all (Kromhout *et al.* 1985). Similarly, a study by Burr *et al.* (1989) revealed a twenty nine percent reduction in mortality from all cardiovascular related causes over a 2 year period in a population who were asked to consume regular fatty fish meals in comparison to a population who were not. More recently, Siscovick *et al.* (1995) elucidated the dietary fish intake of a population by questionnaire and by a biomarker (fatty acid composition of red blood cell membranes). In subjects who consumed more fish, mortality due to myocardial infarction, ischemic heart disease and cardiac arrest were

all reduced. Since these studies there has been growing interest in determining the cardio-protective constituents in fish oil and the mechanisms by which they act.

1.1.2 Fatty Acids

The main class of fatty acids present in fish oil is the n-3 or omega-3 polyunsaturated fatty acids (PUFA). These are believed to be responsible for the cardio-protective actions of fish oil and are the subject of interest in the following studies. n-3 PUFAs are long chain fatty acids containing at least 2 double bonds, the first one positioned after the third carbon from the methyl end. Other classes of fatty acids include the n-6 PUFAs, monounsaturated fatty acids and saturated fatty acids. n-6 PUFAs contain 2 or more double bonds in the carbon chain, the first one positioned after the sixth carbon from the methyl end. Monounsaturated fatty acids contain one double bond in the carbon chain and saturated fatty acids contain no double bonds in the carbon chain (Laposata 1995). Examples of saturated and n-3 and n-6 PUFAs are illustrated in figure 1.

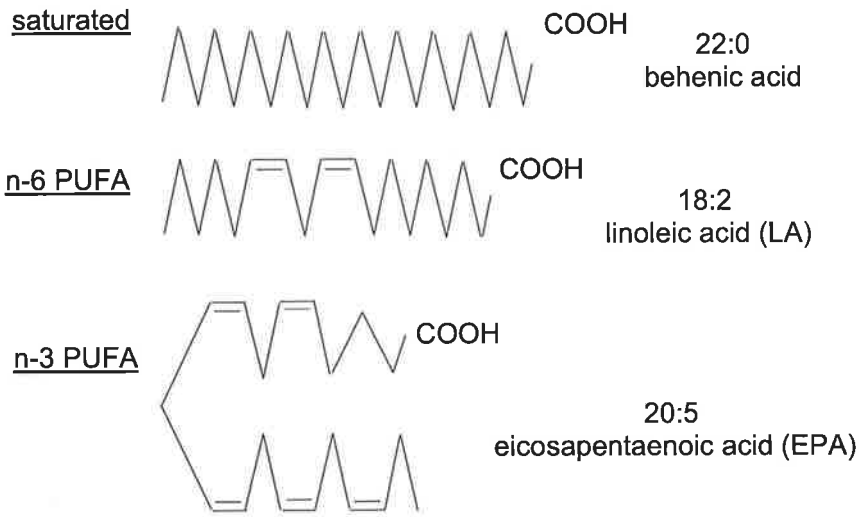


Figure 1: Examples of structures of saturated, n-3 and n-6 polyunsaturated fatty acids.

When acquired through diet, fatty acids are transported in the blood bound to albumin or as triglycerides, which are typically incorporated within chylomicrons and lipoproteins of very low to low density. The precise mechanisms by which fatty acids enter the cells are the topic of some controversy and the details are beyond the scope of this thesis. One model that has been described by which fatty acids enter the cell has been termed the "flip flop" model. This involves the adsorption of fatty acids to the phospholipid membrane exposed to the fatty acids. The fatty acids then distribute (or flip) to the inner leaflet of the membrane where they then release protons to the internal side of the phospholipid membrane reaching an ionization equilibrium. Desorption of the fatty acids from the internal side of the phospholipid membrane then takes place releasing the fatty acids into the cytosol (Kamp and Hamilton 1992, Zhang *et al.* 1996). This model allows for the bi-directional passage of fatty acids both into and out of the cell. However, this model only allows for the transport of unionized fatty acids. Receptor-mediated or protein-mediated transport is another mechanism by which fatty acids may be taken up by the cell. These proteins may work in conjunction with the flip-flop model by aiding desorption of the fatty acids into the cytosol, or may be important in the transmembrane passage of ionized fatty acids (Hamilton 1998).

Many studies performed to determine the actions of fatty acids *in vitro* involve the introduction of free fatty acids to the cell-bathing medium. Fatty acids are generally added to an aqueous medium where they can intercalate between membrane phospholipids, precipitate out in the form of micelles or pass through the cell membrane into the cytosol (as described above). When introduced through diet, the presence of serum albumin binds free fatty acids in the blood. Thus, the adsorption of

fatty acids to the phospholipid membrane becomes dependent on the affinity of the fatty acids for the phospholipids over the albumin (Hamilton 1998). Therefore, the introduction of fatty acids through the diet results in a much lower free fatty acid concentration in the cell bathing solution (or blood) than in *in vitro* studies where the fatty acids are applied acutely.

Once in the cell, the free fatty acids are subject to a number of metabolic pathways dictated by the class of fatty acids to which they belong. The major metabolic pathway for fatty acids of any species is β-oxidation for ATP synthesis by the mitochondria as this provides a large proportion of cell energy, particularly in the heart (Laposata 1995). Fatty acids may be esterified into membrane phospholids, and it is now well recognized that a diet high in fish oil increases the content of n-3 fatty acids in the cell membranes of cardiac myocytes (Charnock *et al.* 1985, McLennan *et al.* 1990, Charnock *et al.* 1992). Fatty acids may also be metabolized into oxygenated species such as thromboxanes and eicosanoids, the nature of these metabolites being dependent on the type of fatty acid. They may also be subject to elongation and desaturation pathways depending on the chain length and degree of saturation of the precursor fatty acid (Laposata 1995); the details of these pathways are beyond the scope of this thesis.

1.1.3 Antiarrhythmic Effects of PUFAs

Epidemiological studies have shown that there is a reduction in mortality due to sudden cardiac death from all causes. The end point of these studies is death where it was assumed that death was the result of cardiac arrhythmia, which leads to the conclusion that a diet high in fish oil (n-3 fatty acids) is protective against cardiac arrhythmias. There has been growing interest in determining whether n-3 fatty acids provide specific protection against cardiac arrhythmias. Since the epidemiological studies, there have been a number of studies determining if both dietary and acutely applied n-3 fatty acids have specific antiarrhythmic effects in isolated hearts.

1.1.3.1 Dietary Studies

In a study by McLennan (1993), the diets of rats were supplemented with olive oil (monounsaturated fat), sheep perirenal fat (saturated fat) or fish oil (n-3 PUFA). Acute myocardial ischaemia was induced in anaesthetized rats by arterial occlusion, followed by the restoration of blood flow (reperfusion). The incidence of reperfusion arrythmias, ischaemic arrhythmias and ventricular fibrillation were all reduced in rats supplemented with fish oil (n-3 PUFAs) but not in rats supplemented with monounsaturated fat or saturated fat. The rats supplemented with fish oil were found to have a greater proportion of n-3 and n-6 fatty acids in the phospholipid membrane compared to the rats supplemented with olive oil or saturated fat. Thus, it was concluded that the observed reduction in arrhythmic activity was likely to be due to the increased n-3 and n-6 fatty acid content in the membrane phospholipids.

Similarly, Pepe and McLennan (1996) supplemented the diet of rats with either saturated fat, fish oil (n-3) or a low fat reference diet. The hearts were removed from the rats and perfused using an isolated working heart model. Lowering the perfusion pressure of the heart induced ischemic arrhythmias and raising perfusion pressure back to control pressure initiated reperfusion. As in the previous study, dietary fish oil supplements prevented reperfusion induced ventricular fibrillation and reduced ischaemic arrhythmias. This study also demonstrated that the threshold of stimuli for the induction of electrically evoked ventricular fibrillation was increased in hearts from rats supplemented with dietary fish oil in comparison to those supplemented with saturated fat. It was concluded that dietary fish oil acts directly on myocardial properties that may contribute to the reductions in cardiac mortality associated with fish consumption.

1.1.3.2 Acute Studies

The n-3 PUFA constituents of fish oil when applied acutely have been shown to be similarly antiarrhythmic compared to those acquired through the diet. A study by Billman *et al.* (1999) has shown that n-3 PUFAs prevented fatal, ischaemia induced arrhythmias in surgically prepared, conscious, exercising dogs. Arrhythmias were induced by the inflation of a cuff placed around the left circumflex coronary artery. The inflation of the cuff resulted in ventricular fibrillation from which the dogs were defibrillated. With the addition of n-3 PUFAs eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or α-linolenic acid (ALA) to the superfusate perfusing the dog's heart prior to the induction of arrhythmias, the expected arrhythmias were prevented. It has been suggested that this form of acute addition of free PUFAs act to

modulate sodium and calcium currents (reducing electrical activity) in the myocytes, thus reducing antiarrhythmic activity.

The above evidence suggests that polyunsaturated fatty acids introduced both through diet and acutely applied via the superfusing solution act to prevent arrhythmias. However, from this information it is not possible to determine the nature of the actions of the PUFAs on the myocardium.

1.1.4 Effects PUFAs on Sarcolemmal Ion Currents

The antiarrhythmic effects of PUFAs are believed to be due to modified activity or blocking of sarcolemmal ion channels responsible for the cardiac action potential (figure 2), thereby reducing membrane excitability and increasing the threshold for action potential firing (Kang *et al.* 1995).

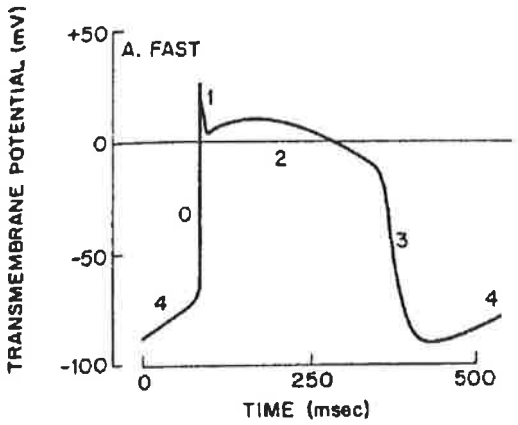


Figure 2: Diagrammatic representation of the action potential in cardiac purkinje fibers
Phase 0: depolarization due to the inward sodium current (I_{Na})
Phase 1: rapid repolarization due to the transient outward current (I_{to})
Phase 2: inward calcium flow via the L-type Ca^{2+} channels contributes to the plateau phase
Phase 3: repolarization due to the delayed rectifier potassium currents
Phase 4: outward potassium current maintains the resting potential
(Goodman and Gilman 1992)

1.1.4.1 Acute Studies

There have been numerous studies to determine the effects of acutely applied PUFAs on cardiac sarcolemmal ion currents. It has been shown that the acute application of EPA and DHA results in a reduction in the size of the cardiac sodium current in both adult and neonatal rat ventricular myocytes (Xiao *et al.* 1995, Leifert *et al.* 1999). Similarly, the addition of EPA was also found to result in a reduction in size of the L-type calcium current in adult rat ventricular myocytes (Xiao *et al.* 1997, Rodrigo *et al.*

1999). In contrast to this finding Pepe *et al.* (1994) found that the exposure of adult rat ventricular myocytes to DHA had no effect on the amplitude of the L-type Ca^{2+} current. However, it was found that the application of DHA inhibited the action of both nitredipine (L-type calcium channel antagonist) and Bay K (L-type calcium channel agonist), suggesting that DHA specifically binds to calcium channels at, or near, the dihydropyridine-binding site and interferes with calcium current modulation.Bogdanov *et al.* (1998) found that the addition of DHA in low concentrations caused a 40% reduction in the size of the transient outward potassium current with little effect on other potassium currents, although at higher concentrations the amplitude of the delayed rectifier potassium current was also reduced.

The above information suggests that the antiarrhythmic actions of PUFAs are due to their direct action on the ion channels responsible for the sarcolemmal ion currents. A reduction in the amplitude of the Na^{+} current could potentially be antiarrhythmic as it would slow the initial rising phase of the action potential (figure 2). This would require a larger electrical stimulus to reach threshold and prolong the time for action potential firing (Kang & Leaf 2000). Similarly the reduction in the amplitude of the L-type Ca^{2+} current would also require a greater electrical stimulus for activation. Electrically "desensitizing" these channels and therefore the cell membrane is beneficial in the protection against arrhythmias that can arise from abnormally elevated cytosolic [Ca^{2+}]. Elevated cytosolic Ca^{2+} can result in the activation of the Na^{+}-Ca^{2+} exchanger (see section 1.2.1) or the dysfunctional behavior of the Na^{+}- K^{+} exchanger. This commonly results in after-depolarizations and premature beats leading to arrhythmias (Kang & Leaf 2000).

The reduction in magnitude of the K^+ currents will reduce the rate of repolarization of the cardiac action potential. This will increase the action potential duration and therefore the refractory period for subsequent action potential firing. This will reduce the incidence of electrical re-stimulation due to possible pathological after-depolarizations thereby reducing the likelihood of arrhythmias (Kang & Leaf 2000).

1.1.4.2 Dietary Studies

The actions of dietary PUFAs on sarcolemmal ion currents have not been fully investigated. Studies by Leifert *et al.* (2000) revealed that membrane incorporation of n-3 PUFAs after dietary fish oil supplementation does not cause the same blocking effects on the sodium current or the transient outward potassium current as does free fatty acids. This suggests that PUFAs exhibit their antiarrythmic activity as free, unesterified fatty acids rather than by incorporation into the membrane phospholipids as with dietary supplementation. This finding is supported in a study by Weylandt *et al.* (1996) who cultured isolated cardiac myocytes in a control medium or one containing either DHA or EPA. Following incubation, isoproterenol (beta-adrenergic agonist) or an elevated concentration of external calcium was applied to the cell to promote arrhythmogenesis. There was no difference found in the induction of arrhythmias by either arrhythmogenic agent in the myocytes grown in PUFA-enriched medium compared to those grown in the control medium. It was therefore concluded that esterified fatty acids do not protect against arrhythmias.

The above information suggests that only free unesterified fatty acids are antiarrhythmic and that membrane-bound esterified fatty acids do not share these

properties. This does not explain how the introduction of PUFAs through the diet is protective against arrhythmias particularly those of ischemic origin.

During ischaemia, upregulation of phospholipase A_2 occurs, resulting in the excision of fatty acids from the phospholipid membranes (Wenzel and Innes 1983, Ford *et al.* 1991). This gives rise to the possibility that free PUFAs are responsible for providing protection against arrhythmias of ischemic origin.

1.2 Calcium Handling in the Heart

The above information describes how PUFAs are protective against arrhythmias due to the electrical disturbances associated with ischaemia. However, a large proportion of arrhythmias also arise from ischemic and non-ischemic induced disturbances in calcium handling of which PUFAs are also likely to offer protection against.

1.2.1 The Calcium Induced Calcium Release System

Contraction of the heart depends on a process known as calcium-induced calcium release (CICR). CICR occurs when calcium enters the cell via the L-type Ca^{2+} channel (dihydropyridine receptor), during the plateau phase of the cardiac action potential (figure 2). The L-type calcium current increases the cytosolic calcium concentration, which then results in activation of the sarcoplasmic reticulum (SR) calcium release channels (the ryanodine receptors; for detailed information on calcium activation of ryanodine receptors see section 1.6). Calcium activation of the ryanodine receptors triggers a global calcium release from the SR. This increase in intracellular calcium, known as a calcium transient, is pivotal for cell contraction (see

section 1.4). The magnitude of the calcium transient is determined by the cytosolic concentration of triggering calcium (Xiao *et al.* 1997). Typically the greater the magnitude of the L-type Ca^{2+}, the higher the cytosolic calcium concentration at the peak of the transient. Post contraction, calcium is primarily taken up by the SR Ca^{2+}ATPase and, to a lesser extent, calcium is extruded from the cell via the sarcolemmal Ca^{2+}ATPase and Na^{+}-Ca^{2+} exchanger (figure 3) (Xiao *et al.* 1997).

During ischemia and reperfusion, cardiac myocytes can loose their ability to control intracellular calcium. The precise mechanisms by which myocytes loose their ability to regulate calcium is unclear, although, it has been proposed that the increase in intracellular calcium associated with ischemia and reperfusion arises from the activation of the Na^{+}-H^{+} exchanger. This occurs on reperfusion due to the ischemia related rise in intracellular H^{+} (Xiao and Allen 2000). The activation of the Na^{+}-H^{+} exchanger leads to an increase in intracellular Na^{+} concentration, which is extruded on the activation of the Na^{+}-Ca^{2+} exchanger. This leads to a subsequent increase in intracellular Ca^{2+} concentration and ultimately calcium overload (Xiao and Allen 2000). The increase in intracellular calcium has been associated with the induction of oscillatory calcium release also known as propagating calcium waves (Brooks *et al.* 1995; Xiao *et al.* 1997; see section 1.5).

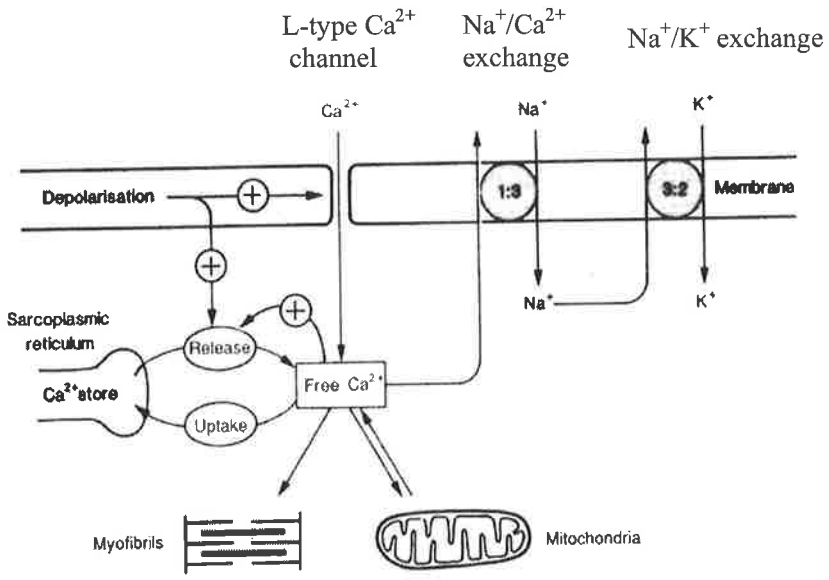


Figure 3: Schematic representation of the calcium-induced calcium release system (Rang *et al.* 1995).

1.2.2 Effects of Polyunsaturated Fatty Acids on the Calcium-Induced Calcium Release System

There is very limited information in the literature on the effects of PUFAs on the specific calcium handling mechanisms in particular PUFAs acquired through the diet. These calcium handling mechanisms include; the SR calcium release channel (the ryanodine receptor see section 1.6), the L-type calcium channel (the effects of acute PUFA application are discussed in section 1.1.4.1), the Na^+-Ca^{2+} exchanger and the SR Ca^{2+}-ATPase. It is the main focus of this thesis to determine if the antiarrhythmic actions of PUFAs are due to, or at least in part due to, the modulation of calcium handling.

In vitro studies looking at the effects of dietary supplementation of PUFAs on the reuptake of Ca^{2+} by the SR have shown that the dietary incorporation of n-3 PUFAs into membrane phospholipids causes a decrease in the reuptake of calcium by the SR. The SR Ca^{2+}-ATPase activity was measured in isolated SR by subtracting values obtained in the presence of EGTA and the absence of $CaCl_2$ from those obtained in the presence of $CaCl_2$. It was found that the activity of the Ca^{2+}-ATPase had decreased in mice whose diet was supplemented with n-3 PUFAs (Croset *et al.*1989). Similarly, Taffet *et al.* (1993) found that the SR Ca^{2+}-ATPase activity was reduced in rats whose diet was supplemented with n-3 PUFAs. These results were obtained by continuously monitoring the oxidation of NADH by SR Ca^{2+}-ATPase with a linked enzyme.

These studies provide a basis for how PUFAs may alter calcium handling. However, it is essential to determine if PUFAs share similar actions in a cellular system compared with the actions at the level of the SR Ca^{2+}-ATPase used in these studies. Normal and pathological intracellular calcium events such as calcium sparks (section 1.3), transients (section 1.4) and waves (section 1.5) are useful in the study of the mechanisms of calcium handling in the heart and may provide information on whether or not PUFAs when applied acutely or acquired through diet, modify CICR. These are discussed separately. It is anticipated that evaluating the effect of PUFAs on calcium sparks, transients, waves and the ryanodine receptor (section 1.6) will provide insight as to if and how PUFAs modulate calcium handling mechanisms.

1.3 Calcium Sparks

Calcium sparks are local transient increases in calcium concentration in the cytosol of cardiac cells (Cheng *et al.* 1993). Calcium sparks are a consequence of small bursts of calcium release from the SR ryanodine receptors (Bers *et al.* 1998, Xiao *et al.* 1997). The concentration of calcium in a calcium spark is approximately 200-300 nM, they are approximately 2 μm in diameter at fifty percent of the maximum intensity and have a half-life of decay of approximately 20 ms (Cannell *et al.* 1995).

Calcium sparks occur spontaneously in the presence and absence of extracellular calcium without the initiation of an L-type Ca^{2+} current (see section 1.2.1 for a description of the CICR cascade). However, SR calcium concentration has been implicated in controlling their frequency of occurrence (Satoh *et al.* 1997). As well as occurring spontaneously, calcium sparks can also be initiated by evoking an L-type Ca^{2+} current. Upon cell membrane depolarization and subsequent activation of the sarcolemmal L-type Ca^{2+} current, the probability of calcium sparks increases exponentially with L-type Ca^{2+} current magnitude (Cannell *et al.* 1995). This has lead to the suggestion that sparks may be the underlying events behind calcium transients and, therefore, muscle contraction (Cannell *et al.* 1995). The sparks initiated by the activation of CICR share similar properties to those that occur spontaneously. There is no variation in the time course of decay, width or amplitude between spontaneous sparks and those generated by L-type calcium current activation (Cannell *et al.* 1995).

Modeling of the conductance of a single ryanodine receptor channel with respect to the calcium flux from the SR during a calcium spark has shown that more than one

ryanodine channel is responsible for a single spark. It has been proposed, from the magnitude of calcium flux from the SR, that a calcium spark is likely to be due to the simultaneous opening of approximately six to seven ryanodine channels (Cheng *et al.* 1993, Bers *et al.* 1998).

Factors implicated in determining the size of a calcium spark are the flux of calcium from the SR and the mechanisms involved in the removal of cytosolic calcium. These mechanisms include calcium diffusion into the surrounding areas, buffering by both intracellular buffers and phospholipids, calcium extrusion from the cytosol by sarcolemmal membrane pumps such as the Ca^{2+}ATPase and the Na^{+}-Ca^{2+} exchanger, and also calcium reuptake by the SR via the SR Ca^{2+}ATPase (Cheng *et al.*, 1993, Bers *et.al.* 1998). Under normal circumstances, calcium sparks remain localized and do not initiate further calcium release. If clusters of sparks occur in a localized area, or they become large in amplitude, they can become initiation sites for pathological regenerative calcium release (Cheng *et al.* 1993). As spontaneous calcium sparks have been implicated in the induction of anomalous calcium handling, they may provide a useful insight into how PUFAs may protect from arrhythmias due to dysfunction in calcium handling.

Previously it has been demonstrated that the application of 15 μM EPA to ventricular myocytes reduces the frequency of calcium spark occurrence but does not alter the duration or width of the sparks. Calcium sparks in this experiment were evoked by the initiation of the L-type calcium current and upon the addition of EPA the magnitude of the L-type calcium current was reduced. It was concluded that the observed reduction in spark frequency was due to the suppression of the L-type calcium current

by EPA (Xiao *et al.* 1997). There have been no previous studies to determine whether PUFAs introduced through the diet result in a similar reduction in spark frequency or whether the application of PUFAs affects spontaneous sparks. A reduction in the magnitude or the frequency of calcium sparks could be beneficial as this could potentially reduce the likelihood of larger pathological spontaneous calcium release. It was the aims of these studies were; (i) To determine the effects of dietary polyunsaturated fatty acids on the spatial characteristics and frequency of spontaneous calcium sparks in rat atrial and ventricular cardiac myocytes. (ii) To determine the contribution of the SR Ca^{2+}ATPase to calcium spark decay. (iii) To observe calcium sparks in rat ventricular myocytes cultured in free polyunsaturated fatty acids.

1.4 Calcium Transients

A calcium transient is a large synchronized global increase in cytosolic calcium concentration due to calcium release from the SR in response to the initiation of the L-type Ca^{2+} current and subsequent activation of the CICR cascade (Cannell *et al.* 1995; see section 1.2.1 for details). The decay of a calcium transient involves the sequestration of calcium by the SR via the SR Ca^{2+}ATPase and the extrusion of calcium from the cell by the Na^{+}-Ca^{2+} exchanger and sarcolemmal Ca^{2+}ATPase (discussed in section 1.2.1) (Xiao *et al.* 1997). The use of calcium transients for investigation into the effects of PUFAs may provide useful insight into how they may affect SR calcium re-uptake in the heart. As previously discussed (section 1.2.2) *in vitro* studies have shown that dietary polyunsaturated fatty acids reduce the activity of the SR Ca^{2+}ATPase (Croset *et al.*1989, Taffet *et al.* 1993). Inhibition of the SR Ca^{2+}ATPase by PUFAs will result in a reduction in SR calcium sequestration and may

alter the duration of the calcium transient. It is, therefore, important to determine the effects of PUFAs on calcium transients in whole cells.

It has been previously shown that the acute addition of 15 μM EPA significantly reduces the amplitude of the calcium transient (Xiao *et al.* 1997). In the same study it was also demonstrated that the acute addition of EPA resulted in a subsequent decrease in the magnitude of the L type Ca^{2+} current, thereby reducing the concentration of triggering calcium for the initiation of a transient. The reduced magnitude of the calcium transient has been attributed to the reduction in the amplitude of the L-type Ca^{2+} current. It was concluded that the reduced calcium transient amplitude was due to changes in sarcolemmal electrical activity by PUFAs (Xiao *et al.* 1997). Whether the introduction of PUFAs through the diet will result in a reduction in the amplitude of the calcium transient similar to that shown by Xiao *et al.* (1997), has not been previously investigated. It is, therefore, the aim of this study to determine the effects of dietary and acute addition of PUFAs on the amplitude and duration of calcium transients in rat ventricular myocytes.

1.5 Calcium Waves

Under normal circumstances, release of calcium from the SR is a smoothly graded event depending on the amount of triggering calcium introduced by the activation of the L-type calcium current (Cheng *et al.* 1996, Lukyanenko *et al.* 1999; see section 1.2.1). In the event of an increased cytosolic calcium concentration or SR calcium concentration, the frequency of spontaneous calcium sparks from the SR increases (Satoh *et al.* 1997). If the magnitude of the calcium sparks becomes large enough, or

they are released simultaneously in close proximity to one another, regenerative calcium waves can be initiated which do not depend on an L-type calcium current for a trigger (Cheng *et al.* 1993, Cheng *et al.* 1996, Lukyanenko *et al.* 1999). The propagation of calcium waves is believed to be due to a combination of local diffusion of calcium to adjacent calcium release sites where this calcium acts as a trigger for further calcium release, and the sensitization of calcium release channels by SR calcium (Cheng *et al.* 1996, Lukyanenko *et al.* 1999).Calcium waves are of importance as they have been implicated in calcium-dependent arrhythmias and arrhythmic activity due to membrane depolarization (Cheng *et al.* 1996).
It has been reported that calcium waves propagate with almost constant amplitude and velocity (Takamatsu and Wier, 1990). Exploitation of this reproducibility may provide a useful insight into whether or not PUFAs alter the sensitivity of the calcium release channels to triggering calcium. By increasing or decreasing the channel sensitivity to calcium, the propagation velocity of the calcium wave is expected to change.

Previously it has been reported that the acute addition of 10 μM EPA to rat ventricular myocytes reduces the frequency and increases both the amplitude and the propagation rate of spontaneous waves. The application of EPA also resulted in an increase in SR calcium concentration and a reduction in resting cytosolic calcium concentration (Negretti *et.al.* 2000, O'Neill *et al.* 2002). From the reduction in spontaneous calcium release combined with the increase in SR Ca^{2+} content it was concluded that the application of EPA inhibits the release of SR Ca^{2+} (Negretti *et.al.* 2000). The reason for the observed increase in propagation velocity is not well understood. It has been reported that inhibition of SR calcium release results in a reduction in wave velocity

(Cheng *et.al.* 1996). In this study, the conclusion was drawn that the increased propagation velocity of the wave may be due to the larger wave amplitude providing a more effective trigger for calcium release at adjacent release sites thereby overcoming the reduced sensitivity of release (Negretti *et.al.* 2000). In a follow-up study it was concluded that the observed reduction in cytosolic calcium concentration was due to a reduced influx of calcium across the sarcolemmal membrane and may be in part responsible for the reduction in wave frequency observed under EPA treatment (O'Neill *et al.* 2002).

The reduction in the frequency of spontaneous calcium waves by EPA may be of potential benefit in the protection against calcium dependent arrhythmias. However, it is important to determine if PUFAs acquired through the diet also modify the amplitude and propagation rate of calcium waves in a similar manner to the observations of Negretti *et.al.* (2000).

The aim of the present study is to evaluate the effect of dietary PUFAs on the amplitude and propagation velocity of spontaneous calcium waves in rat ventricular myocytes.

1.6 Ryanodine Receptor

The SR calcium release channel, referred to as the ryanodine receptor (RyR) recently has been implicated as a potential site of action of PUFAs in the heart. Previous studies have shown that the spontaneous release of calcium is inhibited by the application of EPA (Negretti *et.al.* 2000, O'Neill *et al.* 2002) thereby indicating potential inhibition of the cardiac RyR. The RyR consists of 4 subunits each of

approximately 560kDa and containing approximately 5035 amino acids (Laver, 2001, Franzini-Armstrong and Protasi, 1997, Xu *et al.* 1996). The RyR contains a large hydrophilic amino terminal region that constitutes the cytosolic domain of the channel and a smaller mostly hydrophobic carboxyl terminal region that forms the pore (Franzini-Armstrong and Protasi, 1997). The large cytoplasmic domain is believed to allow for extensive regulation of activity of the RyR.

Regulation of the RyR is complex and numerous regulating ligands have been identified. These regulators include calcium, ATP, Mg^{2+}, calmodulin, protein kinases, phosphatases, fatty acid metabolites and proteins such as triadin and FK506 binding protein (Xu *et al.* 1996). Calcium and ATP are the activating ligands of interest to this series of studies. RyR has four calcium binding domains, the binding of calcium to one or more of these domains will result in channel opening. Calcium in the micromolar range activates RyR activity and in millimolar ranges calcium acts as an inhibitor (Franzini-Armstrong and Protasi, 1997). Milimolar concentrations of ATP are an activator of RyR, although ATP will only activate the channel in the presence calcium. It has been reported that ATP and ADP are both activators of the RyR and compete for the same activation site and it was concluded that ATP activation of the RyR does not involve RyR phosphorylation. It is believed that ATP activates the RyR by binding to sites on or in close association with the channel (Kermode *et al.* 1998).

Previously, in chemically permeabilized ventricular myocytes it has been demonstrated that the frequency of spontaneous calcium release evoked by elevating the concentration of calcium fell upon addition of EPA (Rodrigo *et al.* 1999).

Whether the reduction in spontaneous calcium release was due to inhibition of calcium release or SR re-uptake was unable to be differentiated in this study.

The main aim of the present study is to determine whether the reduction in spontaneous calcium release by EPA observed in previous studies (Rodrigo *et al.* 1999, Negretti *et.al.* 2000, and O'Neill *et al.* 2002), is due to inhibition of the cardiac RyR.More specifically, the present study aimed to determine the effect of (i) cytosolic and (ii) luminally applied EPA on the ATP activation of the RyR, also (iii) to determine the effect of cytosolic EPA on RyR cytosolic calcium sensitivity

2. METHODS

2.1 General Methods

2.1.1 Animals

Animals used in these studies were cared for according to the Australian National Health and Medical Council *Guidelines for the Care and Use of Animals*. All experimental procedures were subject to prior approval by the University of Adelaide Animal Ethics Committees. Dietary experiments were also subject to prior approval by the CSIRO Human Nutrition Animal Ethics Committees.

2.1.2 General Solutions

Tyrode's solution contained: 134 mM NaCl (Sigma), 10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES, Sigma), 4 mM KCl (Sigma), 1.2 mM $MgCl_2$ (Sigma), 1.2 mM NaH_2PO_4 (Sigma), 11 mM glucose (Sigma), and was adjusted to pH 7.4 with 1.0 M NaOH (Sigma).

Culture medium contained: Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 mM $CaCl_2$ (Sigma), 10 mM HEPES, 25 mM $NaHCO_3$, 2 mM carnitine (Sigma), 5 mM creatine (Sigma), 5 mM taurine (Sigma), adjusted to pH 7.2 with 1 M NaOH.

2.1.3 Isolation of Cardiac Myocytes

Cardiac myocytes were isolated using enzymatic digestion described previously by McMurchie *et al.* (1998). Adult Sprague Dawley rats were injected with 2000 units of heparin intraperitoneal to prevent blood clotting in the small coronary vessels. After 30 min, animals were anaesthetised with sodium pentobarbitone (1ml/kg) also injected intraperitoneally. Anaesthesia was determined by the abolition of the righting reflex, the foot withdrawal reflex and the blinking reflex. Following satisfactory anaesthesia, the chest cavity was opened and the heart was rapidly removed. After removal, the heart was washed in cold (to slow metabolism), oxygenated, calcium-free Tyrode's solution to remove blood from the chambers. Following washing, the heart was cannulated via the aorta ensuring the cannula did not penetrate the aortic valve. The heart was perfused for 4 minutes with Tyrode's solution containing 1mM calcium chloride warmed to 37°C. This promoted contraction facilitating the removal of the remaining blood from the coronary arteries. The heart was then perfused with calcium-free Tyrode's for a further 6 minutes in order to remove the extracellular calcium hence resulting in cessation of contraction. After contraction had ceased, the heart was perfused with Tyrode's solution, containing collagenase (0.1 mg ml^{-1}, Yakult Honsha Co., LTD, Tokyo, Japan), protease (0.04 mg ml^{-1}, type XIV, Sigma) to break the collagen and protein matrix linking the cells, delipidated bovine serum albumin (0.1% w/v, fraction V, Sigma) and 2,3-butane-dionemonoxime (BDM, (2.5 mg ml^{-1}, Sigma). The heart was digested when it became flaccid and pale in colour. Digestion time was typically 20 minutes in duration.

Following digestion, either the atria for for studies described in sections 2.2.2 or right ventricle for studies described in sections 2.2.3, 2.2.4, 2.3.3, 2.3.4 and 2.4) was removed and placed into a Tyrode's solution containing 30 mM BDM and delipidated bovine serum albumin (1.5% w/v). The tissue was gently teased apart to separate the cells. Delipidated bovine serum albumin was used in order to reduce the amount of introduced unknown fatty acids other than those introduced experimentally.

The calcium concentration of the cell suspension was gradually increased to 1mM in 4 steps over a 40-minute time span. Initially, the concentration of calcium was raised to 60 μM, then 210 μM, 460 μM, and finally to 1mM. The gradual introduction of calcium to the cell suspension reduced the incidence of spontaneous cell hyper-contracture and subsequent cell death, often associated with the reintroduction of calcium. This phenomenon is known as the "calcium paradox".

Isolated atrial or ventricular myocytes were plated onto laminin-coated (50 μg ml^{-1}, Sigma) coverslips and were allowed to settle for 40 minutes prior to use.The cells were isolated at 37°C, and maintained at room temperature (23-25 °C).

2.1.4 Membrane Fatty Acid Analysis

Atrial tissue (section 2.2.2) or ventricular tissue (sections 2.2.3, 2.3.3 and 2.4) from rats supplemented with dietary oils was subject to fatty acid analysis using modified methods of Bligh and Dyer (1959).

This technique was performed by Assoc. Prof. Edward J McMurchie and Mrs Sharon Burnard, CSIRO division of Health Sciences and Nutrition, Adelaide, Australia. For details of this methodology, refer to appendix C

2.2 The Effects of Polyunsaturated Fatty Acids on Calcium Sparks in Cardiac Myocytes

2.2.1 Recording of Calcium Sparks

Isolated cardiac myocytes from either the atria for 2.2.2 or right ventricle for 2.2.3 and 2.2.4, were loaded with 30 μM of the acetoxymethyl (AM) ester of Fluo-3 (Molecular Probes) in conjunction with 2.5 μl ml^{-1} of 10% (w/v) pluronic acid (Molecular Probes) (dissolved in dimethylsulfoxide, Sigma) for 30 minutes in the dark (to prevent photo-bleaching) at room temperature (22-25 °C) (Gomez *et al.* 1996). Pluronic acid was added to assist the passage of the fluo-3 AM in into the cytosol by slightly increasing cell membrane fluidity. The AM ester of Fluo-3 was used as it is lipid soluble and easily traverses the cell membrane. After this 30-minute period, the dye-containing solution was removed and replaced with Tyrode solution containing 1mM calcium. The cells remained in the dark at room temperature for a further 30 minutes to allow for the de-esterification of Fluo-3 AM. Fluo-3 AM is non-fluorescent whilst existing as its AM ester and de-esterification in required to liberate the fluorescent form of the dye Fluo-3 (see Appendix D for more information). Plated cells were placed in a custom made superfusion bath fitted with platinum field stimulation electrodes then positioned on the stage of a Nikon Diaphot inverted microscope. Calcium sparks were viewed using the fast line scan mode (see figure 4) of a Bio-Rad MRC-1000 Krypton-Argon laser scanning confocal microscope system in fluorescence mode with excitation set at 488 nm and emission at 522 nm for Fluo-3. The objective lens was a ×40 Nikon water immersion lens with a numerical aperture of 1.15. Images were collected using Bio-Rad CoMOS and MPL software.

Prior to the collection of the calcium sparks, Myocytes were stimulated using a Grass SD9 stimulator at 1 Hz for 4 minutes to normalize the SR calcium concentration to a steady state prior to the collection of sparks.The duration of stimulation to normalize SR calcium was determined experimentally, steady state achieved when all transients were similar in amplitude. The laser intensity was set to 10% of full power and the gain was set such that the maximum pixel value of the steady state transient did not exceed 80% of the maximum detectable pixel value. Optimal black level settings for best resolution were determined prior to all experiments and remained constant throughout the course of each experiment to avoid non-dye related changes to image intensity. 70 sequential images were collected saving every image for later analysis. All experiments were performed at room temperature (22-25 °C).

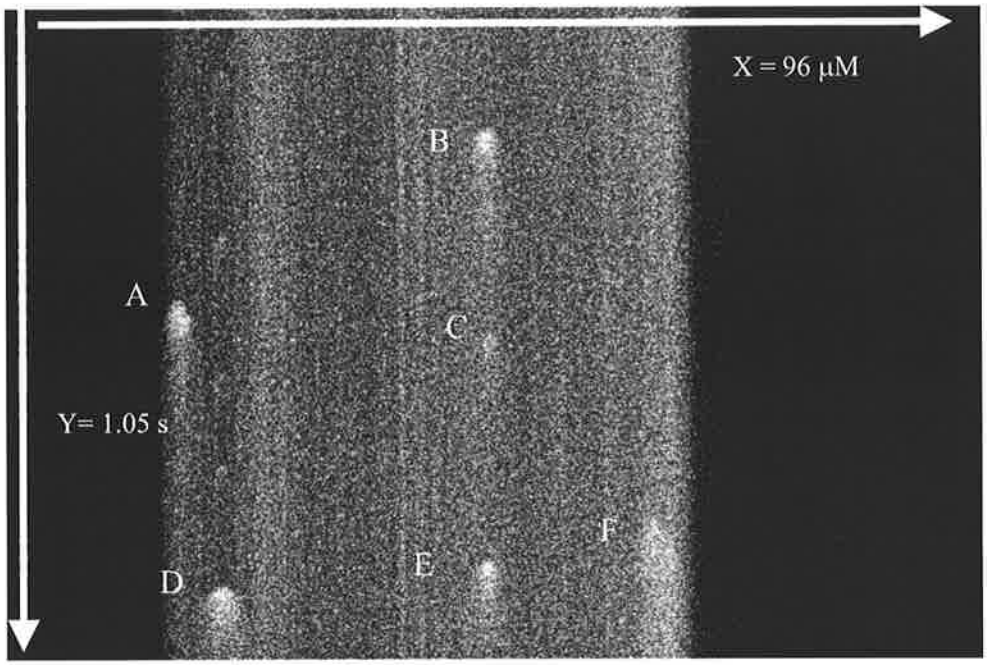


Figure 4: An example of a line scan image filtered by a 3x3 median filter of calcium sparks. This image is obtained by scanning a single line on a single plane repeatedly with each subsequent line scanned stacked below one another to give time increasing down the y axis and cell width across the x axis. Total image width is 96 μm and the height (duration) is 1.05 seconds. Events marked A, B, D & E are considered to be sparks. Event marked C is likely to be a spark however originates from above or below the focal plane and is therefore not included. Event marked F is considered to be propagating to the right of the image and is therefore considered to be a calcium wave rather than a spark. By increasing the black level of this image, the sparks would appear larger and brighter resulting in a larger width and duration and potentially exceed the criteria to be classified as sparks. Similarly if the black level was reduced the sparks would appear smaller and could potentially be missed.

2.2.2 The Effects of Dietary Polyunsaturated Fatty Acids on Calcium Sparks in Rat Atrial Myocytes

2.2.2.1 Diet Supplementation.

Three groups of 7 week old male Sprague-Dawley rats were gavaged with 3 ml of either Lard fat (saturated fat, Metro Quality Foods, Greenacres, NSW.), Canola oil (predominantly monounsaturated and n-6 polyunsaturated fat, Meadow Lea Foods Ltd., Ryde, NSW) or Fish oil (predominantly n-3 polyunsaturated fat, RoPUFA, Hoffmann La Roche, Basel, Switzerland), once daily for a period of three weeks. During this time, rats were maintained on standard rat chow *ad libitum* in addition to the fat supplement. The composition of the supplements is given in Appendix A. After the 3-week gavaging period, the rat atrial myocytes were isolated (as described in section 2.1.3).

2.2.2.2 Collection of Calcium Sparks

Calcium Sparks were collected in Tyrode's solution (section 2.1.2) containing 1 mM $CaCl_2$ at room temperature to the method described in section 2.2.1. Experiments were performed at room temperature (22-25 °C).

2.2.2.3 Data Analysis

Events were identified as sparks if the peak fluorescence of an event exceeded 50% of the average background fluorescence prior to the transient change in fluorescence intensity. To be classified as a spark, the width of the event as plotted on the line scan image was to be equal to or less than 5 μm and no less than 0.5 μm and the duration was no less than 20 ms with the background subtracted. Sparks are non-propagating (Cheng *et al.* 1996) so if an event was identified to be propagating then it was not considered to be a spark. Propagating events often exhibit a shift in their lateral position over time so are easily identifiable from non-propagating events (see figure 4). Other considerations were the shape of the event; commonly sparks can originate from above or below the focal plane and need to be distinguished. The above criteria were able to eliminate many of these events however; the longitudinal profile of a spark is a good indicator of the position of a calcium spark relative to the focal plane. If a spark profile was clearly round in shape (as would be consistent with calcium diffusing into the focal plane), it was not considered to be a spark. Sparks of this nature generally give rise to a "noisy" signal.

Spark duration and width were measured using the computer program Scion Image Beta 4.0.2 (Scion Corp). Prior to analysis, a 3×3 median filter was applied to each image. This filter reduces the unavoidable noise in the image with minimal compromise to the signal. Sparks were analyzed by setting the threshold level to background level. This was determined by the complete removal of the background prior to the calcium spark. This process was often repeated multiple times for images containing multiple sparks or for noisy signals, especially where the background

intensity was not uniform across the with of the image. Spark width was measured as the widest point across the spark and spark duration was measured as the longest length of the spark as it was plotted on the line scan image. Spark intensity was measured from the longitudinal profile of the calcium sparks. As fluo-3 is a non-ratiometric dye (see appendix B for more information) this value was determined as the ratio of the average maximum pixel value at the peak of the spark (F) to the average minimum pixel value prior to the spark (F_o), and is expressed as a percentage. The normalization of the peak of the spark to the background brightness eliminates the variability in brightness between images allowing the cross comparison of sparks between images.

2.2.2.4 Statistical Analysis

The data for a minimum of 10 sparks was averaged for each cell and a minimum of 6 cells were used per rat. The parameters were then averaged for each rat (n equals the number of rats). The averaged data for rats supplemented with fish oil, canola oil and lard were compared using a one way ANOVA. $P<0.05$ was defined as significant. Statistically significant data was subject to a Tukey's Post Hoc analysis to determine the direction of significance. Data is expressed as means ± SEM. Statistical analysis was performed using GraphPad Prism software Version 2 (Graphpad Software Incorporated).

2.2.3 The Effects of Dietary Polyunsaturated Fatty Acids on Calcium Sparks in Rat Ventricular Myocytes.

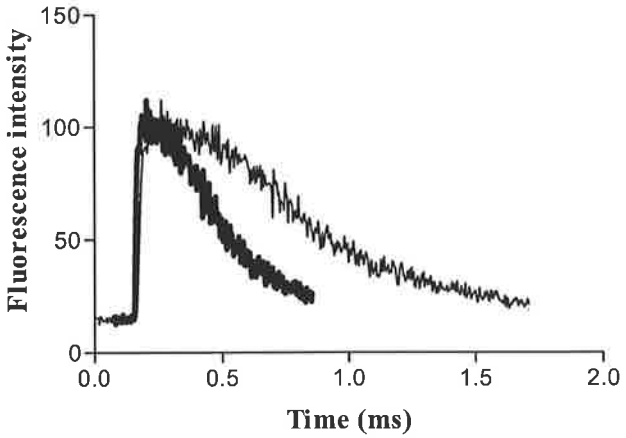
2.2.3.1 Dietary Supplementation.

3 week old, weanling male Sprague Dawley rats were maintained on a modified low fat rat chow diet based on AIN 96 G containing 7% sunflower oil (Meadow Lea Foods Ltd., Ryde, NSW) for 4 weeks. At 7 weeks of age, the rats were divided into 2 groups, one group was maintained on a diet containing 10% saturated fat supplement and the other was maintained on a diet containing a 10% fish oil supplement for a further 3 weeks. In total the experimental diet contained 17% fat. For composition of oils and diets, see appendix A and B. Following the 7-week feeding period, cardiac ventricular myocytes were isolated (method 2.1.3) and calcium sparks were viewed (method 2.2.1).

2.2.3.2 Collection of Calcium Sparks

As in the previous experiment, calcium sparks were recorded in a control Tyrode's solution (see section 2.2.1.2) containing 1 mM $CaCl_2$. Following the collection of calcium sparks in the control solution, the solution was changed to a Tyrode's solution containing 1 mM $CaCl_2$ and 10 μM 2, 5-di(tert-butyl)-1,4-hydroquinone (TBQ, Alomone labs). TBQ prevents the reuptake of calcium by the SR by blocking the SR Ca^{2+}ATPase. The concentration of TBQ used was determined experimentally by stimulating calcium release and determining if the rate of decay of calcium re-uptake was prolonged as previously shown by Gomez *et al.* (1996). Adequate block

of the SR Ca^{2+}ATPase was determined if the decay rate of the calcium transient was significantly prolonged when compared to the control. The supplement figure below illustrates a calcium transient before and after the addition of TBQ. The control transient (heavy line) exhibits a fast decay versus the transient after the cell has been exposed to TBQ (fine line). The prolonged decay of the transient is consistent with the blocking of the SR Ca^{2+} ATPase as shown by Gomez *et al.* (1996). Cells were exposed to TBQ for 3 minutes prior to the collection of sparks. All experiments were performed at room temperature (22-25 °C). Myocytes were stimulated using a Grass SD9 stimulator at 1 Hz for 4 minutes to normalize the SR calcium concentration to a steady state prior to the collection of sparks.



Supplement Figure: an example of calcium transient profiles talen from a line scan image in the absence (——) and after a 3 minute exposure to TBQ (——) of TBQ. Both transients were taken from the same cell.

2.2.3.3 Data Analysis

The criteria for an event to be classified as a calcium spark were the same criterion as described in section 2.2.2.3. In contrast to the methods described in section 2.2.2.3, events were analysed using a combination of built in Scion image macros customised for the image and pixel size, also for line scan duration. Images were subjected to a 3×3 median filter to reduce noise. Following this, the background was subtracted using a scion image built in macro. This macro removes the average background over time to make the background across the width of the image uniform and reduce the need for multiple analyses of events on the same image. After subtraction of the background, threshold was set to remove any remaining background prior to an event. In most cases, the image was uniform although in some instances, the background was not completely even and separate thresholds were set for images containing multiple events and for particularly noisy images.

Prior to the analysis of events, the scale of the image was set accordingly.
The size of the events to be analyzed was preset to ignore any large events, this was set higher than the largest spark size. Events smaller than the minimum spark size were also ignored. This removed any remaining events not likely to be considered as calcium sparks. An ellipse was fitted to all non-excluded events at threshold level, the minor axis was considered to be width and the major axis, length (figure 5). An ellipse has been demonstrated theoretically to fit sparks on the x-t plane of the line scan image. Experimentally an ellipse does not always fit a spark at all amplitudes of fluorescence as the peak of the spark may shift off center due to obstructions in diffusion due to intracellular structures (Cheng *et al.* 1999). By fitting the ellipse at

the level of the background rather than at 50% of peak intensity as performed by Cheng *et al.* (1999), it is intended that any distortions in diffusion are negligible. From the generated data, events that fit the spark criteria were identified and clarified with the original unprocessed image to ensure that false positive identifications were not included or that any possible sparks were not missed, this generally did not occur. The temporal profile of each of the positive events was obtained and used to determine the shape of the spark. The profile was used to determine the point of origin of an event with respects to the focal plane of the image (described in section 2.2.2.3). Spark brightness was determined from images processed with a 3x3 median filter as the peak value of the calcium spark (F) expressed as a percentage of the average background prior to the spark (F_o). These values were taken from the temporal profile of the spark. In order to ensure the integrity of this method of analysis (and the method described in section 2.2.2.3), a colleague analyzed a portion of the data and the results were compared to those obtained by myself. No significant difference was found between my results and theirs indicating that subjectiveness was minimized by the set conditions under which the analysis took place.

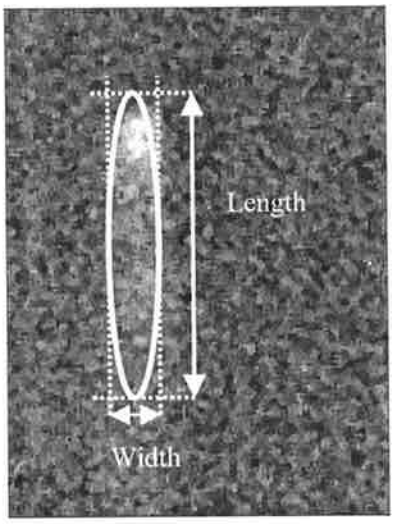


Figure 5: Diagrammatic representation of the fitting of an ellipse to a 3x3 median filtered image of calcium spark. This is for illustrative purposes only and is not an actual fit.

2.2.3.4 Statistical Analysis

The data for a minimum of 10 sparks was averaged for each cell and a minimum of 6 cells were used per rat for both the control and TBQ data sets (n equals the number of rats). Spark parameters from dietary (lard and fish oil) supplemented rats were compared using an unpaired t test. Control and TBQ data were compared using a paired t test. All data is expressed as means ± SEM. Data was assumed to be significant if $P<0.05$. Statistical analysis was performed using Excel 1997 (Microsoft).

2.2.4 The Effect of Free Polyunsaturated Fatty Acids on Calcium Sparks in Rat Ventricular Myocytes.

2.2.4.1 Myocyte Treatment

Rat ventricular myocytes were isolated by enzymatic digestion as previously described in section 2.1.3.Isolated ventricular myocytes were incubated at 37°C (in 95% O_2, 5% CO_2) in either a control culture medium containing 1mM $CaCl_2$ or in a culture medium containing 1mM $CaCl_2$ and 15 μM of either eicosapentaenoic acid (EPA, Sigma), docosahexaenoic acid (DHA, Sigma) or α-linolenic acid (ALA, Sigma) for two hours (see section 2.1.2 for culture medium composition). Fatty acids were added from a stock solution dissolved in 100% ethanol and were stored in nitrogen at -80°C prior to use.

2.2.4.2 Collection of Calcium Sparks

Following the incubation period, calcium sparks were viewed according to the method described in section 2.2.1. Following the collection of sparks in the control Tyrode's solution (see section 2.1.2) containing 1mM $CaCl_2$, the solution was changed to one containing 5 mM thapsigargin (Sigma) and following 3 minutes of stimulation, sparks were recorded. Like TBQ, thapsigargin prevents the reuptake of calcium by the SR due to the inhibition of the SR Ca^{2+}ATPase. The concentration used was determined experimentally as previously described for TBQ in section 2.2.3.2. The reason for using different SR Ca^{2+}ATPase blocking agents to section 2.2.3.2 is due to their cost and the amount required to complete the experiments, typically dietary studies require

a much larger number of experiments thus a cheaper alternative is required compared to the acute studies. Experiments were performed at room temperature (22-25 °C). Myocytes were stimulated using a Grass SD9 stimulator at 1 Hz for 4 minutes to normalize the SR calcium concentration to a steady state prior to the collection of sparks.

2.2.4.3 Data Analysis

Calcium Sparks were analyzed using the method as described in section 2.2.3.3

2.2.4.4 Statistical Analysis

The width, duration and intensity of the calcium sparks were measured and averaged for each cell. A minimum of 10 sparks were recorded per cell and 10 cells were used for each fatty acid treatment. The data was compared using a one way ANOVA and $P< 0.05$ was assumed as significant. Statistically significant data was subject to a Tukey's Post Hoc analysis to determine the direction of significance. Statistical analysis was performed using Graph Pad Prism Version 2 Software (Graphpad Software inc.). All data is expressed as mean ± SEM.

2.3 The Effects of Polyunsaturated Fatty Acids on Calcium Transients in Ventricular Myocytes

2.3.1 Recording of Calcium Transients

Ventricular myocytes were isolated according to the method described in section 2.1.3. Plated ventricular myocytes were loaded with the fluorescent dye fluo-3 AM (Molecular probes) in conjunction with 2.5 $\mu l\ ml^{-1}$ of 10% pluronic acid (dissolved in dimethylsulphoxide) for 30 minutes in the dark at room temperature (22-25 °) as previously described in section 2.2.1. The cells were then placed into a customized bath fitted with 2 parallel platinum field stimulation electrodes then positioned on the stage of a Nikon Diaphot inverted microscope. The cells were then field stimulated at a frequency of 1 Hz with a Grass SD9 stimulator for 4 minutes prior to the collection of transients in order to allow the SR calcium concentration to normalize to a steady state. Following this 4 minutes, using the fast line scan mode of a Bio-Rad MRC-1000 Krypton-Argon laser scanning confocal microscope, set for Fluo-3 (see section 2.2.1). Calcium transients were collected in Tyrode's solution containing 1mM $CaCl_2$. Prior to the recording of calcium transients, the laser intensity was set to ensure the best possible resolution, this was typically 10% laser intensity. The photo-multiplier gain was set to ensure that the peak of the transient did not exceed approximately 80% of the maximal detectable intensity. Black level was set for optimal image resolution and remained constant throughout all experiments. Experiments were recorded at room temperature (22-25 °).

2.3.2 Data Analysis

Images of calcium transients were scaled according to pixel size and line duration then processed with a 3x3 median filter. The profiles of three calcium transients were taken for each cell using the computer program Scion Image Beta 4.0.2 (Scion Corp). The profile was generally taken from a section at the center of the transient to avoid any distortion of the image associated with cell contraction. The profiles were then plotted using the Graph Pad Prism software (Graphpad Software inc.).

In order to determine the rate of rise and decay of the calcium transients, lines were generated with an exponential growth equation (equation 1) fitted to the rising phase of the transient and an exponential decay equation (equation 2) fitted to the decay phase of the transient respectively.

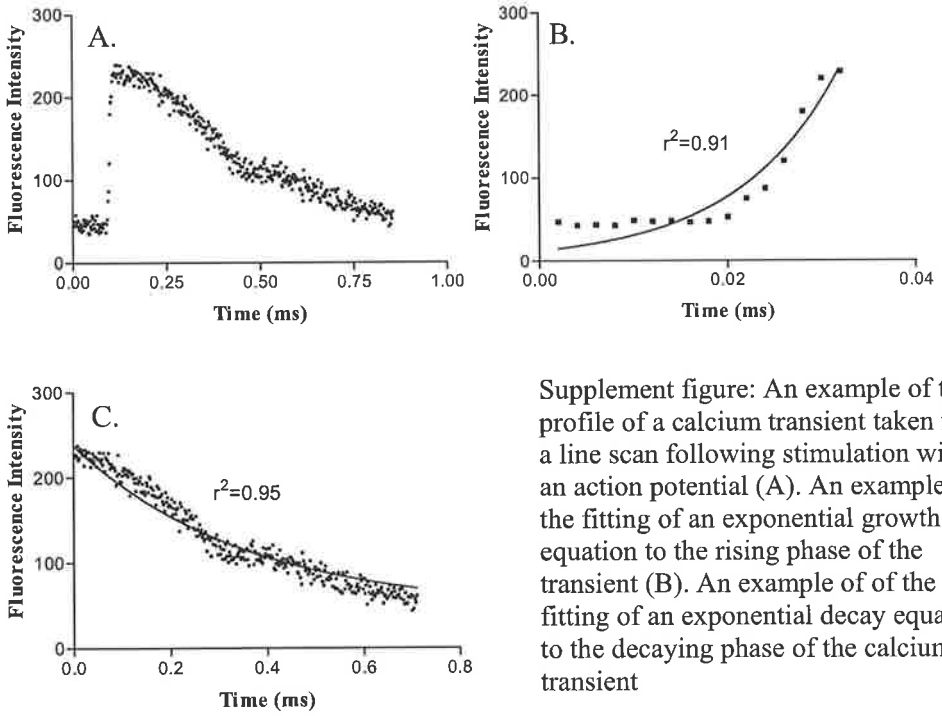
$$Y=Y_0e^{kt} \qquad \text{Equation 1}$$

$$Y=Y_0e^{-kt}+Y_{max} \qquad \text{Equation 2}$$

'Y' is the fluorescence intensity after a time 't', 'Y_0' is the initial fluorescence intensity, 'k' is the growth constant (equation 1) or the decay constant (equation 2), Y_{max} is the maximum fluorescence intensity and 'e' is the base of natural logarithms. Fitting of the above equations to the data was accepted if $r^2>0.9$ as shown in the supplement figure below.The time constant k of rise and decay of the calcium transient was converted to doubling time or half-life, respectively, by the following equation,

Doubling time (or Half-life) =0.6932/k Equation 3

Calcium transient intensity was determined from the profile of the transient as the peak value of the transient (F) expressed as a percentage of the average of the background (F_o) prior to the transient. The normalization of the peak of the transient to the background brightness eliminates the variability in brightness between images allowing the cross comparison of transients between images.



Supplement figure: An example of the profile of a calcium transient taken from a line scan following stimulation with an action potential (A). An example of the fitting of an exponential growth equation to the rising phase of the transient (B). An example of of the fitting of an exponential decay equation to the decaying phase of the calcium transient

2.3.3 The Effects of Dietary Polyunsaturated Fatty Acids on Calcium Transients in Ventricular Myocytes

2.3.3.1 Dietary Supplementation

3 week old, weanling, male, Sprague Dawley rats were maintained on a modified low fat rat chow diet based on AIN 96 G containing 7% sunflower oil for 4 weeks. At 7 weeks of age, the rats were divided into 2 groups. One group was maintained on a diet containing 10% saturated fat supplement and the other was maintained on a diet containing a 10% fish oil supplement for a further 3 weeks. In total the experimental diet contained 17% fat (For composition of oils and diets, see appendix A and B). Following the 7-week feeding period, cardiac ventricular myocytes were isolated (method 2.1.3), and calcium transients were recorded and analyzed according to the method described in section 2.3.2. All experiments were performed at room temperature (22-25°C).

2.3.3.2 Statistical Analysis

The data from the three transients were averaged for each cell and a minimum of 3 cells were averaged for each rat (n equals the number of rats). The averaged data for rats supplemented with lard and fish oil was compared using an unpaired t-test. Statistical analysis was performed using Microsoft Excel 1997. Data is expressed as means ± SEM and was assumed to be significant when $P<0.05$.

2.3.4 The Effects of Free Polyunsaturated Fatty Acids on Calcium Transients in Rat Ventricular Myocytes.

2.3.4.1 Myocyte Treatment

Rat ventricular myocytes were isolated as described in section 2.1.3. Isolated ventricular myocytes were incubated at 37°C (in 95% O_2, 5% CO_2) in a control culture medium containing 1 mM $CaCl_2$, or in a culture medium containing 1 mM $CaCl_2$ and 15μm EPA (Sigma) for 2 hours. A 2-hour incubation time was chosen to enable the fatty acids to enter the cytosol and undergo possible esterification into the phospholipids, this occurs when consumed through the diet. If this time was greatly reduced the effects of PUFAs observed are likely to be mediated by any effects on the sarcolemmal membrane (see introduction).Fatty acids were added from a 10 mM stock solution dissolved in 100% ethanol which was stored in nitrogen at -80°C prior to use. Following the 2-hour incubation period, calcium transients were recorded as described in section 2.3.1 and analyzed according to the method described in section 2.3.2. All experiments were performed at room temperature (22-25°C).

2.3.4.2 Statistical Analysis

The data from three transients were averaged for each cell and 7 cells were used for each treatment. The data was analyzed using an unpaired t-test and $P<0.05$ was taken as significant. Statistical analysis was performed using Microsoft Excel 1997. All data is expressed as mean ± SEM.

2.4 The Effect of Dietary Polyunsaturated Fatty Acids on Calcium Waves in Rat Ventricular Myocytes

2.4.1 Dietary Supplementation

3 week old, weanling, male, Sprague Dawley rats were maintained on a modified low fat rat chow diet based on AIN 96 G containing 7% sunflower oil for 4 weeks. At 7 weeks of age, the rats were divided into 2 groups, one group was maintained on a diet containing 10% saturated fat supplement and the other was maintained on a diet containing a 10% fish oil supplement for a further 3 weeks. In total the experimental diet contained 17% fat. For composition of oils and diets, see appendix A and B. Following the 7-week feeding period, cardiac ventricular myocytes were isolated according to the method previously described in section 2.1.3.

2.4.2 Recording of Calcium Waves

Plated cardiac ventricular myocytes were loaded with 30 μM of the AM ester of fluo-3 (Molecular Probes) in conjunction with 2.5 μl ml^{-1} of 10% pluoronic acid (dissolved in DMSO) for 30 minutes in the dark at room temperature (22-25 °C). Following this, the cells were plated on to laminin coated coverslips and were allowed to incubate for a further 20-30 minutes to allow Fluo-3 AM to de-esterify (see appendix B for more detail). The plated cells were placed in a customized superfusion bath then positioned on the stage of an Optiscan F900E confocal microscope in fluorescence mode with excitation at 488 nm and emission set for 515 nm set for fluo-3. The objective lens was a × 40 Olympus water immersion lens with a numerical appeture of 0.80. Images

were collected using Optiscan F900E software version 1.6.0. Prior to the recording of calcium waves, laser intensity was set to 10% and the gain was set to ensure the maximum pixel intensity of the calcium transient did not exceed 80% of the maximum detectable intensity.

Cardiac myocytes were field stimulated in a solution containing 5 mM Ca^{2+} for 5 minutes, this resulted in SR calcium overload and subsequent SR calcium leak which resulted in the generation of calcium waves.

Calcium waves were recorded in a control Tyrode's solution (see section 2.1.2 for composition) containing 5 mM $CaCl_2$. The solution was then changed to one containing 5 mM $CaCl_2$, 1 μM isoproterenol (Sigma) and 10 mM ascorbic acid (to prevent the oxidation of isoproterenol) and the collection of waves was continued. This solution containing isoproterenol was prepared daily to ensure minimal oxidation of isoproterenol. All experiments were performed at room temperature (22-25°C). Calcium waves were recorded for 80 seconds saving every image. The first 3 waves recorded were used for later analysis, all waves recorded were used in determining wave frequency.

Isoproterenol was used in this experiment to stimulate the β-adrenergic pathway. Stimulation of the β-receptors facilitates the conversion of ATP to cyclic AMP (cAMP) by adenylate cyclase. The increase in [cAMP] results in the activation of cAMP-dependent protein kinase A. Protein kinase A when activated is responsible for the phosphorylation of a number of target proteins, which include the L-type calcium channels, the ryanodine receptors and phospholamban (SR Ca^{2+}ATPase regulatory protein). Beta stimulation is believed to result in an increase in the "gain" of the CICR system (Viatchenko-Karpinski and Gyorke 2001).

2.4.3 Data Analysis

Data was analyzed using the computer program Scion Image Beta 4.0.2 (Scion Corp) and Microsoft Excel 1997. Pixel information containing the calcium wave was exported into Microsoft Excel to add a grid reference to the pixel values. The leading edge of the wave was identified using "conditional formatting" a built in Excel macro. This macro allowed the leading edge of the wave to be identified, by highlighting pixel values greater than the average image background value as determined in Scion Image. Subsequently the propagation velocity could be determined. Wave intensity was measured by determining the maximum pixel value of the wave front (F) and expressing it as a percentage of the average background pixel value (F_o). As previously mentioned, the normalization of the peak of the wave to the background brightness eliminates the variability in brightness between images allowing the cross comparison of waves between images. Wave frequency was determined as the number of waves recorded in each cell over the 80-second scanning period. This is expressed as the number of waves per second.

2.4.4 Statistical Analysis

The data from 3 waves was averaged for each cell and an average of 6 cells was used for each rat. The parameters were then averaged for each rat (n = the number of rats). The data for rats supplemented with fish oil and lard was compared using an unpaired t-test. Data collected in control solution and solution containing isoproterenol was compared using a paired T test. Statistics were performed using Microsoft Excel 1997. $P<0.05$ was considered as significant. Data is expressed as mean ± SEM.

2.5 The Effects of EPA on the Cardiac Ryanodine Receptor

2.5.1 Isolation of Cardiac Ryanodine Receptors

The SR vesicles used in these studies were prepared and supplied by Angela Dulhunty, Suzy Pace and Joan Stivala (Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, Australian National University, Canberra, ACT). RyRs from sheep hearts were isolated as previously described by Laver *et al.* (1995). This method is outlined in appendix E.

2.5.2 Solutions

Cytosolic (*cis*) solution contained 230 mM $CsCH_3O_3S$ (Aldrich Chemical Company), 20 mM CsCl (Aldrich Chemical Company), 10 mM N-tris-(hydroxymethyl0methyl-2-aminoethanesulfonic acid (TES, ICN Biomedicals), 1 mM $CaCl_2$ (BDH Chemicals) and 500 mM Mannitol. Luminal (*trans*) solution contained 30 mM $CsCH_3O_3S$, 20 mM CsCl, 10 mM TES and 1 mM $CaCl_2$.Solutions were adjusted to pH 7.4 with CsOH (ICN Biomedicals).

$CsCH_3O_3S$ was used as the charge carrier as the ryanodine receptor is permeable to Cs^+ and other ion channels present in the SR are permeable to neither Cs^+ nor $CH_3O_3S^-$. The osmotic gradient established between the *cis* and *trans* baths due to the differing $CsCH_3O_3S$ concentrations in the *cis* and *trans* solutions assists in fusion of the SR vesicles into the bilayer (Laver 2001). The introduction of mannitol to the *cis*

solution was used to further enhance the osmotic gradient between the *cis* and *trans* baths.

cis solution used to determine the RyR response to cytosolic calcium contained 230 mM $CsCH_3O_3S$, 20 mM CsCl, 10 mM TES and 1 mM $CaCl_2$. 2 mM 1,2-Bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid (BAPTA, Molecular Probes) was used to buffer [Ca^{2+}] between 0.1 μM and 0.1 mM using an ion meter (Fluka).

2.5.3 Formation of Bilayers

0.8 ml of *cis* solution was added to the *cis* bath and 1 ml of *trans* solution was added to the *trans* bath. By convention, the *cis* bath refers to the cytosolic face of the bilayer and the *trans* bath refers to the luminal face of the bilayer (figure 6).
Lipid bilayers comprised of 50:50 wt/wt 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamone (PE, Avanti Polar Lipids, Alabaster, AL, USA) to 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (PC, Avanti Polar Lipids, Alabaster, AL, USA) in an N-decane or N-tetradecane (ICN) solvent where specified. Bilayers were formed across an aperture of 150-250 μM of a Delrin cup (made in-house). This involves smearing the lipid mix across the aperture of the Delrin cup, which results in a thick film separating the *cis* and *trans* baths. The lipid aggregates to form two monolayers. The solvent then drains from between the two monolayers forming a bilayer. This is known as the film drainage technique (Laver 2001). Following the formation of a bilayer, SR vesicles (1-3 μl) were added to the *cis* bath and were stirred using a magnetic stirrer until fusion occurred. Vesicles added to the *cis* bath will

insert in the bilayer with the cytosolic region of the channel exposed to the *cis* solution.

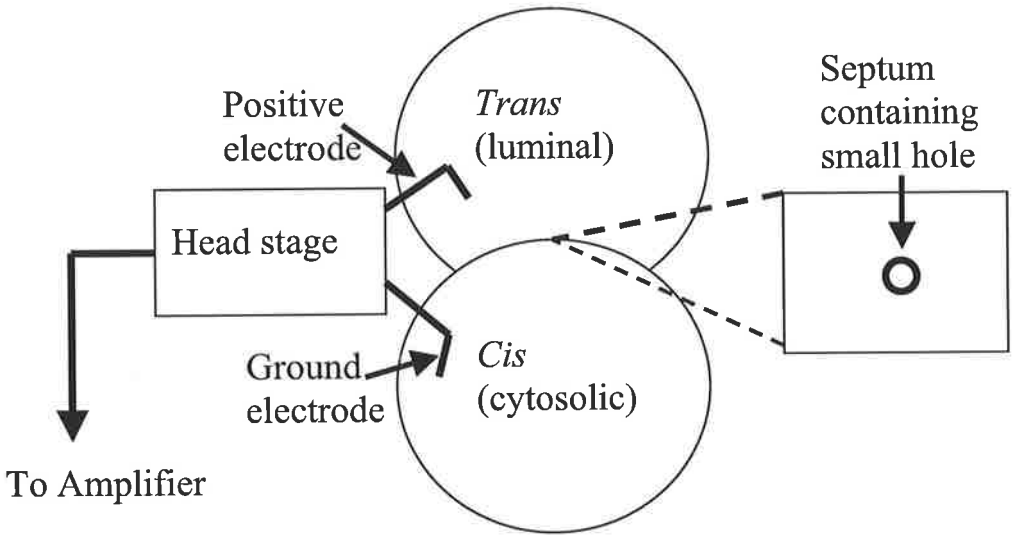


Figure 6: Schematic representation of the bilayer setup

2.5.4 Data Acquisition

Bilayer potential and currents were controlled using an Axopatch 200B amplifier (Axon Instruments) or a Bilayer Clamp-525c (Warner instruments). The *cis* bath was electrically grounded to prevent electrical interference from the perfusion tubes and the potential of the *trans* bath was varied (figure 6). All electrical potentials are expressed using standard physiological convention (cytoplasmic side relative to the luminal side at virtual ground). Ryanodine channel currents were recorded at a bilayer potential difference of –40 mV. Brief voltage pulses of +40 mV were used to prevent ryanodine channel voltage dependent inactivation. During collection, the data was filtered at 5 kHz, sampled at 50 kHz, and stored on computer disk using a data interface (Data Translation DT301) controlled by in-house software (written by Dr Derek Laver, University of Newcastle, NSW).

2.5.5 Data Analysis

For measurement of the open probability (P_o) and channel open (T_o) and closed (T_c) times, Channel 2 software was used (Professor P.W. Gage and Mr. M. Smith, Australian National University). Data was filtered at 1kHz using a Gaussian filter and was sampled at 0.5 kHz. In order to calculate P_o T_o and T_c, a threshold discriminator was set to 50% of the channel amplitude to determine channel opening and closing events in single channel recordings. For bilayers containing multiple channels, P_o was determined by the time-averaged current, divided by the amplitude of a single channel current multiplied by the number of channels present (ie. The maximum conductance of the bilayer). P_o values yielded by this method had similar values to those

determined using the threshold method. T_o and T_c values could not be determined using this method.

2.5.6 The Effects of EPA on the Cytosolic Face of the Ryanodine Receptor

Upon vesicle fusion and the incorporation of a ryanodine receptor, 4.5 mM BAPTA and 2 mM ATP was added to the *cis* bath. BAPTA was added in order to reduce the calcium concentration to 0.1 μM and ATP was introduced in order to activate the channel. The bath was then stirred to ensure adequate mixing. A control current was recorded, and 10, 20, 30, 40 or 50 μM of EPA (ICN) was then added to the *cis* bath and stirred. Typically multiple concentrations could be added to the same channel. However channel longevity often did not permit the recording of channel activity in the presence of all concentrations.Thus concentrations were randomized and tested on separate channels. EPA washout was not possible as the conventional delipidated BSA washout often employed in whole cell experiments resulted in instability and rupturing of the bilayer. As such EPA was added progressively in increasing concentration. This experiment was repeated using N-tetradecane as the solvent for the phospholipids. All experiments were performed at room temperature (22-25°C).

2.5.7 The Effects of EPA on the Luminal Face of the Ryanodine Receptor

Bilayers were formed according to the method described in section 2.5.3, and contained the solvent N-decane. Upon RyR fusion, 4.5 mM BAPTA and 2 mM ATP were added to the *cis* bath then stirred for 30 seconds to ensure adequate diffusion. The RyR current was recorded as a control. 50 μM EPA was added to the *trans* bath and stirred for 30 seconds to allow adequate diffusion. The RyR current was recorded in the presence of EPA. The current was then recorded for as long as the bilayer remained viable. All experiments were performed at room temperature (22-25°C).

2.5.8 Statistical Analysis

P_o, T_o and T_c values for the various EPA concentrations when added to the cytosolic and luminal face of the channel were compared to the values obtained in 0 EPA using t-tests. $P<0.05$ was assumed significant. Values are expressed as means ± SEM.

2.5.9 The Effects of EPA on the Ryanodine Receptor Response to Cytosolic Calcium

Bilayers were formed according to the method described in section 2.5.3; bilayers contained the solvent N-decane. Upon RyR fusion, the channel currents were recorded in the presence of 0.1 μM, 1 μM, 5μM and 0.1mM Ca^{2+} applied to the cytosolic side of the channel in a randomized order. The solution was changed between each recording to the solution containing 0.1 mM Ca^{2+}. This allowed the data to be normalized for changing channel activity through out the course of the

experiment, particularly in the presence of EPA. After data collection in the absence of EPA, 50 μM EPA was added to the *cis* bath and stirred for 1 minute to ensure adequate diffusion. Following the addition of EPA, the above data collection protocol was repeated.

In order to change the solutions, solutions were perfused through a beveled edged tube, which was positioned in close proximity to the bilayer. This allowed for the direct perfusion of solution on to the bilayer. This tube was removed upon the addition and stirring of EPA and was re-positioned after addition. All experiments were performed at room temperature (22-25°C). Data was analyzed according to the methods described in section 2.5.6.

2.5.9.1 Statistical Analysis

In order to determine the effect of EPA on the ryanodine channel sensitivity to calcium, P_o for each of the calcium concentrations was normalized for the P_o collected in 0.1 mM Ca^{2+} following each of the concentrations. Normalizing the data in this manner enabled the differentiation of the effects of calcium on RyR P_o to any other blocking effects EPA has on the channel. This was performed in the absence and the presence of EPA. Calcium concentrations in the absence and presence of EPA were compared using t-tests. In order to determine the effect of EPA on the RyR, P_o values measured in 0.1 mM Ca^{2+} were averaged over time to give a time course for the effects of EPA with calcium as the only activating ligand. Data over each time frame was compared to data prior to the addition of EPA using t-tests.

3. RESULTS

3.1 The Effects of Polyunsaturated Fatty Acids on Calcium Sparks in Rat Cardiac Myocytes

3.1.1 The Effects of Dietary Polyunsaturated Fatty Acids on Calcium Sparks in Rat Atrial Myocytes

3.1.1.1 Membrane Phospholipid Composition of Rat Atrial Myocytes

Atrial membrane phospholipid fatty acid composition of rats following 3 weeks daily lipid supplementation with lard, canola oil and fish oil is shown in table 1. The main difference in the membrane phospholipid profile was that the total n-3 PUFA content of the membranes was increased in the fish oil-supplemented group compared to the lard-supplemented group. The total n-3 PUFA content of phospholipid membranes in atrial myocytes from fish oil supplemented rats was 18.9 ± 0.7% compared to 12.6 ± 0. 2% in the membranes of lard-supplemented rats ($P<0.05$). The larger proportion of n-3 PUFAs in the membranes of the fish oil-supplemented animals was due to an increase in the proportions of membrane EPA and DHA.

The proportion of n-3 fatty acids present in the phospholipids was higher than expected in all 3 groups. The likely reason for this is that rats were maintained on a standard rat chow diet in addition to lipid supplementation by gavaging. The rat chow itself may have contained a relatively high proportion of PUFAs giving rise to smaller

differences in the membrane phospholipid composition between the three groups after the gavaging period. Dietary fatty acid manipulation could be more controlled by the use of fabricated diets incorporating the lipid supplements, as this would reduce the quantity of unknown fatty acids in the rat diets.

3.1.1.2 The Effects of Dietary Polyunsaturated Fatty Acids on Calcium Sparks

Calcium sparks were recorded in Tyrode's solution containing 1mM calcium chloride (see section 2.2.1). Figure 7 shows line scans (x-t scans) of calcium sparks in atrial myocytes from both lard and fish oil-supplemented rats. It is evident that spark size is larger in cells from lard supplemented animals than cells from fish oil supplemented animals.

Spark width was not found to differ significantly from the myocytes of rats supplemented with lard (3.1 ± 0.2 μm), canola oil (3.0 ± 0.3 μm) or fish oil (2.5 ± 0.2) (Figure 8 panel A). Calcium spark duration was found to be reduced in the myocytes from rats supplemented with fish oil (43.5 ± 4.7 ms) in comparison to those from rats supplemented with lard (105.5 ± 18.9 ms; $P<0.05$). The duration of sparks collected in myocytes from animals supplemented with canola oil (56.4 ± 9.0 ms) did not differ from either group. (Figure 8 panel B)

Major FAME	Lard mean	SEM	Canola oil mean	SEM	Fish oil mean	SEM
14:0	0.56	0.09	0.40	0.03	0.19	0.03*
16:0	21.45	1.31	17.15	0.41*	17.69	1.23
17:0	0.49	0.01	0.40	0.02	0.55	0.07
18:0	25.44	0.32	26.04	0.25	26.94	0.37*
18:1 n-9+n-7	10.12	0.29	11.07	0.27	9.66	0.25*
18:2 n-6	7.58	0.36	8.78	0.49	6.08	0.39
18:3 n-3	nd		0.06		nd	
20:0	0.36	0.04	0.46	0.05	0.50	0.10
20:1	0.86	0.25	0.62	0.05	0.25	0.10
20:2	0.37	0.11	0.30	0.04	0.23	0.03
20:3 n-6	0.65	0.11	0.46	0.07	0.51	0.18
20:4 n-6	19.30	0.84	20.48	0.41	17.46	0.65
20:3 n-3	nd		nd		nd	
22:0	nd		0.11	0.07	0.44	0.12
22:1	nd		nd		nd	
20:5 n-3	0.59	0.14	0.60	0.05	1.52	0.11*
24:0	0.61	0.14	0.48	0.03	0.81	0.05
24:1	nd		nd		nd	
22:5 n-3	3.15	0.18	3.07	0.01	3.48	0.16
22:6 n-3	8.90	0.27	9.71	0.37	13.87	0.48*
total saturated	48.91	1.05	44.90	0.63*	47.16	1.08
total mono	10.84	0.42	11.69	0.29	9.74	0.23
total poly	40.25	0.95	43.41	0.52*	43.10	0.97
total n-6	27.42	0.98	29.72	0.68	24.04	0.92*
total n-3	12.64	0.22	13.39	0.49	18.87	0.66*
n-6/n-3	2.17	0.10	2.24	0.13	1.28	0.08*

Table 1:
Fatty acid composition (wt%) of cardiac atrial myocyte total phospholipids (n=6) after dietary lipid supplementation.
FAME, fatty acid methyl esters.
n/d, not detected.
* signifies $P<0.05$ vs. lard
The short hand notation for fatty acid structure is x:y (n-i) where 'x' is the number of carbons in the chain,' y' is the number of double bonds and 'i' is the position of first double bond from the methyl end of the carbon chain. The position of the first double bond is often referred to as "omega".

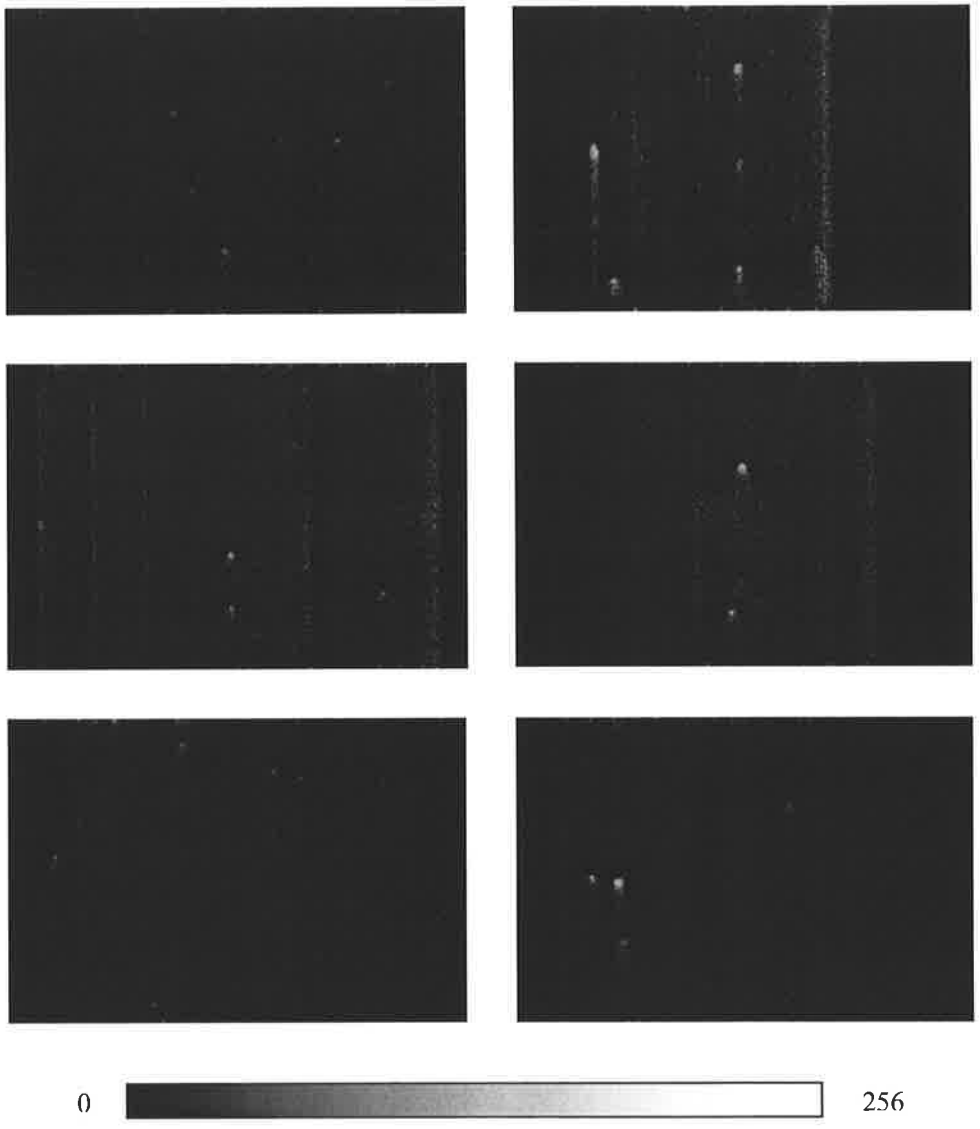


Figure 7. Typical line scan images of calcium sparks of atrial myocytes isolated from rats supplemented with dietary fish oil (left hand side) or lard (right hand column). Total image width is 96 μm and the duration is 1.05 seconds for all images.

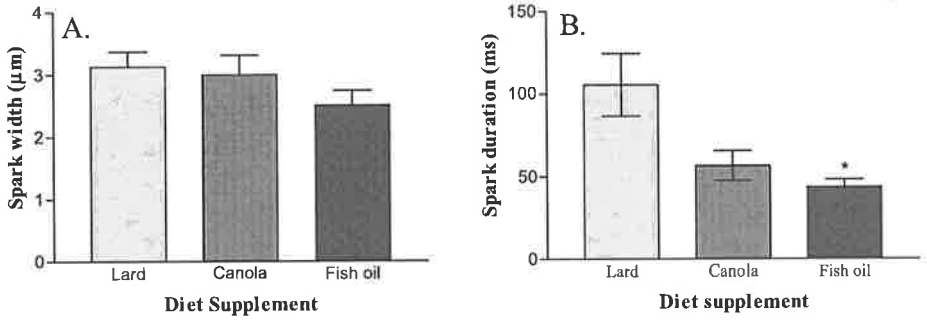


Figure 8: Mean spark width (µm) (panel A) and duration (ms) (panel B) of atrial myocytes from rats supplemented with dietary lard (n=6), canola oil (n=5) and fish oil (n=5). All data is expressed as mean ± SEM. * Significant at $P< 0.05$ compared to the lard-supplemented group.

The frequency of calcium sparks was difficult to determine due to the high variability of spark numbers between myocytes. Aside from the physiological variability between the myocytes, another factor that could have potentially contributed to the large variability in the frequency of sparks, is the positioning of the scan line with respects to the SR. The SR of atrial myocytes is positioned within close proximity to the sarcolemmal membrane due to the absence of T-tubules, and for this reason calcium spark distribution is not uniform and is variable in frequency throughout the cell.

The results of the present study revealed no significant difference in the frequency of the sparks between the three dietary groups. The spark frequency was found to be 8.0 ± 3.2 sparks/min in myocytes from the lard supplemented rats, 4.5 ± 2.4 sparks/min in myocytes from the canola oil supplemented rats and 4.6 ± 1.6 sparks/min in myocytes from the fish oil supplemented animals ($P>0.05$). This data is summarized in figure 9

panel A. Spark intensity was also found to not differ between the three dietary groups, data is summarized in figure 9 panel B.

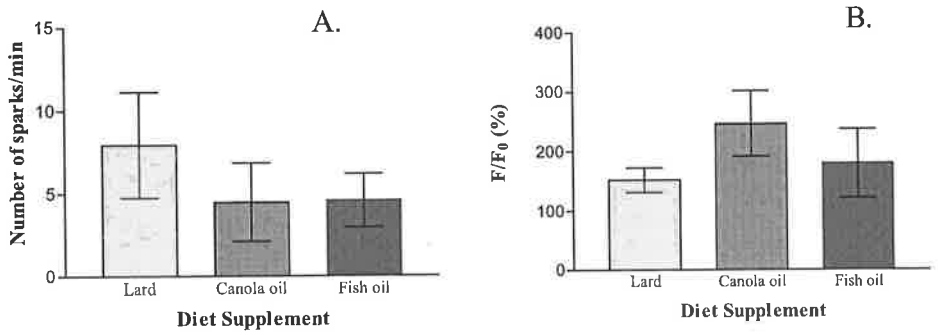


Figure 9: Mean number of sparks/min (Panel A) and mean spark intensity (Panel B). The average peak pixel (F) value expressed as a percentage of the mean background brightness (F_o) of atrial myocytes from rats supplemented with lard (n=6), canola oil (n=5) and fish oil (n=5). Data is expressed as mean ± SEM.

In order to determine the rate of decay of the calcium sparks, the longitudinal profile of the sparks was taken as plotted on the line scan image. These plots revealed that not all sparks shared a similar profile, as illustrated in figure 10. For many sparks, the rate of decay could be measured as they exhibited a typical fast rise and an exponential like decay (type 1 spark). The rate of decay was not able to be determined for a proportion of sparks as the decaying phase was not exponential. Many sparks exhibited a plateau part way through the decaying phase (type 2 sparks). The existence of type 2 sparks is unlikely to be due to image distortion through cell contraction, as calcium sparks do not result in cell contraction. This type of spark was also identified to be non-propagating and as such is unlikely to form a calcium wave. A third distinct profile of spark was also identified, with slow duration followed by an exponential like decay similar to the type 1 spark. This type of spark was not likely to

be originating from below the focal plane, as a peak is evident in the profile whereas those originating above or below the focal plane typically exhibited a rounded profile consistent with calcium diffusion into the region of interest. Following extensive comparison of spark profiles, it was concluded that determination of the rate of decay was not going to provide an accurate measure of spark decay.

Sparks were classed according to whether they had peaked and decayed in an exponential manner, if so they were classed as type 1 sparks. If the sparks exhibited a non-exponential decay or a plateau was present within the decaying phase, they were classed as type 2 sparks. If the sparks exhibited a slow linear rising phase possibly also with a linear decaying phase, they were classed as type 3 sparks. Sparks exhibiting the type 1 profile were undoubtedly the most common. Of the total numbers of sparks in the lard-supplemented group, 63.5% of these sparks were type 1 sparks. In the canola and fish oil-supplemented groups, the percentages of this type of spark were 70.6% and 77.0 %, respectively. Sparks which exhibited a plateau during the decay phase were also common in cells from lard-supplemented animals (19.9%), but relatively scarce in cells from rats supplemented with canola oil (4.8%) and fish oil (1.7%). The third type of spark identified was one of slow uprise duration, these sparks were relatively common in myocytes from canola oil (24.6%) and fish oil (21.3%) the occurrence of this type of spark were slightly less common in rats supplemented with lard (16.6%) (Figure 10).

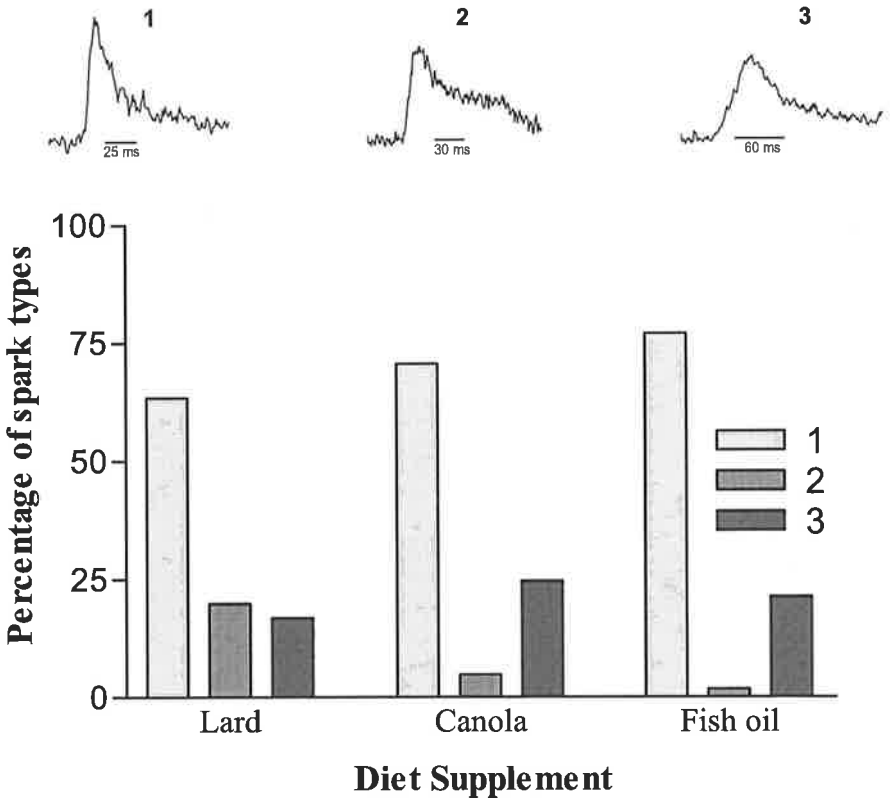


Figure 10: Percentage of spark types illustrated in plot profiles 1-3 from rats supplemented with lard (n=208), canola oil (n=126) and fish oil (n=174).
Definition of spark types.
Spark type 1: steep intensity increase followed by a steep decrease.
Spark type 2: steep intensity increase and sharp peak, then an initial rapid decrease followed by a plateau then decrease to background level.
Spark type 3: gradual intensity increase, short peak followed by a gradual decrease

3.1.2 The Effects of Dietary Polyunsaturated Fatty Acids on Calcium Sparks in Rat Ventricular Myocytes.

3.1.2.1 Membrane Phospholipid Composition of Rat Ventricular Myocytes

In the present study, rats were maintained on a fabricated diet supplemented with either 10% lard or 10% fish oil for 3 weeks. Analysis indicated that dietary fish oil significantly altered the fatty acid profile of the membranes of cardiac ventricular myocytes in comparison to the membranes of lard-supplemented animals. These results are summarized in table 2. Compared to dietary lard, dietary fish oil significantly increased the proportion of n-3 PUFAs present in the membrane phospholipids. The presence of docosahexaenoic acid (22:6, n-3), docosapentaenoic acid (22:5, n-3) and eicosapentaenoic acid (20:5, n-3) were all increased in the membranes of rats supplemented with fish oil in comparison to the rats supplemented with lard. The proportion of n-6 PUFAs present in the cardiac membrane phospholipids of rats supplemented with fish oil was reduced in comparison to those supplemented with lard. This was due to a significant reduction in the proportion of arachidonic acid (20:4, n-6) in the membranes of fish oil supplemented animals. The proportion of monounsaturated fatty acids present in the cardiac phospholipid membrane was slightly lower in rats supplemented with fish oil in comparison to those supplemented with lard. This was due to lower levels of oleic acid (18:1) in the fish oil-supplemented animals. The proportion of saturated fatty acids in the cardiac phospholipid membranes was not found to significantly differ between the two dietary groups.

Major FAME	Lard	Fish oil
16:0	9.4 ± 0.9	11.9 ± 0.5 *
18:0	25.9 ± 1.3	23.1 ± 0.4
18:1 (n-7 + n-9)	14.5 ± 0.6	10.2 ± 0.5 *
18:2 (n-6)	5.9 ± 1.1	3.7 ± 0.6
18:3 (n-3)	n/d	n/d
20:4 (n-6)	33.6 ± 2.4	22.2 ± 2.0 *
20:5 (n-3)	n/d	3.2 ± 0.1
22:5 (n-3)	0.8 ± 0.1	2.5 ± 0.1 *
22:6 (n-3)	6.0 ± 0.8	20.5 ± 0.9 *
Total Sat	37.6 ± 2.4	36.0 ± 0.7
Total Mono	14.9 ± 0.6	10.9 ± 0.5 *
Total Poly	47.2 ± 1.5	53.1 ± 0.9 *
Total n-6	40.1 ± 2.4	26.7 ± 1.7 *
Total n-3	6.9 ± 0.9	26.2 ± 0.9 *
n-6/n-3	50.8 ± 0.6	1.0 ± 0.1 *

Table 2: Fatty acid composition (wt%) of cardiac ventricular myocyte phospholipids after dietary lipid supplementation (n=6). Data is expressed as Mean ± SEM.
* signifies $P<0.05$ vs. Lard
FAME, fatty acid methyl esters
n/d, not detected.
The short hand notation for fatty acid structure is x:y (n-i) where 'x' is the number of carbons in the chain,' y' is the number of double bonds and 'i' is the position of first double bond from the methyl end of the carbon chain. The position of the first double bond is often referred to as "omega".

3.1.2.2 The Effects of Dietary Polyunsaturated Fatty Acids on Calcium Sparks In Ventricular Myocytes

Calcium sparks were collected in a control Tyrode's solution containing 1 mM calcium chloride. Dietary fish oil was found to reduce the width and duration of calcium sparks in comparison to dietary lard supplementation in ventricular myocytes in a similar manner to the reduction observed in atrial myocytes. This data is summarized in figure 11. Spark width was much larger in ventricular myocytes supplemented with lard (3.2 ± 0.1 μm) in comparison to those supplemented with fish oil (2.3 ± 0.1 μm) (figure 11 panel A) ($P<0.05$). Spark duration was also found to be longer in myocytes from lard supplemented animals (67.2 ± 4.8 ms) in comparison to fish oil supplemented animals (39.8 ± 0.7 ms) ($P<0.05$) (figure 11 panel B).

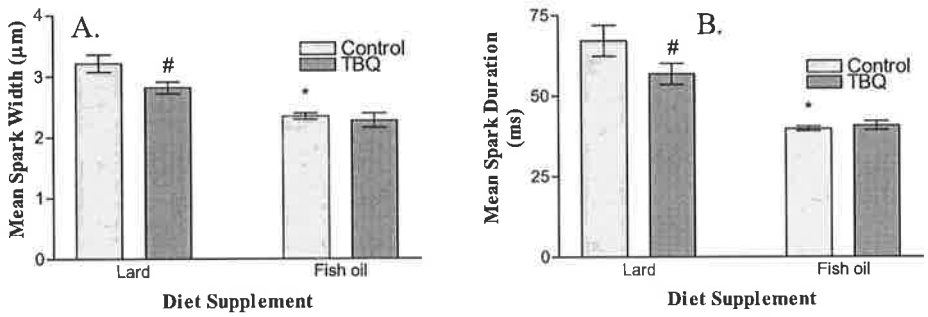


Figure 11: Mean width (μm, panel A) and duration (ms, panel B) (± SEM) of ventricular myocytes from rats supplemented with lard (n=6) and fish oil (n=6) in a control solution and 1 μM TBQ. * signifies $P<0.05$ lard vs. fish oil. # signifies $P<0.05$ control vs. TBQ.

Calcium spark intensity was measured as the mean peak spark pixel values as a percentage of the average background pixel values prior to the spark. Calcium spark intensity was found to be reduced in myocytes from rats supplemented with fish oil (80.7 ± 2.1 % greater than background intensity) in comparison to myocytes from rats supplemented with lard (94.3 ± 2.6 % greater than background intensity) ($P < 0.05$). (Figure 12 control bars)

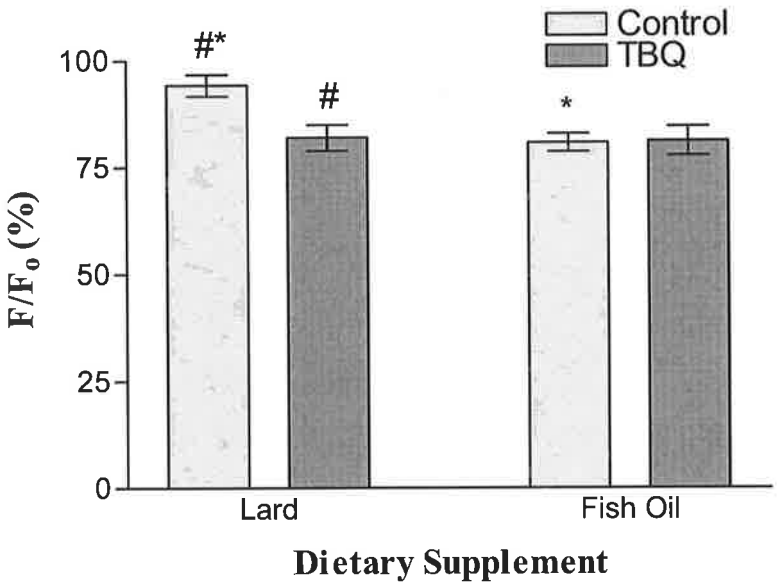


Figure 12: Mean spark intensity (F/F_0 % ± SEM) of spark from rats supplemented with lard (n=6) and fish oil (n=6) before and after the application of TBQ. * signifies significance between lard and fishoil groups, # signifies significance between control and TBQ, $P < 0.05$

As previously shown in rat atrial myocytes, calcium spark profiles do not share similar morphology. Calcium spark profiles are illustrated in figure 10. The dietary supplementation of rats with fish oil altered the proportion of calcium spark profile types in ventricular myocytes compared to myocytes from rats supplemented with dietary lard (figure 13). In the present study it was found that the proportion of type 1 sparks was 75.4 ± 6.4% in myocytes from lard-supplemented rats in comparison to 83.1 ± 5.1% in myocytes from fish oil-supplemented rats. The proportion of type 2 sparks was markedly reduced in myocytes from rats supplemented with fish oil in comparison to those supplemented with lard. The proportion of type 2 sparks in myocytes from lard-supplemented rats was 7.1 ± 3.5% and 0% in myocytes from fish oil supplemented rats ($P<0.05$; unpaired t-test). The proportion of type 3 sparks was 17.1 ± 4.4% in myocytes from rats supplemented with lard in comparison to 16.9 ± 5.1% in myocytes from fish oil-supplemented animals (figure 13).

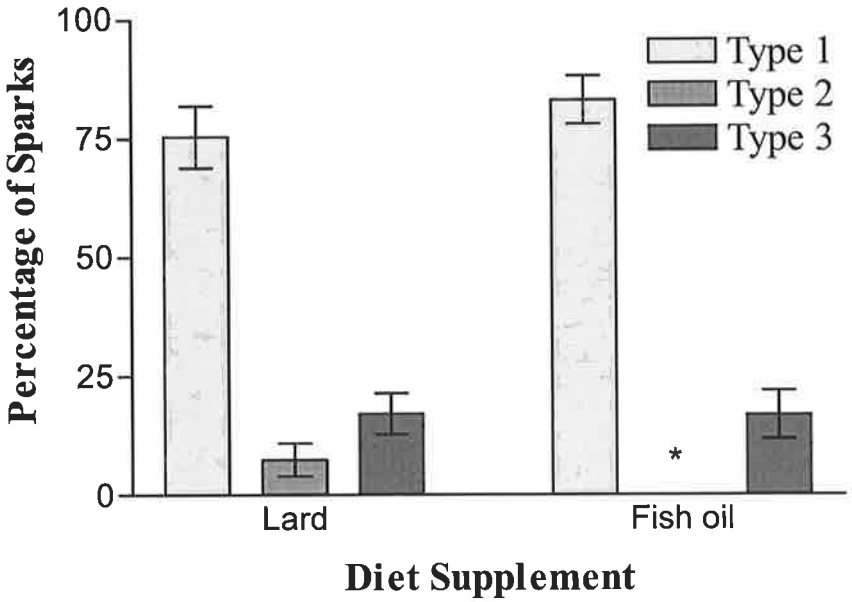


Figure 13: Percentage of spark types from rats supplemented with lard (n=6; 188 sparks) and fish oil (n=6; 146 sparks). * Signifies significance ($P<0.05$) vs. lard (Unpaired t-test).
Definition of spark types.
Spark type 1: steep intensity increase followed by a steep decrease
Spark type 2: steep intensity increase and sharp peak, then an initial rapid decrease followed by a plateau then decrease to background level.
Spark type 3: gradual intensity increase, short peak followed by a gradual decrease

3.1.2.3 The Effects of TBQ on Calcium Sparks in Ventricular Myocytes from Rats Supplemented with Dietary Lard and Fish Oil

The application of TBQ to rat ventricular myocytes did not significantly alter calcium spark width (2.3 ± 0.1 μm) or duration (40.8 ± 1.40 ms) in comparison to the control values in cells from fish oil supplemented rats (figure 11). However the application of TBQ reduced calcium width and duration in comparison to control sparks in cells from rats supplemented with lard. Calcium spark width was reduced from 3.2 ± 0.1 μm to 2.8 ± 0.1 μm ($P<0.05$) and duration was reduced from 67.2 ± 4.8 ms to 56.9 ± 3.9 ms ($P< 0.05$).

The application of TBQ was not found to alter calcium spark intensity in cells from rats supplemented with fish oil (81.1 ± 3.4 % greater than background intensity). However the application of TBQ resulted in a significant reduction in calcium spark intensity from 94.6 ± 2.6% greater than background intensity to 81.9 ± 3.0 % greater than background intensity in myocytes from lard supplemented rats ($P<0.05$, figure 12).

3.1.3.1 The Effects of Free Polyunsaturated Fatty Acids on Calcium Sparks in Rat Ventricular Myocytes

Myocytes treated with EPA were found to have a reduced spark width (2.2 ± 0.1 μm) compared to the control myocytes (3.0 ± 0.2 μm) ($P<0.05$). The width of the calcium sparks was found not to differ in myocytes treated with DHA (2.9 ± 0.14 μm) or ALA (2.8 ± 0.10 μm) compared to the control myocytes (figure 14 panel A). Calcium spark duration was found to be shorter in the myocytes treated with EPA (40.3 ± 1.8 ms) in comparison to the control myocytes (53.6 ± 3.2 ms) ($P<0.05$). Spark duration in the myocytes treated with DHA (57.9 ± 3.9 ms) or ALA (51.8 ± 3.4 ms) were found not to differ from the duration of the control myocytes (figure 14 panel B).

Treatment of the myocytes with EPA, DHA or ALA significantly reduced the calcium spark intensity in comparison to the control values. It was found that the intensity of the calcium sparks was reduced from 104.8 ± 3.2% greater than background intensity in the control myocytes to 81.4 ± 5.8 % greater than background intensity in EPA-treated myocytes, 79.5 ± 4.9% greater than background intensity in the DHA-treated myocytes and 72.6 ± 8.2% greater than background intensity in ALA treated myocytes. This data is summarized in figure 15.

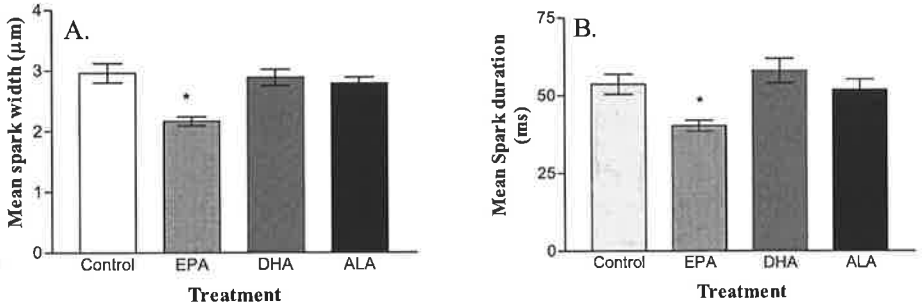


Figure 14: Mean width (panel A) and duration (panel B) of calcium sparks collected from control myocytes (n=10) and myocytes treated with 15 µM EPA (n=10), DHA (n=10) or ALA (n=10). Data is expressed as means ± SEM, * signifies significance

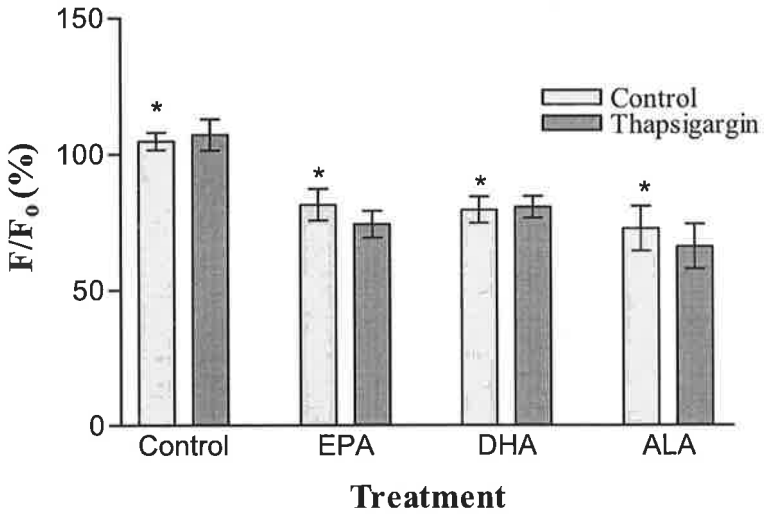


Figure 15: Mean spark intensity (± SEM% of background intensity) from control cells (n=10) and cells incubated with 15 µM EPA (n=10), DHA (n=10) and ALA (n=10) before and after the application of thapsigargin. * signifies significance $P<0.05$ vs. control.

3.1.3.2 The Effect of Thapsigargin on Calcium Sparks from Myocytes Treated with Polyunsaturated Fatty Acids.

The application of thapsigargan resulted in no significant changes in calcium spark width or duration, in comparison to those collected in the control solution. This data is summarized in table 3. Thapsigargin was not found to alter the intensity of calcium sparks in control myocytes (107.2 ± 5.8 % of background intensity) EPA treated myocytes (74.3 ± 4.9 % of background intensity), DHA treated myocytes (80.5 ± 4.0% of background intensity) or ALA treated myocytes (66.0 ± 8.2% of background intensity) in comparison to calcium sparks collected in the control solution (figure 15).

	Width (μm)		Duration (ms)	
Treatment	Control	Thapsigargin	Control	Thapsigargin
Control	2.96 ± 0.16	3.03 ± 0.16	53.63 ± 3.2	55.70 ± 3.26
EPA	2.16 ± 0.07	2.10 ± 0.10	40.30 ± 1.78	39.85 ± 1.23
DHA	2.88 ± 0.14	2.94 ± 0.22	57.93 ± 3.94	55.74 ± 6.14
ALA	2.79 ± 0.10	2.54 ± 0.10	51.76 ± 3.36	45.64 ± 1.11

Table 3:
Mean Calcium spark width and duration from control myocytes (n=10) and myocytes treated with EPA (n=10), DHA (n=10) and ALA (n=10) collected in a control solution and a solution containing 5mM thapsigargin.

3.2 The Effects of Polyunsaturated Fatty Acids on Calcium Transients in Rat ventricular Myocytes

3.2.1 The Effects of Dietary Polyunsaturated Fatty Acids on Calcium Transients in Rat Ventricular Myocytes.

3.2.1.1 Membrane Phospholipid Composition of Rat Ventricular Myocytes

The ventricular myocytes used in this study were obtained from the same rats used in section 3.1.2.1. Refer to table 2 for a summary of the membrane phospholipid profiles. Briefly, Dietary manipulation with fish oil increased the membrane fatty acid composition of n-3 PUFAs to 20.5 ± 0.9% in ventricular cells in comparison to 6.0 ± 0.8% in lard supplemented animals.

3.2.1.2 The Effect of Dietary Polyunsaturated Fatty Acids on Calcium Transients

A typical line scan image (x-t scan) of a calcium transient collected from a rat ventricular myocyte at room temperature (20-25 °C) is illustrated in figure 16, panel A. From the line scan image the longitudinal profile of the transient can be taken, as shown in figure 16 Panel B and an exponential growth and decay equation can be fitted to the rising and decaying phase of the transient, respectively.

In the present study it was found that the doubling time of the calcium transient is reduced in myocytes from rats supplemented with fish oil in comparison to transients

in myocytes from rats supplemented with lard ($P<0.05$). The mean doubling time of the transients was found to be 9 ± 1 ms (n=8) in the lard supplemented group in comparison to 7 ± 0.4 ms (n=8) in the fish oil supplemented group (see figure 17 panel A). No significant difference in the half-life of decay of the calcium transients was observed in the lard-supplemented animals in comparison to the fish oil supplemented animals. The mean half-life was found to be 1.4 ± 1 seconds in the lard supplemented animals compared to 1.1 ± 0.5 seconds in the fish oil supplemented animals (see figure 17 panel B).

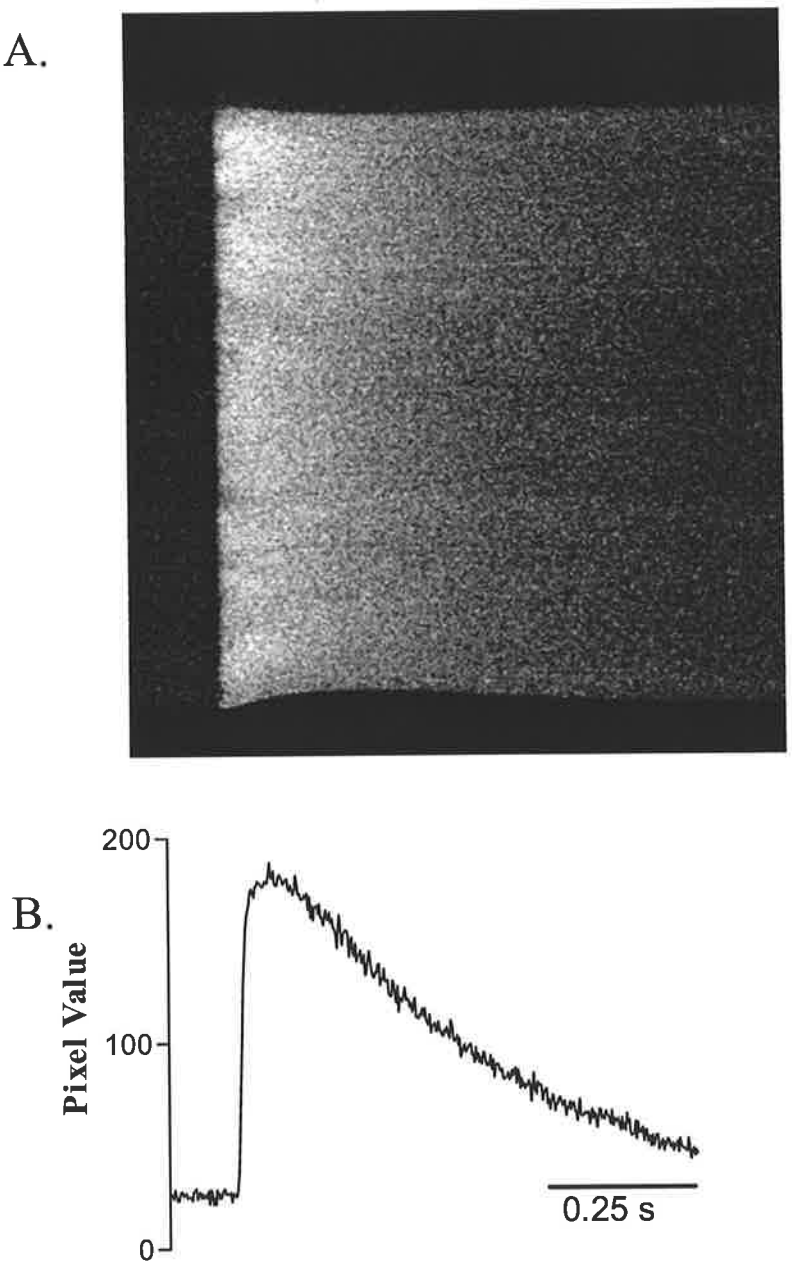


Figure 16: Typical line scan images of a calcium transient following stimulation of an action potential. Total image width is 96 μm and the duration is 1.05 seconds (A.). B. Longitudinal profile of a section of the above calcium transient.

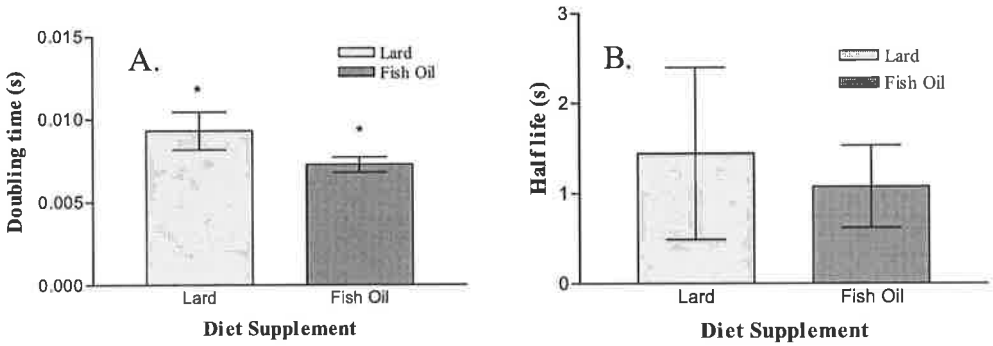


Figure 17: Mean doubling time (± SEM) (A) and half life (B) of calcium transients recorded in ventricular myocytes from lard supplemented (n=8) and fish oil supplemented (n=8) rats. * Signifies $P<0.05$ vs. lard.

The present study revealed no differences in the amplitude of the calcium transients collected in myocytes from fish oil-supplemented animals compared to lard-supplemented animals. This was measured as the peak fluorescence intensity of the transient expressed as a percentage of the background intensity prior to the transient. Mean transient intensity was found to be 1546.1 ± 217.5 % greater than background intensity in the lard-supplemented animals compared to 1135.6 ± 210.1 % greater than background brightness in the fish oil-supplemented animals (see figure 18).

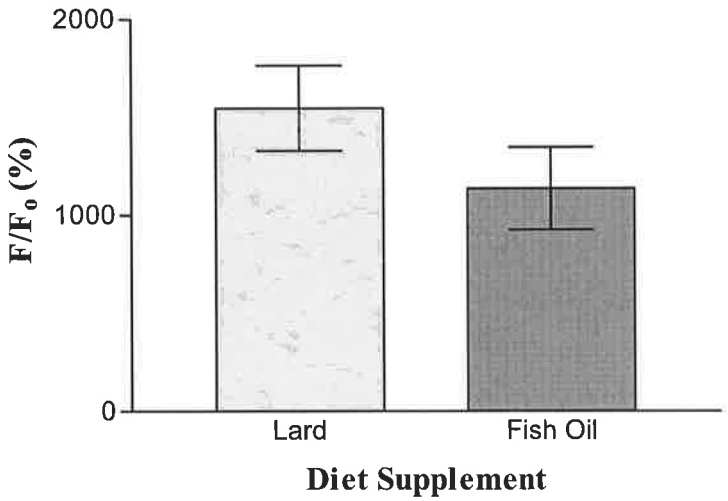


Figure 18: Mean calcium transient intensity (F/F_0 % ±SEM) expressed as a percentage of background intensity in myocytes from rats supplemented with lard (n=8) and fish oil (n=8).

3.2.2 The Effect of Free EPA on Calcium Transients Rat Ventricular Myocytes

The present study revealed that the incubation of cells with 15 μM EPA significantly decreased the doubling time of the calcium transient in comparison to the control cells ($P<0.05$). The doubling time of the transients was found to be 8 ± 0. 4 ms in the control cells compared to 6 ± 0.4 ms in the EPA-treated cells (see figure 19 panel A). Due to large variability between myocytes in the EPA-treated group, there was no significant difference in the half-life of decay between the control and EPA-treated groups. The mean half-life of decay was 0.61 ± 0.19 seconds in the control myocytes and 1.9 ± 1 seconds in the EPA-treated myocytes. This data is summarized in figure 19 panel B.

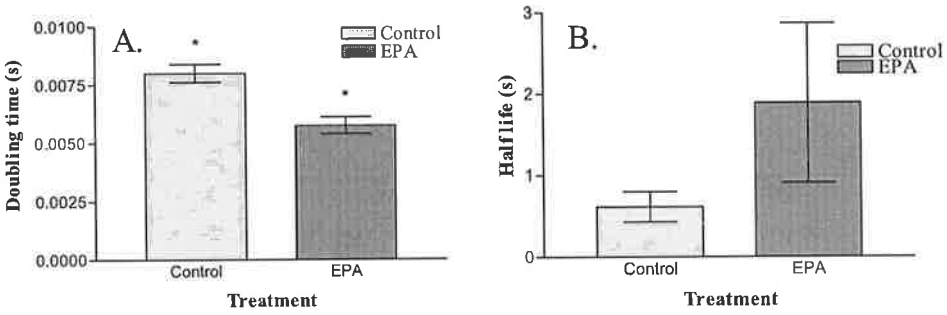


Figure 19: Mean rate constant K (± SEM) of the up rise (A) and decay phase (B) of calcium transients in ventricular myocytes incubated in the control solution (n=7) and in myocytes incubated with 15μM EPA (n=7). * Signifies $P < 0.05$.

The present study revealed no differences in the mean intensity of the calcium transients in the control cells compared to those incubated with EPA. The mean transient intensity was found to be 501.0 ± 75.4 % of the mean background intensity in the control cells and 598.3 ± 96.5 % of the mean background intensity in EPA-treated cells (see figure 20).

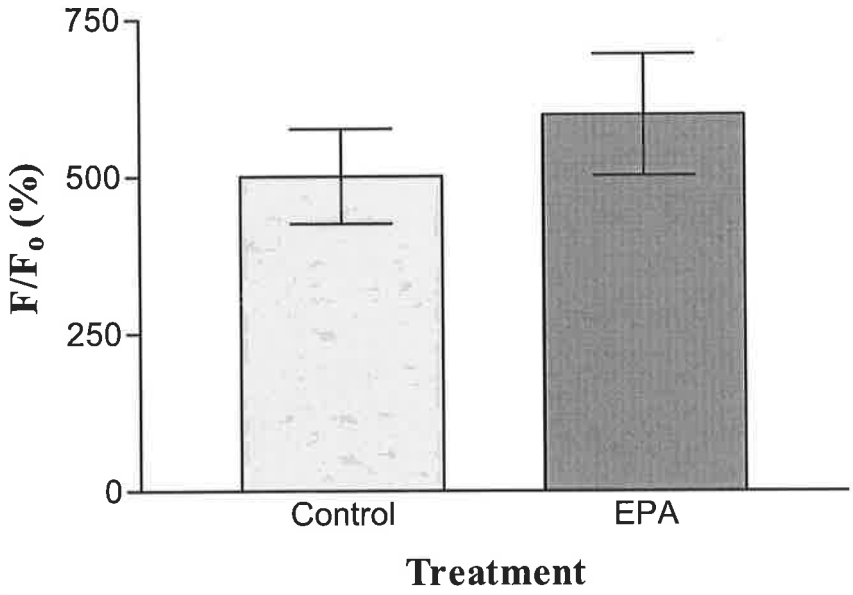


Figure 20: Mean calcium transient intensity expressed as a percentage of background intensity (F/F_o % ± SEM) in control cells (n=7) and in cells incubated with 15μM EPA (n=7).

3.3 The Effects of Dietary Polyunsaturated Fatty Acids on Calcium Waves in Rat Ventricular Myocytes

3.3.1 Membrane Phospholipid Composition of Rat Ventricular Myocytes

Separate phospholipid analysis was not performed for this study, as the protocol for dietary lipid supplementation was identical to that of section 2.2.3.1. including the composition of the diets. See section 3.1.2.1. for a summary of the membrane phospholipid profile of rat ventricular myocytes following dietary lipid supplementation.

3.3.2 The Effects of Dietary Polyunsaturated Fatty Acids on Calcium Waves in Rat Ventricular Myocytes

To determine the effect of dietary fish oil on the gain of the calcium induced calcium release cascade; calcium waves were recorded in Tyrode's solution containing 5 mM calcium chloride. Figure 21 shows a line scan image of a calcium wave propagating from one end of the cell to the other (panel A). Following stimulation with isoproterenol (panel B) the frequency of the waves increased.

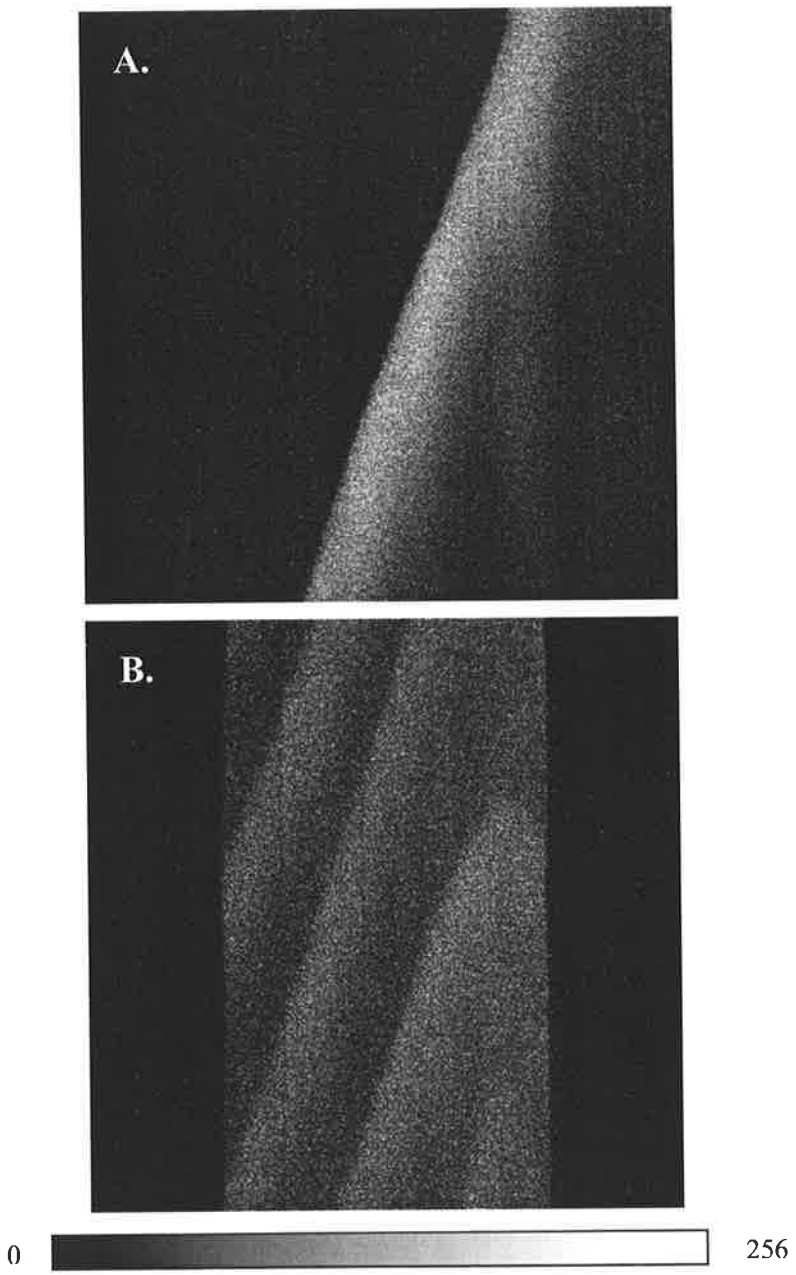


Figure 21: Typical line scan (x-t scan) images of calcium waves collected in 5 mM $CaCl_2$ (panel A) and in the presence of 1 μM isoproterentol (panel B). Each scan is 150 μM in width (x-axis) and 1.54 seconds in duration (y-axis).

The results from this study did not reveal any differences in the propagation velocity of calcium waves between the lard and fish oil-supplemented groups ($P>0.05$). The mean propagation velocity of the calcium waves in the lard and fish oil-supplemented groups were 34.0 ± 1.1 μm/s and 35.9 ± 1.4 μm/s respectively (see figure 22 panel A). The intensity of the calcium waves was also not found to differ between the lard and fish oil-supplemented groups ($P>0.05$). The intensity of the calcium waves were 223.0 ± 38.7 % greater than the background intensity in the lard supplemented animals in comparison to 288.6 ± 39.1 % greater than background intensity in the fish oil supplemented animals (see figure 22 panel B).

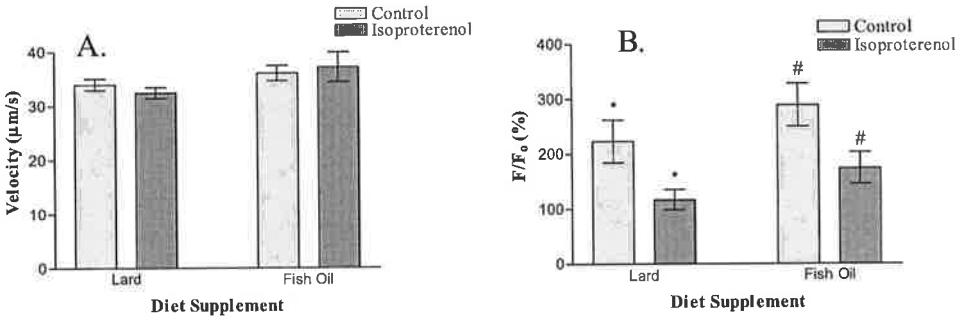


Figure 22: Mean propagation velocity (μm/s ± SEM) (panel A) and mean wave intensity expressed as the percentage of background intensity (F/F_0 %, panel B) of ventricular myocytes from rats supplemented with lard (n=6) and fish oil (n=6) in a control solution and in 1 μM isoproterenol. * and # signify $P<0.05$.

The frequency of calcium waves was found not to significantly differ in myocytes from the lard supplemented rats compared to myocytes from the fish oil supplemented rats ($P>0.05$). The frequency of calcium waves was found to be 0.3 ± 0.0 waves/s in myocytes from the lard supplemented rats and 0.4 ± 0.0 waves/s in myocytes from the fish oil supplemented rats (figure 23).

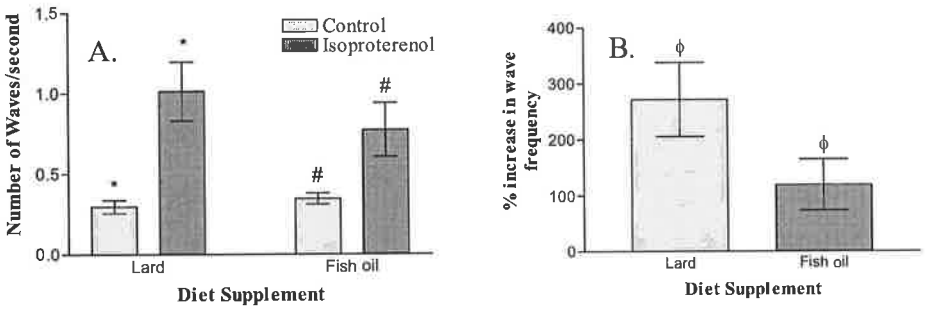


Figure 23: Mean number of calcium waves per second (± SEM) in cardiac ventricular myocytes from rats supplemented with dietary lard or fish oil collected under control conditions and in the presence of 1 μM isoproterenol (Panel A). Panel B illustrates the mean percentage change in calcium wave frequency after the addition of 1μM isoproterenol in ventricular myocytes from lard and fish oil supplemented rats. * and # signify $P<0.05$ vs. control. ϕ signify $P<0.05$ vs. lard.

3.3.3 The Effects of Isoproterenol on Calcium Waves in Ventricular Myocytes From Rats Supplemented With Polyunsaturated Fatty Acids

The application of isoproterenol did not significantly alter the propagation velocity of the calcium waves collected in cells from rats supplemented with lard or fish oil ($P>0.05$, see figure 22 panel A). The propagation velocity of calcium waves in the presence of 1 μm isoproterenol was 32.4 ± 1.0 μm/s in myocytes from lard supplemented animals and 37.1 ± 2.8 μm/s in myocytes from fish oil supplemented animals. Calcium wave intensity was found to decrease from 223.0 ± 38.7 to 116.8 ± 18.7 % greater than the background intensity in the lard-supplemented animals and from 288.6 ± 39.1 to 174.1 ± 28.6 % greater than background intensity in the fish oil-supplemented animals. This data is summarized in figure 22 panel B.

Upon the addition of 1μM isoproterenol, the frequency of the calcium waves increased in both dietary groups as illustrated in figure 21, panel B. Calcium wave frequency increased from 0.3 ± 0.0 to 1.0 ± 0.2 waves/s in myocytes from the lard supplemented group and 0.4 ± 0.0 to 0.8 ± 0.2 waves/s in myocytes from the fish oil supplemented rats ($P<0.05$, figure 23 panel A). The magnitude of change in the frequency of calcium waves following the exposure to 1 μM isoproterenol was greater in myocytes from the lard supplemented rats compared to myocytes from fish oil supplemented rats ($P<0.05$). The increase in wave frequency following isoproterenol exposure was 271.7 ± 66.2 % in myocytes from lard supplemented rats compared to 119.5 ± 45.7% in myocytes from fish oil supplemented rats. This data is summarized in figure 23 panel B.

3.4 The Effects of Polyunsaturated Fatty Acid EPA on Cardiac Ryanodine Receptors

3.4.1 The Effects of Cytosolic Addition of EPA on the Cardiac Ryanodine Receptor

To determine if PUFAs are altering calcium sparks and calcium transients by modulation of the RyR, the effects of PUFAs on RyRs was determined

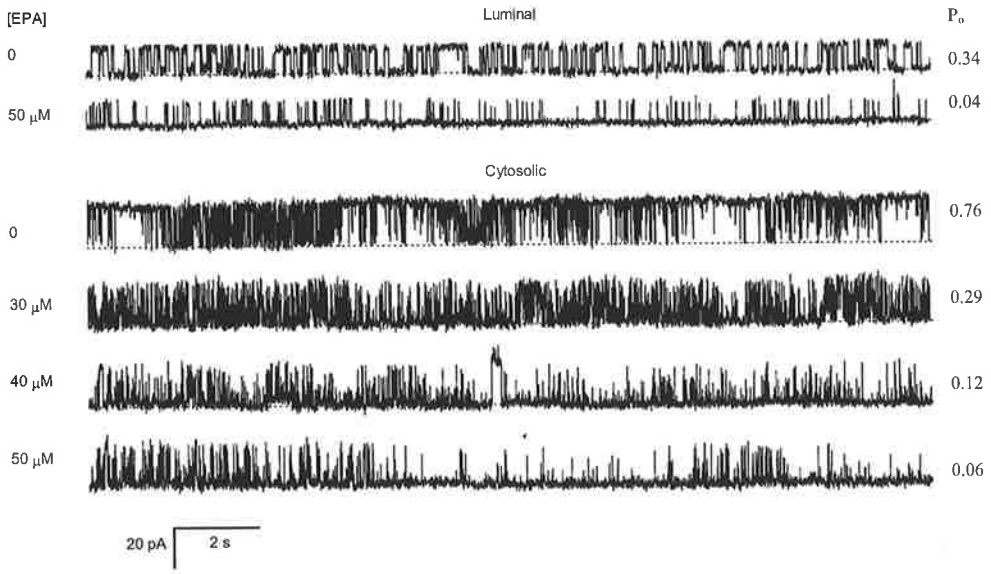


Figure 24: Representative recordings of cardiac RyRs before and after the addition of EPA to the luminal or cytosolic baths. RyRs were activated by the presence of 100 nM Ca^{2+} and 2 mM ATP. The records shown were taken after 60 s exposure to the EPA at concentrations shown at the left of each trace. The open probabilities of the RyRs calculated from these traces are shown at the right. The membrane potential was held at –40 mV and channel openings are shown here by upward deflections of the current from baseline.

The results of the present study show that the addition of 50 μM EPA reduces the open probability P_o of the cardiac RyR in the presence of cytosolic 2 mM ATP and 0.1 μM $CaCl_2$. An example of the effect of cytosolic EPA on the single current trace of the RyR is illustrated in figure 24. Upon the addition 50 μM EPA, the mean P_o of the RyR was reduced from 0.38 ± 0.07 to 0.02 ± 0.02 ($P<0.05$). This data is summarized in figure 25. The addition of 10, 20, 30 and 40 μM EPA did not significantly change the RyR P_o from the control. The P_o for these concentrations are 0.36 ± 0.13, 0.35 ± 0.13, 0.33 ± 0.08 and 0.26 ± 0.14, respectively (see figure 25).

The reduction in RyR P_o resulted from a decrease in the ryanodine receptor open time (T_o) and an increase in the close time (T_c) on the addition of 40 (T_o=2.4 ms, T_c=18.4 ms, n=1) and 50 μM EPA (T_o=2.3, T_c=19.1, n=1) compared to the control (T_o=7.8±3.5, T_c=7.9±1.5, n=9) but not on the addition of 10 (n=4), 20 (n=4) or 30 μM EPA (n=9). Statistical analysis was not performed due to insufficient data at higher EPA concentrations. The above results were obtained from RyRs fused into bilayers containing the solvent N-decane.

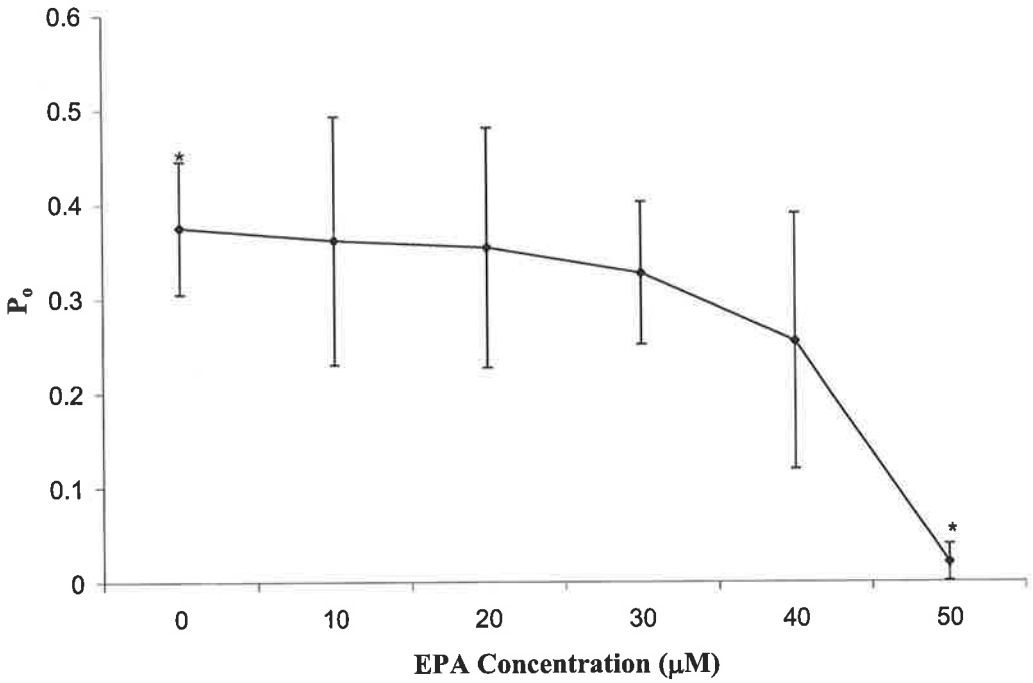


Figure 25: Mean open probability (± SEM) of cardiac ryanodine channels in a 50:50 PE: PC in N-decane bilayer in the presence of 0 (n=12), 10 (n=5), 20 (n=5), 30 (n=10), 40 (n=2) and 50 μM EPA (n=2) when applied to the cytosolic face of the channel. * signifies ($P<0.05$) vs. 0 μM EPA.

To determine if the lack of effect of EPA at concentrations below 50 μM was due to insufficient EPA exposure time, the duration of EPA action was observed. This was determined by measuring the RyR P_o before the addition and at 30-second intervals after the addition, of a single aliquot of either 30 μM or 50 μM EPA. Following 6 minutes of exposure, 30 μM EPA was not found to significantly reduce P_o, (figure 26). However the addition of 50 μM EPA was found to reduce P_o within 30 seconds of exposure (figure 27). This indicates that the exposure time of the RyR to lesser concentrations of EPA was sufficient for an effect to be observed.

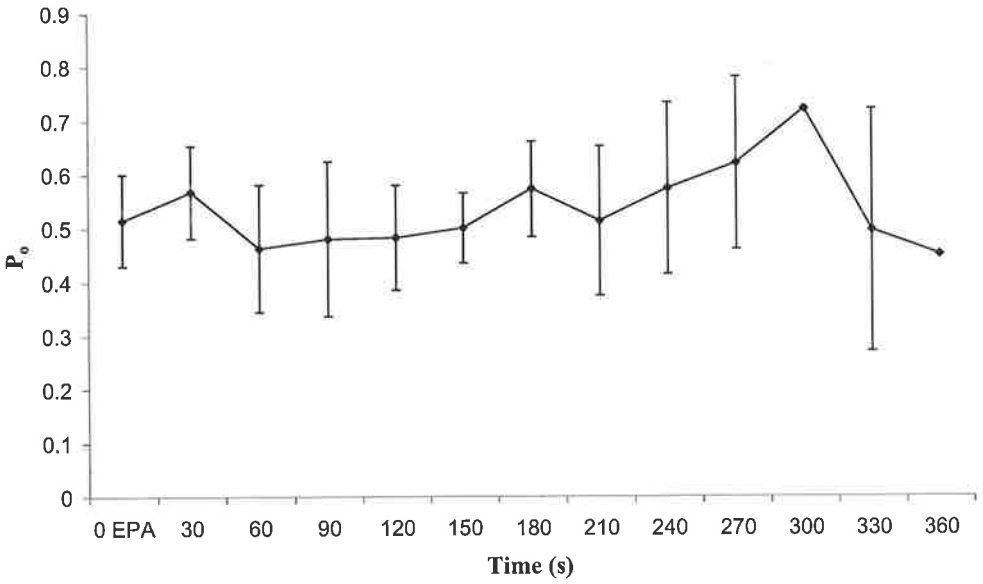


Figure 26: Mean open probability (±SEM) of cardiac RyR sampled at 30 second intervals before and after addition of 30 μM of EPA applied to the cytosolic face of the RyR (n=4).

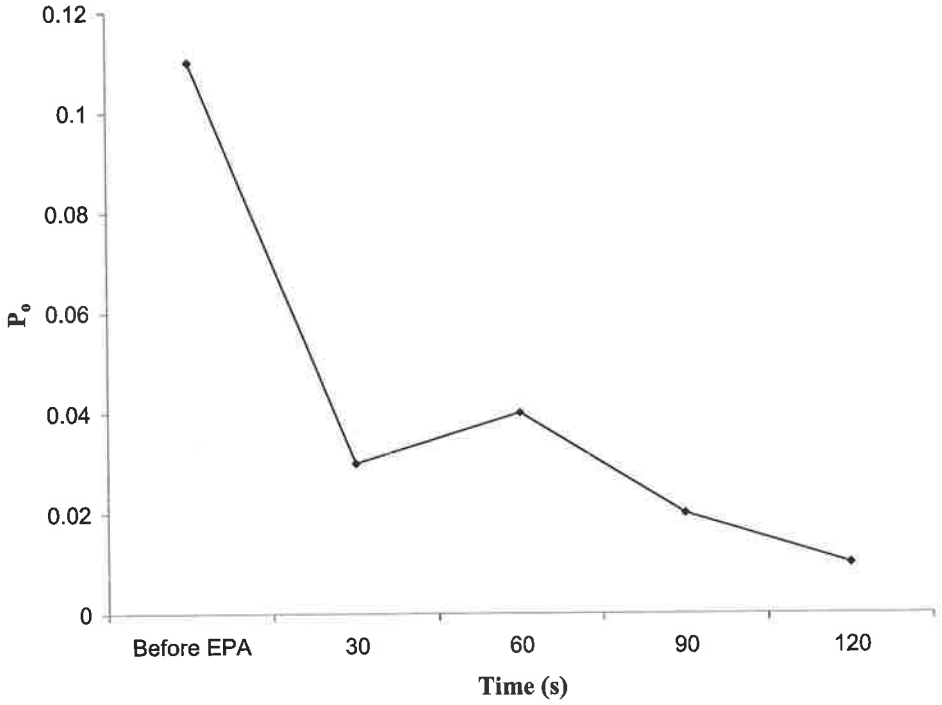


Figure 27: Open probability of a single ryanodine receptor sampled at 30 second intervals in the presence of 50 μM EPA applied to the cytosolic face of the channel.

Phospholipids PE and PC (50:50) were dissolved in an N-tetradecane solvent to determine the influence of the bilayer solvent on the effects of EPA on RyR P_o. The results of this study show that the addition of 30, 40 and 50 μM EPA to the cytosolic face of the RyR result in a significant reduction in P_o compared to the control in the presence of cytosolic ATP (2 mM) and $CaCl_2$ (0.1 μM) ($P<0.05$). The addition of EPA reduced RyR P_o from 0.69 ± 0.08 in the controls to 0.33 ± 0.05 in 30 μM EPA, 0.26 ± 0.14 in 40 μM EPA and 0.04 ± 0.02 in 50 μM cytosolic EPA. This data is summarized in figure 28.

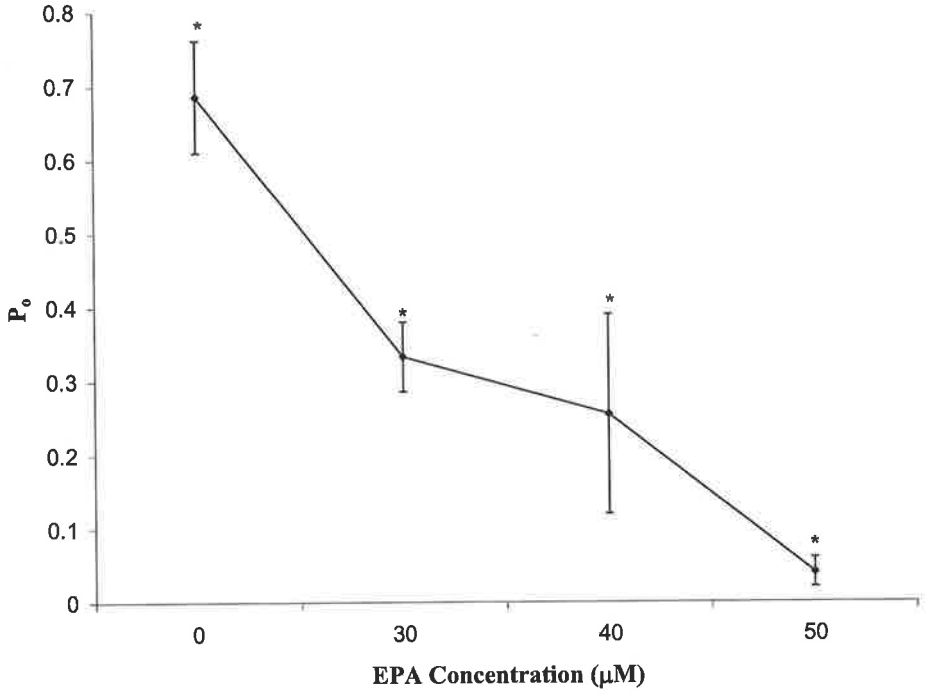


Figure 28: Mean open probability (± SEM) of cardiac ryanodine channels in a 50:50 PE: PC in N-tetradecane bilayer in the presence of 0, 30, 40 and 50 μM EPA when applied to the cytosolic face of the channel (n=2). * signifies significance ($P<0.05$) from control values obtained in 0 μM EPA.

3.4.2 The Effects of Luminal Addition of EPA on the Cardiac Ryanodine Receptor

The results of this study show that the luminal application of 50 μM EPA reduces the P_o of the cardiac RyR in the presence of cytosolic ATP (2 mM) and $CaCl_2$ (0.1 μM). Upon the addition of luminal EPA, the RyR P_o was reduced from 0.32 ± 0.07 to 0.15 ± 0.04 ($P<0.05$) as illustrated in figure 29. These results were obtained from RyR fused into bilayers containing 50:50 PE:PC dissolved in N-decane. As with the application of 50 μM EPA to the cytosolic face of the channel, the luminal application of 50 μM EPA significantly reduced the P_o of the RyR within 30 seconds of EPA exposure ($P<0.05$) as shown in figure 30. As previously shown with the cytosolic addition of EPA, the luminal addition of 50 μM EPA resulted in changes in the ryanodine receptor T_o and T_c. The addition of 50 μM EPA reduced T_o from 9.04 ± 5.44 ms to 4.34 ± 0.94 ms (n=4) and increased T_c from 29.43 ± 12.89 ms to 36.71 ± 8.68 ms (n=4). However, due to the low sample number, these changes were not found to be significant.

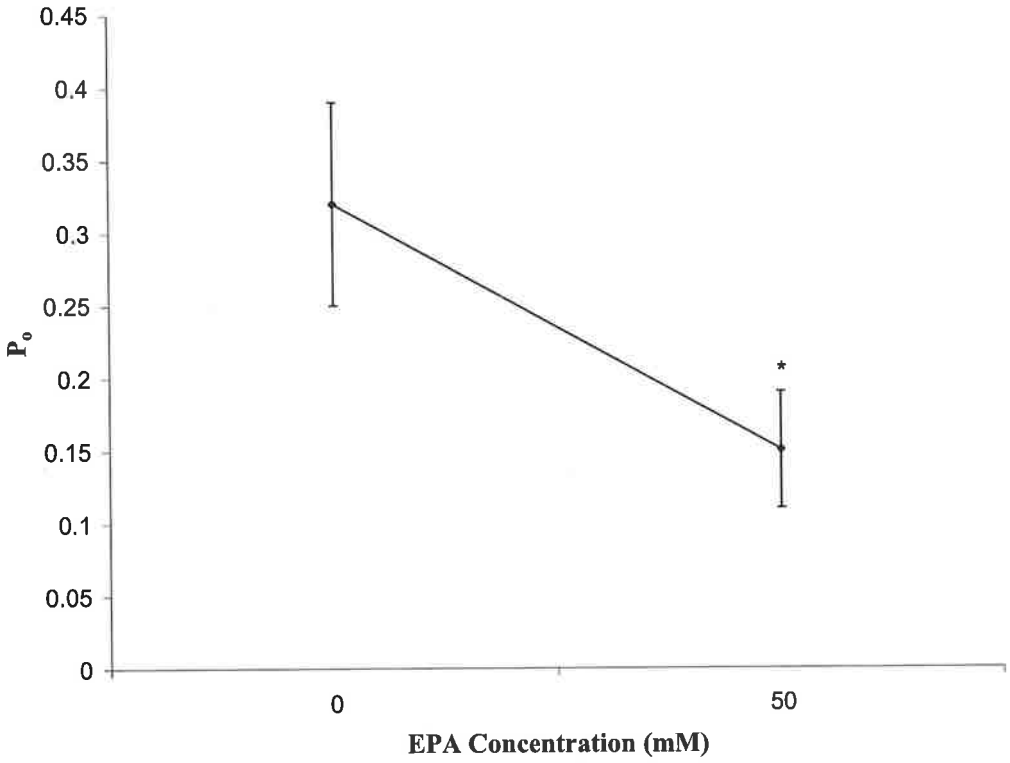


Figure 29: Mean open probability (± SEM) of RyR in a 50:50 PE: PC in N-Decane bilayer before and after the application of 50 μM EPA to the luminal face of the cardiac RyR (n=8). * signifies $P<0.05$ vs. before EPA.

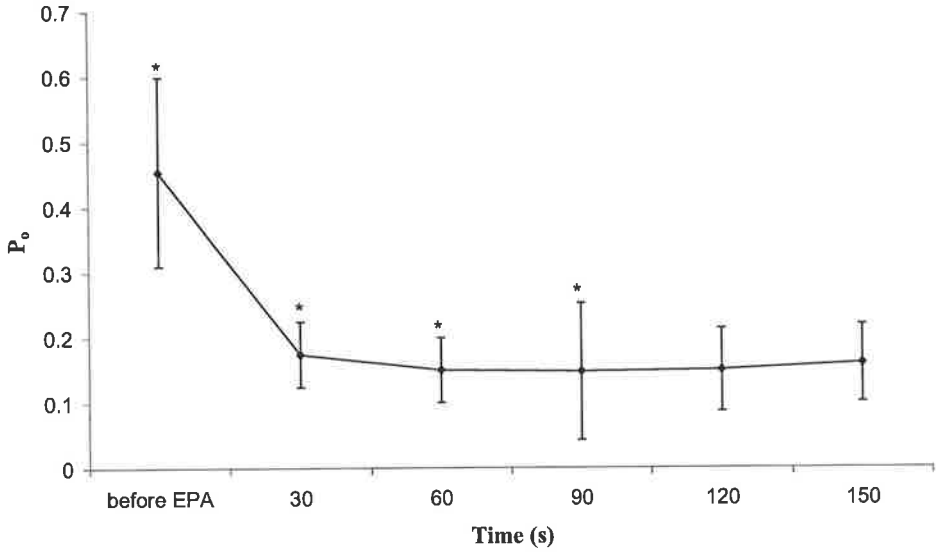


Figure 30: Mean (±SEM) open probability sampled at 30 second intervals in the presence of 50 μM EPA added to the luminal side of the channel (0 and 30 s n=6, 60 and 90 s n=5, 120 and 150 s n=3). * signifies $P<0.05$ vs. before EPA.

3.4.4 The Effect of EPA on Ryanodine Receptor Sensitivity to Cytosolic Calcium

The reduction in cytosolic calcium concentration resulted in a concentration dependent decrease in the P_o of the cardiac RyR in both the absence and presence of EPA. Data was normalized to the P_o of the RyR collected in 0.1 mM $CaCl_2$ between each test concentration. This removes the blocking effect of EPA on the RyR and allows the observation of the changes in magnitude of RyR P_o in response to various cytosolic calcium concentrations and the effect of EPA on this response.

The RyR P_o was reduced from what was considered to be 100% activated in 0.1 mM Ca^{2+} (or normalized $P_{o,n}$=1) to 0.64 ± 0.11 in 10 μM Ca^{2+} (n=7), 0.71 ± 0.13 in 5 μM Ca^{2+} (n=8), 0.35 ± 0.12 in 1 μM Ca^{2+} (n=7) and 0.24 ± 0.16 in 0.1 μM Ca^{2+} (n=3). A similar decrease in RyR P_o was observed in the presence of 50 μM EPA on reducing cytosolic calcium concentration. The RyR P_o was found to be reduced from again what was considered to be full activation in 0.1 mM Ca^{2+} ($P_{o,n}$=1), to 0.89 ± 0.11 in 10 μM Ca^{2+} (n=13), 0.53 ± 0.13 in 5 μM Ca^{2+} (n=11), 0.54 ± 0.13 in 1 μM Ca^{2+} (n=10) and 0.64 ± 0.15 in 0.1 μM Ca^{2+} (n=9). This data is summarized in figure 31. The magnitude of change in P_o of the cardiac RyR was not altered in the presence of 50 μM cytosolic EPA indicating that EPA does not alter the cytosolic calcium sensitivity of the RyR ($P>0.05$).

Following the addition of 50 μM EPA to the cytosolic face of the RyR, the P_o fell over time in the presence of 0.1 mM $CaCl_2$. The addition of 50 μM EPA was found to decrease the P_o from 0.50 ± 0.10 (n=7) to 0.32 ± 0.17 within the first 2 minutes of exposure (n=5), to 0.21 ± 0.08 after 3–5 minutes of exposure (n=6, $P<0.05$ vs. t=0), to

0.27 ± 0.08 after 6-10 minutes of exposure (n=6) and 0.09 ± 0.07 within 11-18 minutes of exposure (P<0.05 vs. t=0, n=7). This is illustrated in figure 32.

This reduction in P_o is unlikely to be due to voltage dependent inactivation of the RyR. This generally occurs at +40 mV where as in the present study currents were recorded at –40 mV. The voltage was routinely switched between + or – 40 mV to further ensure voltage dependent inactivation did not occur.

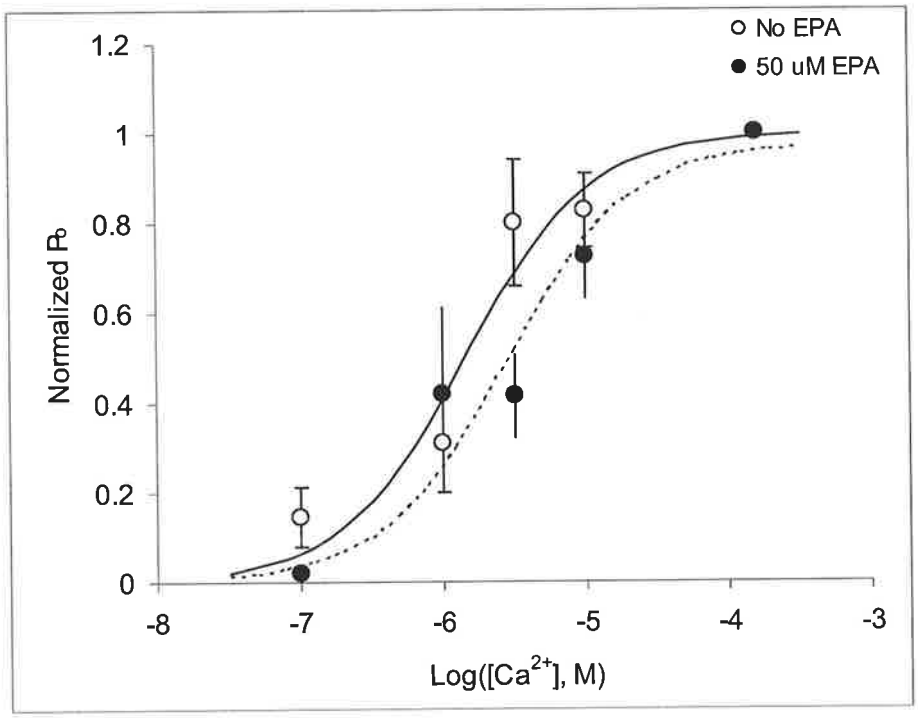


Figure 31: Calcium dependence of open probability (± SEM, n=5-9) for cardiac RyRs in the absence of ATP. Data was normalized to control data at 150 μM Ca^{2+} collected between each [Ca^{2+}] tested. P_o of control in the absence of EPA was 0.54 ± 0.05 (n=28) and with 50 μM EPA was 0.36 ± 0.06 (n=23). The curves show Hill fits to the data with n_a=1 and where K_a= 1.5 ± 1.0 μM, (solid curve) and K_a= 2.8 ± 1.6 μM, (dashed curve).

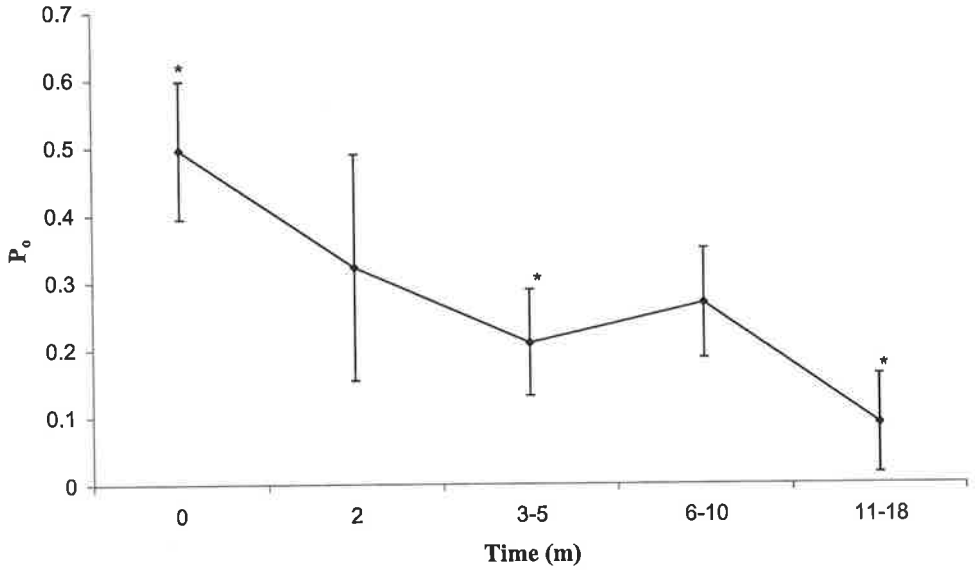


Figure 32: Mean open probability (± SEM) of cardiac RyRs recorded in 0.1μM cytosolic $CaCl_2$ following the addition of 50 μM cytosolic EPA over an 18-minute time interval (n=7). * signifies $P<0.05$ vs. before EPA at T=0.

4. DISCUSSION

4.1.1 The Effect of Dietary Polyunsaturated Fatty Acids on Calcium Sparks in Rat Atrial Myocytes

As previously shown in ventricular myocytes, dietary supplementation of rats with 10% fish oil resulted in an increase in the phospholipid concentration of n-3 fatty acids in rat atrial myocytes (Charnock *et. al.* 1985, McLennan *et. al.* 1990 and Charnock *et al.* 1992) (see Table 1). The increase in membrane n-3 fatty acid content correlates with a reduction in calcium spark duration, which was observed in atrial myocytes from rats supplemented with fish oil in comparison to those supplemented with lard or canola oil (see figure 8). The mechanism by which fish oil may be imposing its effects and subsequently reducing spark duration is not clear. However, possible mechanisms by which dietary fish oil is reducing spark duration may be a reduced calcium flux from the SR or an increased efficiency of the calcium removal and re-uptake mechanisms. These include the RyR, the SR Ca^{2+}ATPase, the sarcolemmal Na^{+}-Ca^{2+} exchanger and the sarcolemmal Ca^{2+}ATPase (Cheng *et al.* 1993, Bers *et al.* 1998).

The reduction in magnitude of the calcium sparks in myocytes from fish oil supplemented rats could be due to a reduction in the SR calcium concentration. Although this was not measured directly in the present study, it is recognized that the amplitude and frequency of spontaneous calcium sparks correlates with the SR calcium concentration (Lukyanenko *et. al.*1996, Satoh *et al.* 1997 and Santana *et al.*

1997). The results presented in this study have shown both the intensity and frequency of the calcium sparks to be similar across the three dietary groups (figure 9) indicating that the SR calcium concentration is unchanged following the dietary manipulation with lard, canola oil or fish oil.

Another factor implicated in the magnitude of flux from the SR contributing to the calcium spark is the properties of the RyR gating. The calcium flux from the SR responsible for a single spark is greater than that of a single RyR and it has been reported that the initiation of a calcium spark is due to simultaneous opening of a number of RyR (Cheng *et al.* 1993, Bers *et al.* 1998). This indicates that the number of RyR responsible for a calcium spark is an important determinant in the magnitude and duration of the spark.

Whether dietary fish oil reduces the width and duration of the calcium spark by altering the properties or recruitment of the RyR can not be deduced from this study. However, this study has shown that the profiles of all calcium sparks do not share the same morphology and that the proportion of sparks displaying prolonged decay profiles is reduced in myocytes from fish oil-supplemented animals in comparison to those from rats supplemented with lard or canola oil (figure 10). This could imply that dietary fish oil alters the gating and the local recruitment of the RyRs. Prolonged spark decay could arise from a greater opening duration of the RyRs responsible for the calcium spark (Cheng *et al.* 1993) or potentially by the reactivation of RyRs throughout the decay of the spark.

It has been reported in isolated RyRs that multiple RyRs when coupled exhibit simultaneous gating effectively behaving as a single unit. This has been termed as "coupled gating". The protein identified to be responsible for this coupled gating is the FK506 binding protein (FKBP12.6). The removal of this protein functionally uncouples the RyRs allowing only single channel behavior (Marx *et al.* 2001). Previously it has been shown in FKBP12.6 knock out mice that the calcium sparks are larger in amplitude and duration (Xin *et al.* 2002). It is therefore possible to consider that a spark exhibiting a fast transient up-rise and a fast exponential like decay (type 1 spark, figure 10) to be the result of the simultaneous opening and closing of the cluster of RyR, similar to "coupled gating". Calcium sparks exhibiting a plateau during the decay phase (type 2, figure 10) of their profile may be due to RyRs being functionally uncoupled and behaving as single channels or a combination of functionally coupled and uncoupled RyR. In the absence of FKBP12, RyR channels exhibit increased gating frequency (Gaburjakova *et al.* 2001), this could potentially give rise to sparks with prolonged up rise or decay profiles. Whether the presence of a greater proportion of n-3 PUFAs in the SR membrane phospholipids affects this coupling and uncoupling process can not be determined by this study but could provide a possible direction for further investigation.

In summary this study has shown that dietary fish oil reduces the duration widthof calcium sparks in rat atrial myocytes compared to dietary lard and canola oil. The proportion of spark exhibiting prolonged decay profiles was also reduced in myocytes from fish oil-supplemented rats compared to lard and canola oil-supplemented rats. This indicates that dietary fish oil may alter the gating of the RyRs.

The present study does not indicate whether or not the calcium re-uptake mechanisms are implicated in the reduction in spark size in myocytes from fish oil supplemented rats and this possibly is the subject of further investigation.

4.1.2 The Effect of Dietary Polyunsaturated Fatty Acids on Calcium Sparks in Rat Ventricular Myocytes

In accordance with previous studies, the dietary supplementation of rats with 10% fish oil resulted in an increase in the percentage of n-3 fatty acids in the membrane phospholipids of ventricular myocytes (Charnock *et. al.* 1985, McLennan *et. al.* 1990 and Charnock *et al.* 1992) (see Table 2). In atrial myocytes, dietary fish oil was found to significantly reduce the width and duration of spontaneous calcium sparks in rat ventricular myocytes compared to those collected from myocytes of rats supplemented with lard (see figure 11).

In contrast to the previous study on atrial myocytes, the present study revealed that dietary supplementation of fish oil results in a reduction of calcium spark intensity in ventricular myocytes compared those collected in myocytes from rats supplemented with lard (see figure 12). As discussed in section 4.1.1, an indicator of SR calcium concentration is the amplitude of the calcium sparks (Satoh *et al.* 1997). As calcium spark intensity in this study was reduced by dietary fish oil, it is conceivable that the SR calcium concentration may be reduced in these cells. However, a study by Leifert *et al.* (2001) found no difference in the SR calcium concentration between myocytes from rats supplemented with lard compared to myocytes from rats supplemented fish oil. SR calcium concentration was determined by the rapid application of caffeine to empty the store, with the resultant increase in cytosolic calcium concentration measured using fluorescence microscopy and the calcium indicator Fura-2 (see Appendix D for more information on Fura-2). This study was performed in ventricular myocytes from the same animals as the present study, thereby eliminating

the implication of SR calcium concentration as a possible mechanism for the reduction in width and intensity of calcium sparks due to dietary fish oil.

The reduction in spark intensity due to dietary fish oil is unlikely to result from a decrease in triggering calcium activating the RyR, as the sparks observed in this study arise spontaneously. Spontaneous sparks do not require activation of the CICR cascade and can occur in the absence of external calcium (Satoh 1997). As a reduction in calcium spark intensity was observed with no subsequent change found in SR calcium concentration by Leifert *et al.* (2001), it is plausible that RyR gating is altered by dietary fish oil. To test this notion, the proportion of spark exhibiting prolonged decay profiles was reduced in the fish oil supplemented group in comparison to the lard supplemented group (figure 13) indicating the possible manipulation of the stochastic nature of the RyR by dietary fish oil intervention (see section 4.1.1).

Enhanced activity of the SR Ca^{2+}ATPase by dietary fish oil may pose a possible mechanism for the observed reduction in spark width and duration seen in myocytes from fish oil supplemented rats. It has previously been reported that calcium spark decay is in part due to the re-uptake of calcium by the SR and that SR calcium re-uptake contributes significantly to the duration and width of calcium sparks (Gomez *et al.* 1996). This was determined by the change in spark properties before and after the block of the SR Ca^{2+}ATPase by thapsigargin. The application of TBQ in the present study did not reveal the same increase in duration or width of the calcium sparks as shown by Gomez *et al.* (1996).

The application of TBQ to cells from rats supplemented with fish oil resulted in no change to width, duration or intensity of the sparks where as in the lard supplemented group, the application of TBQ reduced width and intensity (figure 11 & 12).
The reduction in spark properties observed in the lard supplemented group on the application of TBQ is likely to be due to the progressive reduction SR calcium concentration due to leak over time. The decrease in SR calcium concentration subsequently leads to smaller less intense sparks (Satoh *et al.* 1997). Why this was not observed in the fish oil supplemented group is unknown but could potentially be due to a reduction in the magnitude of calcium leak from the SR as previously suggested by the reduction in both the duration and width of the sparks under control conditions.

The contradictory findings of this study compared to those of Gomez *et al.* (1996) could have arisen from the conditions under which the calcium sparks were collected. Gomez *et al.* (1996) collected their sparks in the absence and presence of SR Ca^{2+}ATPase blockade in a sodium and calcium free solution to abolish the calcium efflux by the Na^{+}-Ca^{2+} exchanger. The prevention of cytosolic calcium extrusion whilst SR calcium re-uptake is inhibited will result in an increase in cytosolic calcium and saturate the buffering capacity of the cell. A reduction in the buffering capacity and the inhibition of the Na^{+}-Ca^{2+} exchanger of the cell may contribute to the larger sparks observed by Gomez *et al.* (1996) as all decay mechanisms are compromised. In the present study, only SR calcium re-uptake was inhibited. Therefore, calcium could be extruded from the cell via the Na^{+}-Ca^{2+} exchanger. As calcium in the present study did not accumulate in the cytosol, it can be expected the buffering capacity of the cell is near normal. Hence, the sparks decay in a similar manner to those collected under

control conditions. As the blocking of SR Ca^{2+}ATPase does not alter calcium spark decay, it is concluded that the primary mechanism for spark decay is diffusion and buffering within the cytosol.

As the inhibition of SR calcium re-uptake does not alter the duration or width of the calcium sparks in myocytes from fish oil-supplemented rats, it can be concluded that SR calcium re-uptake is an unlikely mechanism by which dietary fish oil is reducing the properties of the calcium sparks in comparison to lard supplementation. Possible mechanisms by which dietary fish oil may be imposing its effects on calcium sparks include inhibition of the RyR, a reduction in the local recruitment of RyRs contributing to the spark, or a reduction in RyR sensitivity to triggering calcium.

4.1.3 The Effects of Free Fatty Acids on Calcium Sparks

The main constituents that have been implicated in the antiarrhythmic actions of fish oil are EPA and DHA. To determine whether the reduction in spark magnitude due to the dietary intervention with fish oil as observed in the previous two studies is due to these components of fish oil, ventricular myocytes were incubated with either EPA, DHA, ALA (n-3) or in a control medium.

The findings of this study show that the incubation of myocytes with EPA results in a decrease in calcium spark width and duration compared to the controls. No change in spark width or duration was seen in DHA or ALA-treated cells compared to the controls (figure 14). A reduction in calcium spark intensity was observed in the EPA, DHA and ALA treated myocytes in comparison to the controls (figure 15). This finding indicates that these fatty acids may reduce the flux of calcium from the SR via the RyRs although this would not explain why there was no associated reduction in spark duration or width in the ALA or DHA treated groups. A possible explanation could be that when calcium is released from the SR, K^+ enters the SR via SR K^+ channels to maintain electroneutrality (Rodrigo *et al.* 1999). If the SR K^+ current is reduced then less calcium can be released from the SR potentially contributing to the reduction in calcium spark intensity. It is not unreasonable to consider that n-3 PUFAs could reduce the SR K^+ current in a similar manner they reduce the sarcolemmal K^+ currents (Bogdanov *et al.* 1998). This could provide an avenue for further investigation.

The application of free PUFAs has been previously been identified to increase membrane fluidity (Jahangiri *et al.* 2000). Increased membrane fluidity has been demonstrated to reduce or block various sarcolemmal ion currents. It is plausible that similar changes in membrane fluidity may occur at the SR level. The incubation of ventricular myocytes with EPA, DHA or ALA would be expected to modify the fluidity of the phospholipid membrane. If increased membrane fluidity was responsible for the reduction in the width and duration of the calcium sparks collected from ventricular myocytes incubated with EPA, then a similar decrease in the properties of the sparks would be expected with DHA and ALA treatment as these also increase membrane fluidity.

It was expected that all three fatty acid treatments would result in alteration to the calcium sparks in comparison to the control. Why this did not occur is uncertain. Previously, it has been shown that the application of EPA reduces the magnitude of the L-type Ca^{2+} current (Xiao *et al.* 1997, Rodrigo *et al.* 1999), whereas the magnitude of the current was not effected by DHA (Pepe *et al.* 1994). The differences in the actions of EPA and DHA on the L-type Ca^{2+} highlight the possibility that the pharmacological actions of EPA, DHA and possibly ALA may differ in other aspects of calcium handling. This could provide a possible explanation as to why EPA reduces the width and duration of the spontaneous sparks whereas both DHA and ALA did not. Alternatively, during the incubation period the fatty acids may have been metabolized. A metabolite of EPA that is not common to either DHA or ALA may be responsible for the reduction in width and duration of the calcium sparks. However, this is only speculation.

It has been previously demonstrated that the 'flip-flop' of fatty acids across phospholipid membranes results in a concomitant decrease in internal pH (Kamp and Hamilton 1992). A reduction in cytosolic pH has been implicated in decreasing spontaneous spark frequency and causing a slight prolongation in spark duration without affecting the amplitude (Balnave and Vaughan-Jones 2000). In the present study, prolongation of the calcium sparks was not observed. DHA and ALA were found not to alter spark duration, whereas EPA was found to reduce spark duration. The lack of similarity in the results of this study compared to those of Balnave and Vaughan-Jones (2000) implies that pH was not a factor contributing to the decrease in width, duration and intensity of calcium sparks following EPA treatment.

It has been reported that acute application of 15 μM EPA reduced the frequency of calcium sparks evoked by the initiation of the L-type Ca^{2+} current, with no alteration to their duration or width (Xiao *et al.* 1997). The findings of Xiao *et al.* (1997) are contradictory to the observations made in the present study. A possibility for this discrepancy could lie in that calcium sparks were evoked by the initiation of an L-type Ca^{2+} current in the study by Xiao *et al.* (1997), whereas the present study evaluated the effects of EPA in spontaneous sparks. Although it has previously been recognized that evoked and spontaneous calcium sparks share a similar width and time course (Cannell *et al.* 1995), their initiation differs. Evoked sparks are a result of the activation of CICR whereas it has been reported that spontaneous sparks do not require triggering calcium for their initiation, and factors such as SR calcium concentration determine their frequency and amplitude (Satoh *et al.* 1997). Xiao *et al.* (1997) discovered an associated reduction in the magnitude of the L-type Ca^{2+} current upon the application of EPA (likely due to membrane fluidity changes). They

concluded this was responsible for the observed reduction in spark frequency. As spontaneous sparks do not rely on the initiation of the L-type Ca^{2+} current, the actions of EPA are likely to differ in the present study.

An alternative explanation for this discrepancy is different durations of exposure of the myocytes to EPA in this study and the study of Xiao *et al.* (1997). Rodrigo *et al.* (1999) found that the contraction strength of guinea pig ventricular myocytes declined in two phases in response to the application of EPA. The first phase of action occurred after 60 seconds of exposure and was reversed by 0.2% BSA washout. It was concluded that this phase was due to the actions of EPA on the sarcolemmal membrane. With continued exposure they found slight recovery, followed by a greater reduction in contraction strength, which was attributed to EPA entering the cell. In the present study it is likely that the effect of prolonged EPA exposure was observed where as Xiao *et al.* (1997) observed the initial effects of EPA.

In order to determine the whether the reduction in the width, duration and intensity of the calcium sparks resulting from EPA incubation was due to manipulation of SR Ca^{2+}ATPase activity, the SR Ca^{2+}ATPase was blocked with thapsigargin. SR Ca^{2+}ATPase blockade did not alter spark properties in the controls or the EPA, DHA or ALA-treated groups (table3, figure 15). It can, therefore, be concluded that the reduction in spark properties due to EPA treatment was not due to the manipulation of the SR Ca^{2+}ATPase activity by EPA and is more likely due to modulation of the RyR.

In summary these studies show that the introduction of 10% fish oil to the diet of rats results in a reduction in calcium spark duration in atrial myocytes and a reduction in

calcium spark width and duration in ventricular myocytes. This reduction in spark size correlates with an increase in membrane n-3 polyunsaturated fatty acid content in comparison to the membrane composition of myocytes from lard supplemented rats. The observed reduction in spark width and duration was not found to result from modified SR Ca^{2+}ATPase activity. The differences in the distribution of spark profiles between the dietary groups suggest that the gating of the RyR may be altered by dietary fish oil. Dietary fish oil may be altering RyR sensitivity to cytosolic calcium, manipulating the channel gating properties via the membrane, or by direct action on the channel (see section 4.1.1 for more detail). The reduction in the width and duration of the calcium sparks obtained in myocytes from fish oil supplemented rats is not due to increased membrane fluidity, as dietary PUFAs have been previously found to not alter membrane fluidity (Leifert *et al.* 2001). The latter study shows that the application of EPA reduces spark properties in a similar manner to those seen in the fish oil-supplemented animals. The reduction in the magnitude of spontaneous calcium release by EPA but not DHA or ALA may indicate that EPA may be the constituent of fish oil providing protection against calcium-dependent arrhythmias at the SR level.

The overall reduction in the magnitude of the calcium sparks in response to dietary and free PUFAs may contribute to the lower resting cytosolic calcium concentration observed in studies by Negretti *et al.* (2000), Leifert *et al.* (2001) and O'Neill *et al.* (2002). A lower resting cytosolic calcium concentration may reduce the activation of the Na^{+}-Ca^{2+} exchanger thereby reducing the inward flux of Na^{+}. This could potentially reduce premature depolarization of the myocytes and therefore protect against arrhythmic activity.

4.2.1 The Effects of Dietary Fish Oil on Calcium Transients in Rat Ventricular Myocytes

Results of the present study show that a 10% fish oil supplement to the diets of rats reduces the doubling time (figure 17) of the calcium transient without altering the intensity (figure 18) or the half-life of decay in ventricular myocytes. The reduction in the doubling time of the transient in the fish oil supplemented group correlates with an increased proportion of membrane n-3 fatty.

A calcium transient is initiated when calcium enters the cell upon the activation of the L-type calcium current during the cardiac action potential. This rise in intracellular calcium binds to the RyR, which initiates SR calcium release (section 1.2.1). Factors that can influence the transient include; the L-type calcium current, the sensitivity of the RyR to cytosolic calcium and SR calcium concentration.

The correlation between the increase in the proportion of n-3 fatty acids in the lipid membrane and the decreased doubling time of the transient indicates that the n-3 fatty acids may be altering the properties of the SR membrane. This could result in altered gating of the RyR giving rise to more rapidly rising transients. Properties of the RyR that may potentially result in a more rapidly activating transient include an increased RyR sensitivity to triggering calcium, this can occur particularly during instances of elevated SR calcium concentration (Xu and Meissner 1998). An increase in the conductance of the RyRs or a greater proportion of RyRs opening simultaneously ("coupled gating" discussed in section 4.1.1) can also result in a faster activating transient or a larger triggering L-type calcium current. Which, if any of these

possibilities are responsible for the faster transient up rise can not be determined by this study.

Previously it has been reported that the amplitude of the calcium transient is graded according to the amplitude of the L-type calcium current (Cheng *et al.* 1996, Xiao *et al.* 1997, Lukyanenko *et al.* 1999). The results of the present study did not reveal any differences in the amplitude of the calcium transient between the two dietary groups (figure 18). If the amplitude of the L- type calcium current was significantly altered in this study, it would be expected that similar changes in the amplitude of the transient as observed by (Cheng *et al.* 1996, Xiao *et al.* 1997, Lukyanenko *et al.* 1999). As no variation in the amplitude of the transients was observed, it is possible that the reduction in doubling time of the transients in the fish oil-supplemented group was not due to alterations in the calcium current. Previous findings have shown that dietary fish oil does not alter the sodium current (Leifert *et al.* 2000). It is therefore possible that dietary fish oil does not alter the L-type calcium current either.

A factor that could contribute to the reduction in the doubling time of the calcium transient in the fish oil-supplemented group is the coupling of the ryanodine receptors and their sensitivity to triggering calcium. If the receptors are functionally coupled (see section 4.1.1 for a full description) (ie, FKBP12 is present) the RyR behave as clusters rather than individual channels. When functionally coupled the channels are regulated together such that if they are activated with calcium, they will all open giving rise to a faster transient. If the RyR are functionally uncoupled then each channel must be activated separately, prolonging the rise of the transient (Marx *et al.* 2001). RyR sensitivity to cytosolic calcium can also alter the doubling time of the

transient. One mechanism which modifies RyR calcium sensitivity is the SR calcium concentration. RyR have a binding site on the lumenal side of channel, that detects calcium. Under conditions of high SR calcium concentration, cytosolic binding site calcium sensitivity is increased.

In the present study the transient half-life of decay was not found to differ between the two dietary groups (figure 17). Previously Leifert *et al.* (2001) investigated the amplitude and time course of decay of calcium transients at stimulation frequencies ranging between 0.2 and 2.0 Hz. They found no difference in the amplitude of the transients in this frequency range although the time constant for decay was found to increase in transients stimulated at 0.2 Hz in the fish oil-supplemented group. Leifert *et al.* (2001) did not observe a significant difference in the decay time constant or amplitude in transients when stimulated at 1 Hz, which is consistent with the findings of this study. The transients recorded by Leifert *et al.* (2001) exhibited a faster decay than the transients observed in this study. The most likely reason for this is that this study was performed at room temperature (22-25 °C) in comparison to 37 °C in the study of Leifert *et al.* (2001). Calcium re-uptake by the SR is temperature dependent (Bers *et al.* 1989, Engel *et al.* 1995) and is greater at 37 °C than at room temperature. From this it is expected that the rate of decay of the transients would be reduced in this experiment compared to those of Leifert *et al.* (2001).

In conclusion, this study shows that the dietary manipulation of rats with 10% fish oil results in a decrease in the doubling time of the calcium transient without affecting either the intensity or the half-life of decay. The decrease in the doubling time could

be due to modification of the physical properties of the phospholipid membrane manipulating RyR properties.

4.2.2 The Effects of Acute EPA Addition on Calcium Transients in Rat Ventricular Myocytes

The present study shows that incubation of ventricular myocytes with EPA significantly decreases the doubling time of the calcium transient (figure 19) without affecting the half-life of decay or the amplitude (figure 20) when compared to the controls. The mechanisms by which EPA may be imposing its effects on the calcium transient are unknown. However, modifications of membrane properties altering the gating of the RyRs or direct actions on RyRs are likely.

As previously mentioned the amplitude of the calcium transient is graded according to the amplitude of the L-type calcium current (Cheng *et al.* 1996, Xiao *et al.* 1997, Lukyanenko *et al.* 1999). It is unlikely that calcium influx across the sarcolemmal membrane has been altered, as there was no associated change in transient amplitude. It has previously been reported that the rapid application of 15 μM EPA results in a significant reduction in the amplitude of the calcium transient (Xiao *et al.* 1997). Xiao *et al.* (1997) attributed the reduction in transient amplitude by EPA to the blocking of the L-type Ca^{2+} current. In the present study, the cells were incubated with EPA for two hours prior to use and the solution containing EPA was removed prior to the collection of transients whereas Xiao *et al.* (1997) rapidly applied EPA and collected transients in its presence. It has been previously been shown by Rodrigo *et al.* (1999) that the effects of EPA on isolated myocytes differ throughout the course of exposure

where they have identified 3 different phases of action. It is therefore likely that the results of this study and those of Xiao *et al.* (1997) are looking at different phases of EPA action. Another consideration is that during the incubation process, EPA is likely to be metabolized within the cell where as with rapid short-term exposure, EPA is more likely to have its effects in its free non-esterified form on the surface membrane (this is previously discussed in section 4.1.3).

From this study it can be concluded that EPA increases the rate of rise of the calcium transient without affecting the amplitude or rate of decay in ventricular myocytes. The mechanisms by which EPA is acting are unknown. However, its actions could be due to modification of the RyR lipid environment thereby modifying its gating properties.

In summary, the present studies show that both dietary fish oil and EPA result in a similar decrease in the doubling time of the calcium transient with no associated change in amplitude or half-life of decay. As both studies exhibit similar findings, it is likely that the incubation of cardiac myocytes with EPA result in similar metabolism, as do the PUFAs introduced through the diet. This may be indicative of similar actions by the two methods of introduction of PUFAs. How both dietary PUFAs and EPA are decreasing the doubling time of the transient is unknown. However they may modify the properties of the lipid membrane altering calcium release from the SR.

4.3 The Effect of Dietary Polyunsaturated Fatty Acids on Calcium Waves in Rat Ventricular Myocytes

Previous studies have shown that the acute application of EPA to ventricular myocytes reduces the frequency and increases both the amplitude and propagation velocity of calcium waves (Negretti *et al.* 2000, O'Neill *et al.* 2002). The results of the present study have revealed that the incorporation of PUFAs following dietary manipulation with fish oil resulted in no differences in the propagation velocity, intensity (figure 22), or frequency (figure 23 panel A) of calcium waves in rat ventricular myocytes compared to those from lard-supplemented animals.

It was expected that the properties of the calcium waves would be altered in response to dietary PUFAs as both dietary and free PUFAs were identified to reduce the properties of calcium sparks. A possible reason for the absence of an effect may be due to the high SR calcium concentration activating the RyR and potentially overriding cytosolic regulation. This effect can only be speculated but could provide an avenue for future investigation. As there was no observed changes in the propagation velocity of the wave it is unlikely that dietary fish oil alters the gain of the CICR cascade. These results indicate that dietary PUFAs do not modulate the mechanisms involved in wave initiation and propagation, as do acute PUFAs.

The above information suggests that dietary PUFAs do not provide protection against arrhythmias due to calcium handling dysfunction. However, it has recently been reported that dietary fish oil reduces the asynchronous contractile behavior of ventricular myocytes induced by β- stimulation with 1 μM isoproterenol (Leifert *et al.*

2001). Spontaneous calcium waves often result in arrhythmic activity due to membrane depolarization and may serve as a possible reason for the asynchronous contractile behavior associated with isoproterenol stimulation. The results of the present study show that 1μM isoproterenol increases the frequency (figure 23 panel A), and reduces the amplitude (figure 22 panel B), of the calcium waves without affecting the propagation velocity (figure 22 panel A) in ventricular myocytes from both the fish oil and lard-supplemented rats. The magnitude of increase in the frequency of waves was found to be less in ventricular myocytes from fish oil-supplemented animals in comparison those from the lard-supplemented animals (figure 23 panel B). The reduction in the magnitude of the response to isoproterenol in cells from the fish oil-supplemented animals could be due to a reduction in the activation of the β-adrenergic pathway, or a reduction in the response of the signaling pathway to β-aderenergic stimulation.

In order to resolve how dietary fish oil may reduce the response to stimulation with isoproterenol it is essential to evaluate the events occurring upon β-stimulation. Upon stimulation of the β-receptors, a cascade of events occurs leading to the conversion of ATP to cAMP by the membrane associated enzyme adenylate cyclase. Manipulation of the membrane with dietary fish oil could potentially change the physical properties of the membrane thereby affecting the activity of membrane bound enzymes or the β-receptor signaling system itself. This could potentially result in the reduction of cAMP synthesis down regulating the response to β-stimulation in fish oil-supplemented animals. Following the activation of cAMP, protein kinase A phosphorylation of the L-type Ca^{2+} channels, the ryanodine receptors and

phosphorylation of phospholamban by calcium-calmodulin dependent protein kinase occurs (Viatchenko-Karpinski and Gyorke 2001).

The phosphorylation of the L-type Ca^{2+} channels enhances the magnitude of the calcium current which has been implicated in increasing the amplitude of calcium transients on the application of isoproterenol (Leifert *et al.* 2001, Viatchenko-Karpinski and Gyorke 2001). A reduction in the phosphorylation of the L-type Ca^{2+} channels is an unlikely mechanism by which dietary fish oil may reduce the effects of isoproterenol on calcium waves. Calcium waves do not require activation of the L-type Ca^{2+} current for their initiation and propagation (Cheng *et al.* 1993, Cheng *et al.* 1996, Lukyanenko *et al.* 1999).

In the present study it was observed that the amplitude of the calcium waves was reduced in the presence of isoproterenol in myocytes from both lard and fish oil-supplemented rats, although it was not found to differ between the two dietary groups. Upon β-stimulation, the phosphorylation of phospholamban (a regulatory protein of the SR Ca^{2+}ATPase) takes place. This results in the removal of the inhibitory effects of phospholamban on the SR Ca^{2+}ATPase. Enhanced activation of the SR Ca^{2+}ATPase provides more efficient calcium sequestration by the SR possibly contributing to the observed reduction in amplitude of calcium waves in this study.

Previously, it has been demonstrated in phospholamban knock out mice that greater SR Ca^{2+}ATPase activity increases the frequency of calcium waves in ventricular myocytes (Huser *et al.* 1998). It is likely that the isoproterenol-induced increase in wave frequency observed in this study is due to enhanced SR Ca^{2+}ATPase function

(or reduced inhibition). As the magnitude of increase in wave frequency was less in the myocytes from the fish oil-supplemented group compared to the lard, it is possible that dietary fish oil is having an effect on the SR Ca^{2+}ATPase. Dietary fish oil may be reducing the effects that B-stimulation has on the SR Ca^{2+}ATPase by reducing the level of activation of the B-cascade, by modulating SR Ca^{2+}ATPase directly or via the surrounding membrane or by reducing the effects of B-stimulation of the SR Ca^{2+}ATPase regulatory (eg. Phospholamban). If enhanced SR Ca^{2+}ATPase activity were solely responsible for the isoproterenol-induced increase in wave frequency differences in SR calcium concentration between the two groups would also exist if the modulation of the SR Ca^{2+}ATPase differs. i It is unlikely that isoproterenol exposure increased SR calcium concentration in this study as a concomitant increase in wave amplitude would be expected and was not observed. As this study demonstrated a reduction in wave amplitude, any increase in SR calcium sequestration is likely to be matched by a similar SR calcium leak.

The reduction in the magnitude of increase in the frequency of calcium waves from the fish oil-supplemented group compared to the lard supplemented group may be a result of alterations in the response of the RyR to isoproterenol. As previously mentioned, activation of the β-adrenergic pathway results in the phosphorylation of RyR by protein kinase A. A greater frequency and a reduction in the width of calcium sparks have been observed in ventricular myocytes from phospholamban knock out mice (Huser *et al.* 1998). In a study by Li *et al.* (2002) it was found that protein kinase A-dependent- phosphorylation of ryanodine receptors in ventricular myocytes from phospholamban knock out mice, did not alter the width of the calcium sparks compared to sparks collected in the absence of protein kinase A. From this the authors

concluded the changes in spark properties are entirely attributable to enhanced SR Ca^{2+}ATPase activity (Li *et al.* 2002). This evidence could indicate that the changes observed in wave properties observed in this study evoked by isoproterenol might also be primarily due to enhanced SR Ca^{2+}ATPase activity. Enhanced SR Ca^{2+}ATPase activity results in an increase in free calcium in the SR, which has been previously implicated in increasing the open probability of the RyR (Lukyanenko *et al.* 1999, Ching *et al.* 2000). Rather than RyR function being enhanced by phosphorylation during β-stimulation, from the above information it is plausible that SR calcium release is closely matched to the rate of SR calcium accumulation by the luminal detection of free calcium by the RyRs. Alterations to the RyR sensitivity to luminal calcium, or RyR inhibition by dietary PUFAs, could throw imbalance to this system, potentially reducing the frequency of the calcium waves although this effect would be expected to be observed under control conditions and wasn't.

In summary, this study shows that dietary fish oil does not alter the frequency, rate of propagation or amplitude of calcium waves in the instance of SR calcium overload compared to dietary lard. The application of isoproterenol was found to increase the frequency and reduce the amplitude of calcium waves in ventricular myocytes from both lard and fish oil-supplemented animals. The magnitude of increase in wave frequency was found to be reduced in fish oil-supplemented animals compared to lard-supplemented animals. Although it cannot be concluded as to how this down regulated response may have arisen, it is possible that modification to the properties of the phospholipid membrane by dietary PUFAs may induce down regulation of the β-receptor stimulation pathway by altering the activity of membrane associated enzymes.

4.4 The Effects of EPA on the Cardiac Ryanodine Receptor

The present study shows that the addition of 50 μM EPA to the cytosolic face of the RyR reduces the open probability (P_o) in the presence of 2 mM ATP and 0.1 μM Ca^{2+}. The application of 10, 20, 30 or 40 μM EPA was not found to significantly reduce P_o of the RyR (figure 25). The failure of lesser concentrations of EPA to affect the RyR P_o was not due to inadequate exposure time. There was no significant decline in P_o on the application of 30 μM EPA over a 6-minute time span (figure 26) whereas 50 μM EPA was found to reduce P_o within the 30 seconds of exposure (figure 27). In bilayers where N-tetradecane was used as the solvent, the P_o of the RyRs approximately halved on the addition of 30 μM EPA (figure 28) whereas this concentration did not significantly alter the P_o of the RyRs in bilayers where N-decane was used as the solvent. The likely reason for this difference is that N-decane easily intercalates between the phospholipids and often resides within the bilayer resulting in bilayer swelling and possibly may induce changes to the bilayer fluidity. N-tetradecane is a larger molecule than N-decane and is extruded to a greater extent than N-decane on the formation of a bilayer, giving rise to a more "relaxed" bilayer. The presence of N-decane in the bilayer may have similar actions to EPA on RyR P_o thus a greater concentration of EPA is required to overcome the effect of the solvent. Bilayers where N-tetradecane was used as the solvent contains less residual solvent hence an effect can be observed lesser concentrations of EPA. Bilayers where N-tetradecane was used as the solvent were difficult to achieve and maintain thus N-decane was used for the remaining experiments discussed.

As the P_o of the RyR on EPA exposure was influenced by the solvent used in the bilayer, it is likely that the effects of EPA are mediated via the membrane. To further support this notion, the application of 50 μM EPA to the luminal face of the RyR resulted in a similar reduction in the P_o of the RyR as the application of 50 μM EPA to the cytosolic face in the presence of cytosolic ATP (2 mM) and Ca^{2+} (0.1 μM) (figure 29). This reduction in P_o occured within 30 s of application, similar to the cytosolic application of EPA of the same concentration (figure 30).

One could postulate that the reduction in P_o in response to both cytosolic and luminal EPA is associated with a decrease in pH due to fatty acid ionization. Previously in both cardiac and skeletal muscle RyR, P_o was found to fall by half when the cytosolic pH was dropped from 7.4-6.5 whereas a similar luminal pH change had no effect on channel P_o (Rousseau and Pinkos 1990, Xu *et al.* 1996, Laver *et al.* 2000). In order to inhibit the skeletal muscle RyRs luminal pH was required to be dropped to 5.5 or below (Laver *et al.* 2000). In the present study, the addition of 50 μM EPA reduced pH of the bath solutions from 7.4 to 7.34 which, in itself, does not significantly inhibit RyRs, thus pH change provides an unlikely modulator of the observed reduction in P_o observed in these experiments.

The more rapid reduction in P_o in the presence of ATP (2 mM) and $CaCl_2$ (0.1 μM) compared to the gradual reduction in P_o observed in the presence of 0.1 mM $CaCl_2$ and absence of ATP on the application of 50 μM EPA indicates that EPA is influencing the ATP activation of the RyR. ATP is a ryanodine receptor agonist in the presence of physiological cytosolic [Ca^{2+}]. ATP has been previously been demonstrated to activate the RyR by binding to a binding site on the RyR (Kermode

et al. 1998). EPA may modulate ATP activation of the RyR by binding directly to the ATP binding site potentially inhibiting the binding of ATP or by indirectly influencing the binding of ATP by manipulating the properties of the bilayer and therefore the ATP binding site. If EPA was to bind directly to the ATP binding site, the rate of EPA induced inhibition of the RyR would be expected to differ depending on which face of the channel EPA was applied. EPA applied to the luminal face would be required to pass through the bilayer to act on ATP binding sites present on the cytosolic face of the channel, this would result in a gradual response to EPA in comparison to cytosolic EPA application. As the time course of the decline in P_o was similar whether EPA was applied to the cytosolic or luminal side, it is probable that EPA modulates the ATP activation of the RyR via the bilayer.

Where 0.1 mM $CaCl_2$ was the only activating ligand, the reduction in RyR P_o observed on the application of 50 μM occurred after 3-5 minutes (figure 32). This reduction in P_o was not found to be due to a reduction in the sensitivity to calcium. The P_o of the RyR increased in response to increasing cytosolic Ca^{2+} in a concentration dependent manner as previously shown by Sitsapesan *et al.* (1995). However the magnitude of the response to the various concentrations was not altered by cytosolic EPA (figure 31). This indicates that EPA does not alter RyR gating by modifying the properties of the cytosolic calcium binding sites.

Previously it has been demonstrated that the application of free EPA increases membrane fluidity, which has been implicated in the blocking of sarcolemmal ion channels (Jahangiri *et al.* 2000, Leifert *et al.* 2001). It is likely that the intercalation of EPA into the bilayer will increase bilayer fluidity also and potentially alter the gating

of the RyR. The relatively slow reduction in RyR P_o is likely to be due to the presence of residual N-decane solvent. As previously discussed, N-decane may already induce bilayer fluidity changes and a relatively high bilayer concentration of EPA could be required to overcome these effects causing a slowed reduction in P_o in response to EPA.

In summary the addition of 50 μM EPA to either the cytosolic or luminal face of the RyR results in a reduction in the channel P_o in the presence of cytosolic ATP. It is likely that EPA reduces RyR P_o by altering the properties of the bilayer and potentially affecting the binding of ATP and the subsequent activation of the channel. The application of EPA was also found to reduce RyR P_o in the presence of 0.1 mM cytosolic $CaCl_2$. This reduction in P_o was not due to a reduction in the sensitivity of the RyR to activating calcium but could be due to changes in bilayer fluidity associated with EPA intercalation amongst the phospholipids.

The reduction in RyR P_o on EPA application observed in these studies may be functionally important in reducing arrhythmic, mishandling of calcium handling that occurs particularly during ischemia.

5. GENERAL CONCLUSIONS

Since it became evident that the n-3 PUFAs provide protection against cardiac arrhythmias, the actions of both dietary and acutely added PUFAs have been the subject of intense investigation. Free PUFAs have been demonstrated to provide protection against arrhythmic activity in whole heart and single cell studies, primarily due to the reduction in electrical excitability of the sarcolemmal membrane. Until recently this was believed to be how PUFAs protect against arrhythmias. However not all arrhythmias are due to disturbances of membrane electrical activity. A large class of arrhythmias arising from abnormal calcium handling is also well known. The aim of these studies was to determine if both dietary and free PUFAs provide protection against arrhythmias by modifying calcium handling in the heart.

The findings of these studies show that the width and the duration of calcium sparks is reduced in myocytes from rats supplemented with fish oil in comparison to those supplemented with lard. This reduction in the magnitude of the sparks are unlikely to be due to modification to the calcium sensitivity of the RyR, as the propagation velocity of calcium waves collected in ventricular myocytes from fish oil supplemented rats would be expected to differ from those collected from lard-supplemented rats and was not found to.

The incubation of ventricular myocytes with EPA was found to reduce the width and duration of calcium sparks in a similar manner similar to that seen in myocytes from fish oil supplemented rats. Both DHA and ALA were not found to influence the width or duration of the sparks. The results of bilayer studies have shown that EPA, when

applied to the both the cytosolic and luminal face of the RyR, reduces the open probability of the RyR. This may provide a possible explanation for the reduction in the properties of the calcium sparks in response to EPA. To confirm that EPA reduces the magnitude of the calcium sparks by acting directly on the RyR, it would be interesting to determine the effects of DHA or ALA on the open probability of the RyR. The cytosolic application of EPA to the RyR in bilayer experiments was not found to alter the sensitivity of the RyR to cytosolic calcium. Therefore, this is an unlikely mechanism by which EPA is reducing the magnitude of the calcium sparks.

The results of these studies reveal no evidence that calcium re-uptake by the SR is altered by either dietary or free PUFAs. Blockade of the SR Ca^{2+}ATPase did not significantly alter the duration or width of calcium sparks in myocytes from fish oil-supplemented rats or in myocytes incubated with free PUFAs. This indicates that enhanced SR Ca^{2+} re-uptake was not responsible for the reduction in the duration and width of the sparks due to dietary or free PUFAs. The rate of decay of calcium transients was also found not to differ in myocytes from rats that were supplemented with lard or fish oil. The incubation of ventricular myocytes with EPA was also found not to effect rate decay of the calcium transient. If free or dietary PUFAs affected SR calcium re-uptake, it would be expected that the decay rate of the transient would be altered and this was not found. These findings suggest that calcium sequestration by the SR is unlikely to be affected by both dietary and free PUFAs, although further investigation into the effect of PUFAs on other cytosolic calcium removal mechanisms such as the Na^{+}- Ca^{2+} exchanger need to be investigated.

It has been demonstrated that sparks differ in their profiles. The proportion of sparks displaying prolonged up-rise or decay profiles were less common in both atrial and ventricular myocytes from rats whose diet was supplemented with fish oil in comparison to those supplemented with lard. This may indicate that the recruitment and/or gating of the RyR differ in response to dietary fish oil. Bilayer studies have shown that the cytosolic and luminal application of EPA to the RyR results in reduction in open probability compared to the controls when activated with 2 mM ATP and 0.1 μM Ca^{2+}. When the RyR was activated with 0.1 mM cytosolic Ca^{2+}, the magnitude of the reduction in the open probability upon cytosolic EPA exposure was similar to when activated by ATP and Ca^{2+}. However, the rate of reduction was much slower. These findings could indicate that EPA modulates RyR gating by altering the channel response to activating ligands. It would be interesting to determine if EPA effects RyR gating by altering the regulatory function of other regulating ligands.

In conclusion, both dietary and free PUFAs have been demonstrated in these studies to modify spontaneous calcium sparks. These findings indicate that PUFAs may provide protection against arrhythmias by reducing spontaneous calcium release thereby reducing spontaneous membrane depolarization resulting from calcium extrusion from the cytosol by the Na^{+}-Ca^{2+}exchanger. The lack of effect of dietary fish oil on spontaneous calcium waves indicates that dietary fish oil may not provide protection from calcium overload arrhythmias in the instance of raised cytosolic calcium concentration. The reduction in RyR open probability by EPA may be functionally important in providing protection against the potentially arrhythmogenic calcium handling disturbances during ischemia. n-3 PUFAs attained through the diet and incorporated into the membrane phospholipids may provide a potential store for

the generation of free n-3 PUFAs on the activation of phospholipases activated during ischemia. These free PUFAs may have similar actions on the RyR in a cellular system as demonstrated in the bilayer studies.

Appendix A: Fatty Acid Composition of Oil Supplements

Fatty acid type	lard	canola oil	fish oil
14:0	8.36		0.18
14:1	1.56		
16:0	32.00	4.55	0.16
16:1	2.81	0.18	0.30
unk			4.84
18:0	16.89	1.51	0.39
18:1 n-9	35.71	58.80	4.63
18:1 n-7			
18:2 n-6	1.34	21.76	
18:3 n-3	0.68	12.06	0.48
20:0	0.65	0.17	0.16
20:1		1.06	10.18
20:2			
20:3 n-6			0.15
20:4 n-6			1.63
20:3 n-3			
22:0			
22:1			0.70
20:5 n-3			48.03
24:0			0.83
22:5 n-3			1.22
22:6 n-3			26.21
total saturated	57.89	6.23	1.72
total mono	40.08	60.04	15.81
total poly	2.02	33.82	77.70
total n-6	1.34	21.76	1.77
total n-3	0.68	12.06	75.93
n-6/n-3	1.97	1.81	0.02

Fatty acid composition (%) of the lard, canola oil and fish oil dietary supplements. The short hand notation for fatty acid structure is x:y (n-i) where 'x' is the number of carbons in the chain,' y' is the number of double bonds and 'i' is the position of first double bond from the methyl end of the carbon chain. The position of the first double bond is often referred to as "omega".

Appendix B: Composition of Rat Diets

Ingredients		kg/kg
Sucrose		0.1
Starch		0.3975
Dextrinised starch		0.132
α-Cellulose		0.05
Casein (acid)		0.2
Sunola Oil		0.07
DL-Methionine		0.003
*Mineral mix		0.035
*Vitamin mix		0.01
Choline chloride		0.0025
Total		1
Vitamin Mix	(AIN_93G)	
Vitamin A (500,000UI/g)		0.0008
Vitamin D (500,000UI/g)		0.0002
Vitamin E		0.0075
Vitamin K		0.0001
Niacin		0.003
Riboflavin		0.0006
Ca^{2+} Pantothenate		0.0016
Vitiman B12 (1% m/m)		0.001
Thiamine		0.0006
Pyridoxine		0.0007
Folic acid		0.0002
Biotin (2%m/m)		0.001
Sucrose		0.983
Total		1
Mineral Mix	(AIN_93G)	
Potassium Iodate		0.000004
Copper Sulphate		0.00065
Ferrous Sulphate		0.00498
Magnesium Oxide		0.024
Manganese Chloride		0.00032
Zinc Chloride		0.001245
Lime (fine calcium carbonate)		0.375
Salt (fine sodium chloride)		0.074
Chromium potassium sulphate		0.00055
Potassium phosphate		0.196
Lithium Chloride		1.74E-05
Potassium sulphate		0.0466
Potassium citrate		0.0708
Glucose (dextrose)		0.2059
Total		1
for 10% lipid supplementation		
fish oil		0.104
or lard		0.104

Appendix C: Membrane Fatty Acid Analysis

Thank you to Assoc. Prof. Ted McMurchie and Mrs. Sharon Burnard for performing the membrane phospholipid fatty acid analysis on both the atrial and ventricular tissue.

Total lipids were extracted from atrial tissue (section 2.2.2) from rats supplemented with lard, canola oil and fish oil and from ventricular tissue (section 2.2.3) from rats receiving either the lard or fish oil supplemented diet. The method used was a slightly modified method from that of Bligh and Dyer (1959) and is described by Leifert *et al.* (2001).

Atrial or ventricular tissue was partially digested according to the method described in section 2.1.3. The section of tissue of interest was homogenized in 1 ml of water using a hand held homogenizer (Tenbröeck). Following homogenization, 8 ml of 2-propranolol was added to the mixture and the mixture was boiled for 30 seconds. The mixture was allowed to cool then 16 ml of chloroform was added and mixed. The organic phase was collected and dried with nitrogen. The phospholipids were separated from other lipid classes by thin layer chromatography (TLC) on silica gel 150A-LK5D plates (Whatman, Clifton, NJ, USA). The plates were developed using petroleum ether:acetone (3:1, v/v). Phospholipids remaining at the origin were scraped from the plate. Phospholipid fatty acid methyl esters (FAMEs) were prepared by heating the samples over night at 50°C in 2 ml of 1% (v/v) sulphuric acid in methanol. Following this, 200 µl of water was added and the FAMEs were extracted using hexane and contaminants were removed using a Biosil (silicic acid) column.

The antioxidant butylated hydroxytoluene (0.05%) was added to all solvents used for lipid extraction. FAMEs from the lipid extracts and phospholipid extracts were analyzed by gas liquid chromatography (GLC). GLC was performed using a Hewlett Packard HP 5710 gas chromatograph (Hewlett Packard, CA, USA) fitted with a 50 m BPX70 capillary column (Scientific Glass Engineering, VIC, Australia). The FAMEs were separated using a carrier gas (hydrogen) flow of 35 cm/s with a temperature gradient of 130°C to 230°C at 4°C per minute. A cold on-column injector was used with the flame ionization detector temperature set at 250°C. FAMEs were identified using authentic lipid standards (Nu-Chek-Prep Inc., MN, USA) by GLC. The proportions of the total fatty acids were normalized to 100%.

Students unpaired t-test or two way ANOVA were used to compare differences between the various dietary lipid treatments.

Appendix D: Calcium Probes

The calcium indicator used in all confocal studies in this thesis was the acetoxymethyl (AM) ester derivative of fluo-3 (fluo-3 AM). Calcium sparks can only be visualized using fluo-3 as its large dynamic range and its high calcium dissociation constant (K_d=390nM) render it highly sensitive to small fluctuations in calcium concentration.

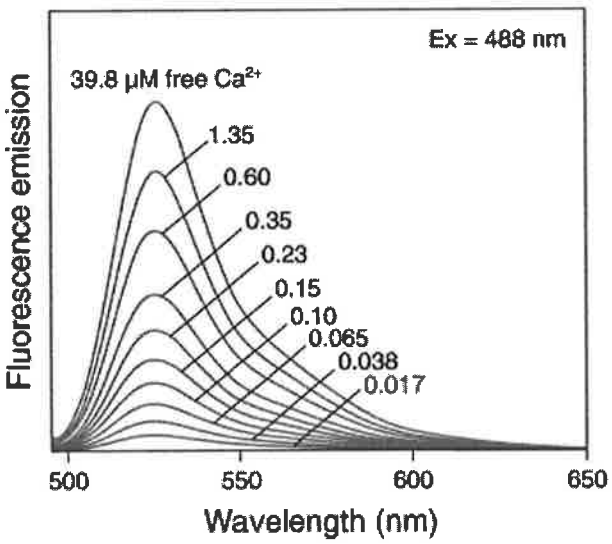


Figure A: Ca^{2+}-dependent fluorescence emission spectra of fluo-3. The spectrum for the Ca^{2+}-free solution is indistinguishable from the baseline.

Fluo-3 is a non-ratiometric dye that can be excited at 488nm and fluoresces at 525 nm (figure A). This property of Fluo-3 is problematic if attempting to determine a concentration of calcium, for this application a ratiometric dye is more appropriate. Problems that can arise are that the fluorescence increase with increasing calcium concentration do not necessarily give rise to a linear relationship also the amount of

dye in the cell can not be accounted for, this could give rise to an over or underestimation of the calcium concentration. A problem with fluo-3 AM with regards to determining calcium concentration is that while in the AM ester form, fluo-3 is non-fluorescent, the AM ester must cleave in order for the dye to fluoresce once calcium is bound. Allowing the freshly loaded cells to incubate and de-esterify the dye prior to use can minimize this problem. Failure to do this can result in an increase in the fluorescence signal irrespective of the calcium concentration over time as fluo-3 is uncaged.

Ratiometric dyes fluoresce two wavelengths of which a ratio can be taken of the two intensities (for example indo-1, figure B). This ratio corresponds to a given calcium concentration. The amount of dye in the cell and any non-linearity between calcium concentration and fluorescence intensity is eliminated when using ratiometric dyes, as this does not alter the ratio between the two fluorescing wavelengths.

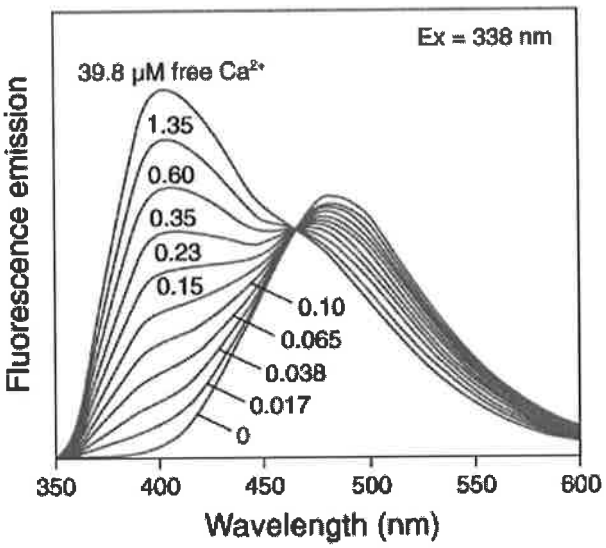


Figure B: Fluorescence emission spectra of indo-1 (I-1202) in solutions containing 0–39.8 µM free Ca^{2+}

In these studies, a ratiometric dye could not be used to determine calcium concentration or fluorescence intensity, as currently there is not a ratiometric dye with a high enough calcium affinity to visualize calcium sparks. Hence peak fluorescence was expressed as a ratio of background fluorescence to allow cross comparison between cells. Determining an actual calcium concentration is not possible using this method hence fluorescence intensity was used.

Information and figures A and B were sourced from
Molecular Probes hand book, Chapter 20, Indicators for Ca{2+}, Mg{2+}, Zn{2+} and Other Metal Ions, http://www.probes.com/handbook/.

Appendix E: Isolation of SR Vesicles

Thanks to Angela Dulhunty, Suzy Pace and Joan Stivala (Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, Australian National University, Canberra, ACT) for supplying SR vesicles.

Sheep hearts were excised from anaesthetized ewes (5% pentobartitone (IV) followed by oxygen/halothane) and rinsed in ice cold homogenizing buffer containing 20 mM imidazole, 300 mM sucrose; pH 7.4 with HCl. Ventricular material was stripped of fat and connective tissue, minced and homogenized in a Waring blender. The homogenate was centrifuged at 11,000 g for 20 min. The supernatant was then centrifuged at 110,000 g for 120 min. The crude vesicle pellet was re-suspended in homogenizing buffer containing 10 μM leupeptin and 2 mM DTT, snap frozen and stored in either liquid nitrogen or at -70 ^{o}C.

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Journal of Nutritional Biochemistry, v. 13(6), pp. 322-329

Submitted to the Journal of Membrane Biology. In Press

Suppression of calcium sparks in rat ventricular myocytes and direct inhibition of sheep cardiac RyR channels by EPA, DHA and Oleic acid.

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The anti-arrhythmic effects of long chain polyunsaturated fatty acids (PUFAs) may be related to their ability to alter calcium handling in cardiac myocytes. We investigated the effect of eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) on calcium handling in rat cardiac myocytes and the effects of these PUFAs and the monounsaturated oleic acid on cardiac calcium release channels (RyRs). Visualization of sub cellular calcium concentrations in single rat ventricular myocytes showed that intensity of calcium sparks was reduced in the presence of EPA and DHA (15 μM). It was also found that calcium sparks decayed more quickly in the presence of EPA but not DHA. Sarcoplasmic vesicles containing RyRs were prepared from sheep hearts and RyR activity was determined by either [^{3}H]ryanodine binding or by single channel recording. Bilayers were formed from phosphatidylethanolamine and phosphatidylcholine dissolved in either n-decane or n-tetradecane. EPA inhibited [^{3}H]ryanodine binding to RyRs in SR vesicles with K_I = 40 μM. Poly- and mono-unsaturated free fatty acids inhibited RyRs activity in lipid bilayers. EPA (cytosolic or luminal) inhibited RyRs with K_I =32 μM and Hill coefficient, n_I = 3.8. Inhibition was independent of the n-alkane solvent and whether RyRs were activated by ATP or Ca^{2+}. DHA and oleic acid also inhibited RyRs, suggesting that free fatty acids generally, inhibit RyRs at micro molar concentrations.