Antiarrhythmic mechanisms of omega-3 polyunsaturated fatty acids in rat ventricular cardiomyocytes

Wayne R. Leifert

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Department of Physiology
Medical School North
Adelaide University, Australia

and

CSIRO Health Sciences and Nutrition
Diagnostics and Therapeutics Program
Adelaide, Australia

March 2001

Abstract

Recent evidence suggests that the consumption of polyunsaturated fatty acids (PUFAs) of the omega 3 (or n-3) class (of marine origin) reduce both the incidence of and mortality from coronary heart disease and this may be related to the antiarrhythmic activity of these PUFAs. The studies described in this thesis investigated the mechanisms underlying the antiarrhythmic effects of n-3 PUFAs using adult rat ventricular cardiac myocytes. The voltage-dependence of Na^+ current activation and inactivation was associated with an increase in sarcolemmal membrane fluidity following acute addition of micromolar amounts of n-3 PUFAs. Dietary supplementation with fish oil (FO) significantly increased the proportion of total n-3 PUFAs in ventricular membrane phospholipids compared with saturated fat (SF) supplementation and this was independent of membrane fluidity. The n-3 PUFAs were selectively increased in both phosphatidylcholine and phosphatidylethanolamine. The level of non-esterified n-3 PUFAs was significantly increased by up to 40-fold following fish oil supplementation for 3 weeks. The voltage-dependence of inactivation of whole-cell Na^+ currents was significantly shifted to more hyperpolarsied potentials in the FO group compared with the SF group (as determined by patch-clamp techniques). The voltage-dependence of activation and inactivation of whole-cell K^+ currents was similar in both groups. Cardiomyocytes isolated from the FO group were significantly less susceptible to isoproterenol-induced spontaneous and asynchronous contractile activity and this was associated with a significantly increased time constant for decay of Ca^{2+} transients and a generally lower diastolic $[Ca^{2+}]_i$. These results demonstrate that the antiarrhythmic effects of dietary fish oil may partly involve electrophysiological changes in rat cardiac myocytes, including inactivation of the fast Na^+ channel and modification of sarcolemmal Ca^{2+} efflux mechanisms following increases in myocardial non-esterified n-3 PUFAs and phospholipid n-3 PUFAs.

Declaration

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

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying.

Wayne R. Leifert (March 2001)

Chance favours only the prepared mind.
Louis Pasteur

We cannot learn everything from "general principles"-there may be exceptions.
Talmud

Acknowledgements

I express my sincerest thanks to my Supervisor, Associate Professor Ted McMurchie, for his friendship, encouragement, guidance and support throughout this project and to my co-supervisor, Dr Mavis Abbey, for her genuine interest and encouragement during the course of this project and to the CSIRO Health Sciences and Nutrition for providing facilities to enable this work to be performed.

I would also like to thank the following:

Mrs Sharon Burnard and Associate Professor Ted McMurchie for carrying out some of the phospholipid fatty acid analyses as well as teaching me the procedures involved in phospholipid fatty acid analysis and Mr Mark Mano for ongoing assistance and technical guidance with chromatography techniques.

Ms Camilla Dorian for conducting some of the cardiomyocyte asynchronous contractility scoring experiments.

Mrs Sharon Burnard, Ms Anisa Jahangiri and Associate Professor Ted McMurchie for assistance with the cardiomyocyte anisotropy (fluidity) experiments.

Dr David Saint (Department of Physiology, Adelaide University) for allowing me to spend much of my time analysing Na^+ and K^+ currents in his laboratory and for contributing to the roster of gavaging and administering diets to the animals used for this study.

Drs Peter Kenny and Roger Lainson (SDR Clinical Technologies, Sydney, Australia) for their helpful input into establishing the high-speed fluorescence microscopy analysis set-up used for determining intracellular Ca^{2+} concentration and surface membrane potential.

To the Department of Physiology, University of Adelaide, I am grateful for the financial support I received for travel as a result of their generous and encouraging Postgraduate Publication Award Scheme and to CSIRO Health Sciences and Nutrition, Diagnostics and Therapeutic Technologies Program, for contributing to my travel costs to present results from this thesis and for my Scholarship supplement.

I was supported by a *National Heart Foundation of Australia Postgraduate Research Scholarship* for which I am extremely grateful to be a recipient.

Academic Prizes and Travel Awards

National Heart Foundation of Australia Travel Grant, 2001.

University of Adelaide, Department of Physiology Travel Award (2001) for publication in *Journal of Nutritional Biochemistry*.

Industry Award - Meadow Lea Foods Ltd. Travel Award (received July, 2000). To present at the World meeting of the International Society for Heart Research, Winnipeg, Canada, July 2001.

University of Adelaide, Department of Physiology Travel Award (2000) for two publications in *Journal of Nutritional Biochemistry*.

Walter and Dorothy Duncan Trust Award. Administered by the University of Adelaide (2000).

University of Adelaide, Department of Physiology Travel Award (2000) for published article in *Journal of Physiology*.

University of Adelaide, Department of Physiology Travel Award (1999) for published review article, *Journal of Nutritional Biochemistry*, 1999.

Ross Wishart Memorial Award for most outstanding presentation at Australian Society for Medical Research (ASMR) conference, Adelaide, SA, Australia, June 1999.

CSIRO Health Sciences and Nutrition, Travel Award (to meet costs of travel to Auckland, New Zealand, August 1999) to present at ISHR meeting.

WG Nayler Prize for Best Presentation. International Society for Heart Research (ISHR), Australasian Section, Sydney, August 1998.

Publications and Abstracts Arising From This Thesis

Wayne R. Leifert, Anisa Jahangiri, David A. Saint and Edward J. McMurchie (2001). Dietary fish oil alters the voltage-dependence of Na^+ currents in rat cardiomyocytes. *Journal of Molecular and Cellular Cardiology*. Abstract (in press).

Anisa Jahangiri, **Wayne R. Leifert** and Edward J. McMurchie (2001). Dietary fish oil reduces free radical damage in isolated cardiomyocytes. *Journal of Molecular and Cellular Cardiology*. Abstract (in press).

#**Wayne R. Leifert,** Camilla L. Dorian, Anisa Jahangiri and Edward J. McMurchie. (2001). Dietary fish oil prevents asynchronous contractility and alters Ca^{2+} handling in rat cardiomyocytes. *Journal of Nutritional Biochemistry.* Full paper (in press).

Wayne R. Leifert, Anisa Jahangiri, David A. Saint and Edward J. McMurchie (2000). Dietary n-3 polyunsaturated fatty acids alter the voltage-dependence of Na^+ currents in rat cardiac myocytes. *Proceedings of the Nutrition Society of Australia*. **24**: 141.

Wayne R. Leifert, Camilla L. Dorian, Anisa Jahangiri and Edward J. McMurchie (2000). Dietary fish oil prevents arrhythmic contractility and increases Ca^{2+} transient decay time in adult rat cardiac myocytes. *Proceedings of the Nutrition Society of Australia*. **24**: 100.

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#**Wayne R. Leifert**, Anisa Jahangiri, David A. Saint and Edward J. McMurchie. (2000) Effects of dietary n-3 fatty acids on contractility, Na^+ and K^+ currents in a rat cardiomyocyte model of arrhythmia. *Journal of Nutritional Biochemistry.* **11**: 382-392.

#**Wayne R. Leifert**, Anisa Jahangiri and Edward J. McMurchie. (2000) Membrane fluidity changes are associated with the antiarrhythmic effects of docosahexaenoic acid in adult rat cardiomyocytes. *Journal of Nutritional Biochemistry*. **11**: 38-44.

#Anisa Jahangiri, **Wayne R. Leifert**, Glen S. Patten, and Edward J. McMurchie. (2000) Termination of asynchronous contractile activity in rat atrial myocytes by n-3 polyunsaturated fatty acids. *Molecular and Cellular Biochemistry*. **206**: 33-41.

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#**Wayne R. Leifert**, Anisa Jahangiri and Edward J. McMurchie. (1999). Antiarrhythmic fatty acids and antioxidants in animal and cell studies. (Review). *Journal of Nutritional Biochemistry.* **10**: 252-267.

#**Wayne R. Leifert**, Edward J. McMurchie and David A. Saint. (1999) Inhibition of cardiac Na^+ currents in adult rat myocytes by n-3 polyunsaturated fatty acids. *Journal of Physiology*. **520.3**: 671-679.

Wayne R. Leifert, Edward J. McMurchie and David A. Saint. (1999) Inhibition of cardiac Na^+ currents in adult rat myocytes by n-3 polyunsaturated fatty acids. *Journal of Molecular and Cellular Cardiology*. **32**(3): A5.

David A. Saint, **Wayne R. Leifert** and Edward J. McMurchie (1999). Block of I_{Na} in adult rat cardiac myocytes by N-3 polyunsaturated fatty acids. *Journal of Physiology*. **521P**: 19P.

Wayne R. Leifert and Edward J. McMurchie. (1999) Protective effects of n-3 PUFAs in adult rat ventricular cardiomyocytes. *Journal of Molecular and Cellular Cardiology*. **31**(1): A5.

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David A. Saint, **Wayne R. Leifert** and Edward J. McMurchie. (1999) Block of INa in adult rat cardiac myocytes by N-3 polyunsaturated fatty acids. *Proceedings of the Australian Physiological and Pharmacological Society*. 30(2) 75P.

#Full papers

Presentations

(by the author)

National

Omega Discussion Workshop, oral presentation, Wollongong, New South Wales, Australia, February, 2001.

Nutrition Society of Australia, 24th Annual Scientific Meeting, oral and poster presentation, Fremantle, Western Australia, 3-6 December, 2000.

International Society for Heart Research (ISHR) Australasian Section, poster presentation. Melbourne, Australia, August 5-7, 2000.

The Australian Society for Medical Research (ASMR), oral presentation. Adelaide, South Australia, June 9 2000.

The Australian Society for Medical Research (ASMR), oral presentation. Adelaide, South Australia, June 4 1999.

International Society for Heart Research (ISHR) Australasian Section, poster presentation. Sydney, Australia, August 21-23, 1998.

International

International Society for Heart Research (ISHR) Australasian Section. Oral presentation. Pakatoa Island (Auckland), New Zealand, August 4-6, 1999.

Abbreviations

AA	arachidonic acid (20:4, cis-Δ5,8,11,14-eicosatetraenoic acid)
ALA	α-linolenic acid (18:3, cis-Δ9,12,15-octadecatrienoic acid)
ANOVA	Analysis of variance
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
Bay K8644	(1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]pyridine-3-carboxylic acid methyl ester)
BHA	butylated hydroxyanisole
BSA	bovine serum albumin
Ca^{2+}	calcium ion concentration ion
$[Ca^{2+}]_i$	intracellular calcium ion concentration
CHD	coronary heart disease
CICR	calcium-induced calcium release
CLP	cardiolipin (diphosphatidyl glycerol)
CO	canola oil
CON	control diet (containing 17% sunola oil)
DA	docosanoic acid (22:0)
DBHQ	2,5-Di-tert-butylhydroquinone
DHA	docosahexaenoic acid (22:6, cis-Δ4,7,10,13,16,19)
$DiBAC_4(3)$	bis-(1,3-dibutylbarbituric acid)trimethineoxonol
DMEM	Dulbecco's modified Eagles medium
DPA	docosapentaenoic acid (22:5, cis-Δ7,10,13,16,19)
ECG	electrocardiogram
EGTA	ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid
EPA	eocisapentaenoic acid (20:5, cis-Δ5,8,11,14,17)
E_{rev}	the reversal potential for the Na^+ current
F_{340}	fluorescence emission at 340 nm
F_{380}	fluorescence emission at 380 nm
$F_{340/380}$	fluorescence ratio of 340nm/380nm
FAME	fatty acid methyl ester
FO	fish oil
GLC	gas-liquid chromatography
G_{max}	maximum conductance
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
IPOH	isopropanol
I	ion current
I_{max}	maximum ion current
I-V	current-voltage
k	is a slope factor
K^+	potassium ion
LA	linoleic acid (18:2, cis-Δ9,12-octadecadienoic acid)
LPC	lysophosphatidylcholine
MeOH	methanol
Mono.	monounsaturated fatty acid
Na^+	sodium ion
NEFA	non-esterified fatty acid
OA	oleic acid (18:1, cis-Δ9-octadecenoic acid)
PC	phosphatidylcholine

PI	phosphatidylinositol
Poly.	polyunsaturated fatty acid
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
REF	reference diet (containing 7% sunola oil)
r_{ss}	steady-state fluorescence anisotropy value
SA	stearic acid (18:0, octadecanoic acid)
Sat.	saturated fatty acid
SEM	standard error of the mean
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
SF	saturated fat
SPM	sphingomyelin
SR	sarcoplasmic reticulum
SSFA	steady-state fluorescence anisotropy
TES	(N-tris-(hydroxy-methyl)-methyl-2-aminoethanesulphonic acid)
TLC	thin layer chromatography
TMAP-DPH	N-((4-(6-phenyl-1,3,5-hexatrienyl)phenyl)propyl) trimethyl-ammonium p-toluenesulfonate
V	the test membrane potential
V'	membrane potential for half activation or inactivation of ion channels
VSRM	voltage-sensitive release mechanism
Σ	sum of all parameters

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CHAPTER 1

1 "Literature Review"

1.1 CORONORAY HEART DISEASE

Coronary heart disease (CHD) remains one of the leading causes of mortality in many industrialised countries (Leaf & Kang, 1996). The major clinical manifestations of CHD include myocardial infarction, cardiac arrhythmias and sudden cardiac death. Cardiac arrhythmia can occur during the early phase of ischaemia and, in certain situations, following the restoration of normal blood flow (reperfusion) to the ischaemic region of the myocardium. Individual heart cells are electrically coupled both to each other and to the conducting pathways by gap junctions that allow electrical conductance to pass from one cell to the next (Verrecchia & Herve, 1997). During ischaemia, the electrical properties of the heart are changed resulting in arrhythmias such as ventricular tachycardia and ventricular fibrillation (VF) which can lead to sudden cardiac death. Whilst arrhythmias can be of many types and vary in their aetiology, the inability of individual cardiomyocytes to function properly is fundamental to the generation of arrhythmias.

Useful strategies for reducing the incidence of CHD mortality in the population can be directed either at disease prevention, or at improving the treatment for patients with known symptoms. Many of the risk factors associated with CHD are nutrition-related and therefore modifiable. Risk factors such as the intake of saturated fat, high blood pressure, age, smoking, obesity and diabetes are known to be related to the development of CHD (Kuller, 1983). More recent evidence suggests that consumption of certain types of

polyunsaturated fatty acids (PUFAs) in preference to saturated fats may reduce both CHD incidence and mortality (Siscovick *et al.*, 1995; Singh *et al.*, 1997).

1.2 CARDIAC ARRHYTHMIAS

Myocardial ischaemia exists when the reduction of coronary flow is so severe that the supply of oxygen (and other substrates) to the myocardium is inadequate for the oxygen demands of the tissue (Opie, 1998b). Lack of oxygen supply to the mitochondria results in a rapid decrease in ATP synthesis which impacts on many processes underlying the normal excitation-contraction coupling cycle of the myocardium. A number of sequelae develop as a result of ischaemia. Internal Na^+ concentration increases rapidly at the time of ischaemia. The Na^+/H^+ exchanger operates to expel intracellular H^+ (which can impair contractility) in exchange for extracellular Na^+ (Meng & Pierce, 1990; Pierce *et al.*, 1993). This accumulation of intracellular Na^+ leads to further increases in intracellular Ca^{2+} via the Na^+/Ca^{2+} antiporter (Figure 1.1). Following ischaemia, potassium is released from cardiomyocytes and there is an increase in intracellular lactate and inorganic phosphate levels and a decrease in intracellular pH. Changes in the activity of K^+ channels can also occur as a result of the decline in the cellular ATP level. The increased Ca^{2+} will also affect the action of phospholipases enhancing the liberation of free fatty acids from membrane phospholipids. In addition, the accumulation of lipid metabolites is increased and may have adverse lytic effects on the cell. Delayed afterdepolarisations and triggered automaticity caused by increased β-adrenergic receptor activation by catechoalmines have also been described in the genesis of ischaemic arrhythmias (Coetzee & Opie, 1987; Opie, 1989; Priori & Corr, 1990; Du *et al.*, 1999; Akiyama & Yamazaki, 2001). Characteristic changes in the electrocardiogram (ECG) pattern include

shortening of the action potential duration and ST-segment deviations during ischaemia (Opie, 1998b).

Many clinically encountered arrhythmias result from the phenomenon of re-entry and this can arise equally in many areas of the heart such as from a bundle of conducting fibres to an area containing working myocardial cells (Katz, 1977). The phenomenon of re-entry and subsequent generation of re-entrant arrhythmic activity in the form of premature systoles and tachycardia, results from the presence of an area of decremental conduction exhibiting slowed impulse conduction together with a unidirectional block. The normal propagation of the impulse conduction wave through areas of the functional syncytium, be they conducting fibres or muscle cells, can therefore be perturbed in such areas due to damage arising from, for example, heart failure or the imposition of ischaemia. Through secondary processes such as summation and inhibition of the conduction wave arising from these conduction blocks, re-entrant pathways are established and the syncytium no longer functions as an effective integrated unit, but rather displays through these mechanisms many of the types of arrhythmic activity encountered clinically (Katz, 1977).

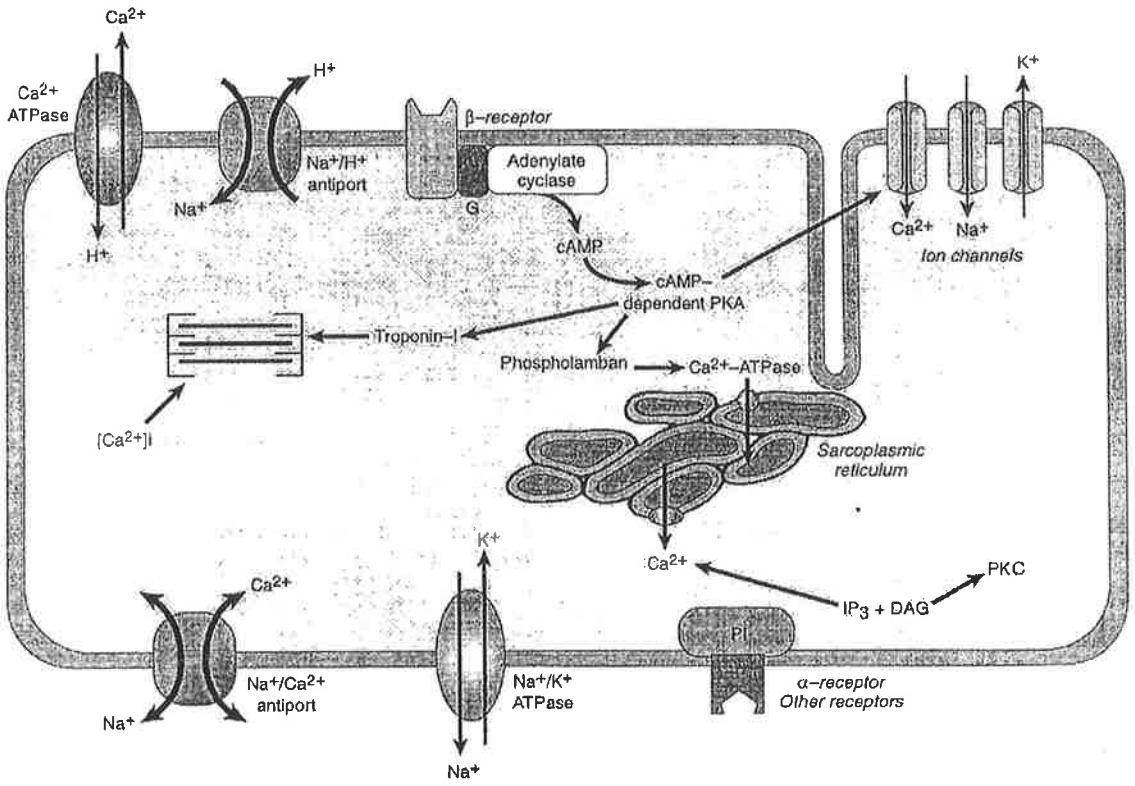


Figure 1.1 Ion transporters in cardiomyocytes.
Some of the major ion transporters affected during myocardial ischaemia resulting in accumulation of intracellular Ca^{2+} and subsequent contracture. Abbreviations: G, G-protein; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; cAMP, cyclic AMP; PKA, protein kinase A; PI, phosphoinositide. Reproduced from Leifert *et al.* (1999).

1.3 THE SYNTHESIS OF POLYUNSATURATED FATTY ACIDS

Fatty acids are typically classified into saturated, monounsaturated and polyunsaturated fatty acids (PUFAs)[1]. Animal fats are the major dietary source of saturated fatty acids which include palmitic (16:0) and stearic acids (18:0). Oleic acid (18:1, n-9) is a monounsaturated fatty acid found in most of the common edible oils such as olive oil, sunflower oil, safflower oil and canola oil. Naturally occurring PUFAs typically contain an even number of carbon atoms (between 18 and 24) and are incorporated into the

[1] The shorthand notation for fatty acid structure is "a:b, n-c". The letter "a" represents the total number of carbon atoms in the fatty acyl chain; "b", the number of double bonds separated by single methylene groups; and "n-c", also written as "omega-c" (or ω-c), denotes the number of carbon atoms between the first double bond and the methyl end of the chain. The n-3 fatty acids are those in the linolenic acid family; n-6 fatty acids are in the linoleic acid family.

phospholipids within the membranes of cells where they are esterified onto hydroxyl groups in the phospho-glycerol backbone (Katz & Messineo, 1981b). Mammals are able to synthesise all fatty acids *de novo* except the "essential" parent fatty acids, linoleic acid (LA, 18:2, n-6) and α-linolenic acid (ALA, 18:3, n-3). These fatty acids are categorised as essential since humans lack the enzymes necessary to insert double bonds between the terminal methyl carbon and the 9th carbon atom. LA and ALA must therefore be obtained from the diet. Linoleic acid is found in plant seed oils such as sunflower, safflower, olive and cottonseed oil, and ALA is found in canola, soybean, flaxseed and linseed oils.

The synthesis of PUFAs proceed via a series of reactions involving classes of enzymes that insert carbon atoms (normally two) into the fatty acid chain (elongases), and those that insert double bonds at specific regions of the fatty acid chain (desaturases) leading to increased unsaturation. The original concept of Brenner (Brenner, 1982) that a delta-4 desaturase may be involved in this process has now been largely superseded by the recent work of Sprecher and his group who have reported that during long chain PUFA synthesis, peroxisomes and β-oxidation participate in the synthesis of the long chain n-3 PUFAs, docosahexaenoic acid (22:6, n-3, DHA) and eicosapentaenoic acid (20:5, n-3, EPA) by way of C24 PUFA intermediates (Voss *et al.*, 1991). Figure 1.2 summarises the pathways for metabolism of the n-6 and n-3 PUFAs.

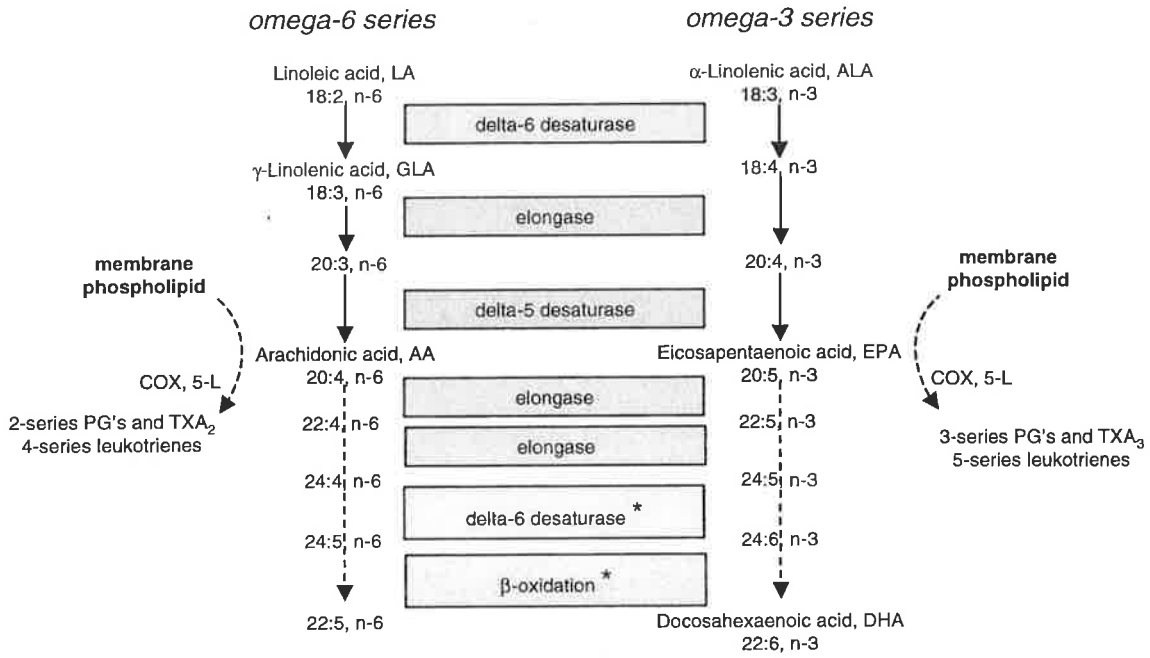


Figure 1.2 Schematic representation of the n-6 and n-3 series metabolic pathway.
Representation of eicosanoid production from the 20-carbon n-6 and n-3 precursors, arachidonic acid (AA) and (eicosapentaenoic acid) EPA, respectively. Both AA and EPA can be released from membrane phospholipids by the action of phospholipase and further metabolised to the 2- or 3-series eicosanoids, as indicated. Abbreviations: COX, cyclo-oxygenase; 5-L, 5-lipoxygenase, *established pathway (Voss *et al.*, 1991). Adapted from Leifert *et al.* (1999)

Arachidonic acid (AA, 20:4, n-6) and EPA also act as precursors for the eicosanoids (bioactive metabolites of AA and EPA). The generation of these latter components in cardiac tissue particularly with regard to the prostaglandin to thromboxane ratio have been implicated in arrhythmogenesis (Abeywardena & Charnock, 1995) and will be discussed later. The n-3 fatty acid family includes the essential fatty acid ALA and the very long chain PUFAs, EPA, docospentaenoic acid (DPA, 22:5, n-3) and DHA found in marine phytoplankton consumed by fish and in fish *per se* (Sinclair *et al.*, 1998). In the presence of dietary ALA, humans are only able to synthesise EPA and DHA *de novo* at a relatively slow rate by elongation and desaturation of ALA (Figure 1.2). The consumption of fish or fish products substantially increases the amounts of EPA and DHA available for

membrane incorporation and cellular processes (Leaf & Weber, 1988). Lack of a dietary supply of essential fatty acids leads to elevation of Mead acid (20:3, n-9), a marker for essential fatty acid deficiency, possibly as a consequence of a compensatory mechanism to offset the reduced levels of long chain PUFAs.

1.4 DIETARY PUFAS IN HUMAN STUDIES

Scientific interest in the health benefits of the n-3 PUFAs was generated by the epidemiological studies of Bang and Dyerberg (Bang *et al.*, 1971; Bang & Dyerberg, 1972; Bang *et al.*, 1980) who reported that Greenland Eskimos, whose dietary intake from marine sources averaged 500g/day, had extended bleeding times. Later studies revealed that this population had significantly lower levels of total and low density lipoprotein cholesterol and a relatively lower incidence of CHD when compared with a Danish study population (Bang & Dyerberg, 1980). The potential benefits of consuming fish and fish oil have been described in several population and clinical studies with particular reference to their potential role in cardiac arrhythmias and sudden cardiac death. In a 20-year and 30-year follow-up study, mortality from CHD was found to be inversely related to fish consumption (Kromhout *et al.*, 1985; Daviglus *et al.*, 1997). In the diet and reinfarction trial (DART study) the incidence of mortality due to ischaemic heart disease was significantly lower in a group of post-myocardial infarction patients advised to include fish in their diet (Burr *et al.*, 1989). Similarly, the beneficial cardioprotective effects of n-3 fatty acids have been reported in cardiac patients (de Lorgeril *et al.*, 1994; Siscovick *et al.*, 1995; Sellmayer *et al.*, 1995; Christensen *et al.*, 1996; Valagussa *et al.*, 1999). Recently, Singh et al. (Singh *et al.*, 1997) concluded that fish oil protects against reperfusion injury in patients suffering an acute myocardial infarction. Although the recent "US Physicians' Health Study" demonstrated that fish intake was associated with a

significant reduced risk of total mortality, the authors point out that fish consumption (at least in their study) may be a marker for a healthier lifestyle (Albert *et al.*, 1998), and that fish consumers were more likely to exercise regularly and take antioxidant vitamin supplements. Table 1.1 briefly summarises some of the major findings investigating the potential cardioprotective effects of fish and fish oil. Collectively, the results of clinical and epidemiological studies indicate that fish consumption may have a direct protective effect on the myocardium. Therefore, dietary n-3 PUFAs may improve the chances of surviving an episode of myocardial ischaemia and prevent primary cardiac arrest, and they may do so by their intrinsic antiarrhythmic activity.

Table1.1 Summary of human studies: outcomes of dietary fish and n-3 PUFA intake on cardiac related mortality

Reference	Subjects	Intervention	Intake	Outcome
Kromhout *et al.*, 1985	852 men	20 year follow up	200g fish/week	<50% mortality from CHD
Burr *et al.*, 1989	2033 post MI patients	2 years fish advice	200-400g fish/week	33% in mortality due to IHD
de Lorgeril *et al.*, 1994	302 post MI patients	1 year fish diet	αLNA rich diet (increased fish consumption)	75% ↓ in recurrence of cardiac events
Siscovick *et al.*, 1995	334 patients	dietary fish intake examined	one fish meal/week	50% ↓ in risk of primary cardiac arrest
Sellmayer *et al.*, 1995	68 patients with frequent VPCs	fish oil	1.5 g DHA daily	48% ↓ in VPCs, antiarrhythmic effects
Christensen *et al.*, 1996	49 post MI patients	fish oil	5.2 g n-3 PUFAs daily	↑ in heart rate variability
Daviglus *et al.*, 1997	1822 men	30 year follow up	≥35 g fish daily	42% ↓ in death rate from MI
Singh *et al.*, 1997	360 post MI patients	MaxEPA capsules	1.8 g EPA and DHA daily	≈50% ↓ in arrhythmias and total cardiac deaths
Albert *et al.*, 1998	20551 healthy US physicians	dietary fish intake examined	≥1 fish meal/week	↓ risk of total mortality
GISSI–Prevenzione Investigators: Valagussa *et al.*, 1999	2836 post MI patients	n-3 PUFA administration	daily for 3.5 years	17-30% ↓ risk in cardiovascular death
Christensen *et al.*, 1999	60 healthy patients	n-3 PUFA administration	2.0-6.6 g n-3 PUFA daily for 12 weeks	dose-dependent effect of n-3 PUFA and heart rate variability

Abbreviations: CHD, coronary heart disease; MI, myocardial infarction; IHD, ischaemic heart disease; ↓, decrease; ↑, increase; VPCs, ventricular premature complexes.

1.5 ANIMAL MODELS OF ARRHYTHMIAS

Different animal models of cardiac arrhythmia have been used for studies on the relationship between dietary lipids, cardiac membrane lipid composition, myocardial

function and the biochemical mechanisms underlying antiarrhythmic effects in relation to nutritional components. A number of studies have investigated the protection afforded by various nutritionally-derived or related agents on arrhythmias induced in the isolated or ligated heart model using both dietary and acute addition of these putative antiarrhythmic compounds. Antiarrhythmic properties of PUFAs were first reported in animal models of arrhythmia by Murnaghan (Murnaghan, 1981). Subsequently, McLennan et al. (McLennan *et al.*, 1985) reported that diets high in saturated fats were associated with a relatively higher incidence of ventricular fibrillation in rats when myocardial ischaemia was induced by coronary artery ligation *in situ*. Dietary sunflower oil (LA rich), reduced the incidence of ischaemia-induced ventricular arrhythmias by approximately 30% compared with animals maintained on a diet supplemented with saturated fat, whereas tuna fish oil high in n-3 PUFAs completely prevented both ischaemia and reperfusion-induced arrhythmias (McLennan *et al.*, 1985; McLennan *et al.*, 1988; McLennan *et al.*, 1992; McLennan *et al.*, 1993). These findings were confirmed in the non-human primate marmoset model when animals were fed diets containing a mixture of sheep fat (saturated fat) and sunflower seed oil as compared with animals fed sheep fat combined with fish oil for 16 weeks (McLennan *et al.*, 1993). The ventricular fibrillation threshold under programmed electrical stimulation was significantly elevated in the fish oil group compared with the sunflower seed oil group and this was associated with increased levels of n-3 fatty acids incorporated into the myocardial membrane phospholipids.

McLennan (McLennan & Dallimore, 1995) reported that following 15 min of ischaemia induced by *in vivo* coronary artery ligation, rats fed an olive oil supplemented diet for 12 weeks exhibited a high incidence of ventricular fibrillation (VF) in comparison with rats fed a canola oil enriched diet where no VF events and a lower arrhythmia score were

recorded. If the duration of ischaemia was shortened to 5 min, the canola oil fed animals again exhibited a lower arrhythmia score, a tendency to fewer VF events and no fatal VF in comparison with animals fed olive oil. Although protection afforded by the n-3 PUFAs may be due to the coincident effect of the reduced saturated fatty acid content, it has been reported that animals fed a fish oil supplemented diet are consistently protected from developing arrhythmias compared with animals fed n-6 PUFA and saturated fatty acid diets, such that their effects do not correlate with the relative amounts of saturated and polyunsaturated fatty acids *per se* (McLennan *et al.*, 1985; McLennan *et al.*, 1988; McLennan *et al.*, 1993). Additionally, canola oil, which like olive oil is composed mainly of oleic acid (which is not antiarrhythmic) but also contains approximately 8% ALA, does offer significant antiarrhythmic protection (McLennan & Dallimore, 1995). The antiarrhythmic effect of the canola oil can not be solely attributed to the presence of the n-3 PUFA, ALA, since soybean oil which contains similar concentrations of ALA is not antiarrhythmic (McLennan & Dallimore, 1995), but rather to the fact that the LA present in soybean oil competes with ALA preventing its conversion to the longer chain n-3 PUFAs, which are potently antiarrhythmic.

Rats fed a fish oil diet for 16 weeks were protected against the development of arrhythmias following ischaemia and reperfusion when blood-perfused, electrically-paced working hearts were investigated (Pepe & McLennan, 1996). This protection by a fish oil diet was associated with an increase in n-3 PUFA incorporation into the myocardial phospholipids. In hearts allowed to spontaneously contract, VF could be induced by programmed stimulation. Rats fed fish oil required a significantly higher stimulation current to induce VF compared with saturated fat fed rats (Pepe & McLennan, 1996). Hock et al. (Hock *et al.*, 1990) reported that the protection afforded against arrhythmias

induced *in situ* by coronary artery ligation and reperfusion by a fish oil diet (4 weeks) was associated with reduced leukocyte infiltration into the left ventricular wall. Leukocytes can release a number of potentially deleterious substances including free radicals that would promote myocardial necrosis. They suggested that the fish oil diet may be providing protection by selective incorporation of n-3 PUFAs into leukocyte membrane phospholipids leading to inhibition of phospholipase activity with a resultant reduction in lipoxygenase metabolite production (Hock *et al.*, 1990). However, in isolated hearts following reperfusion, Yang et al. (Yang *et al.*, 1993) showed a protective effect of fish oil independent of its effects on plasma. Using a perfusate free of plasma and circulating cellular elements such as platelets and leukocytes, reperfusion injury was lower in hearts from animals fed a fish oil diet for five days.

More recent studies by McLennan (McLennan *et al.*, 1996), demonstrated that dietary supplementation of purified DHA mimics the actions of fish oils. The antiarrhythmic effects of free n-3 PUFAs have also been observed following slow intravenous infusion (40-60 min) of the free n-3 PUFAs in arrhythmia susceptible, conscious dogs (Billman *et al.*, 1997). Furthermore, the antiarrhythmic effect of the infused free n-3 PUFAs was associated with a reduction in heart rate and prolongation of the ECG atrial-ventricular conduction time.

1.6 ARRHYTHMIA STUDIES USING ISOLATED CARDIOMYOCYTES

1.6.1 Cardiomyocytes as a model of cardiac arrhythmia

Studies using isolated rat heart cells (cardiomyocytes) to investigate mechanisms by which dietary components exert their antiarrhythmic properties have both advantages and

disadvantages compared with whole animal *in situ* heart studies or isolated heart studies. Dietary studies aimed at determining effects on the heart at the *in vivo* level may be confounded by neural and humoral influences, variable blood pressure and heart rate, circulating fatty acids, or other extracardiac effects influenced by the dietary lipid intake. Studies at the cellular level enable an investigation of the direct effect of fatty acids on the cardiomyocyte as well as determining the possible cellular mechanism(s) involved in cardioprotection by both infused and incorporated fatty acids. Isolated ventricular cardiomyocyte preparations have the potential to be used for arrhythmia studies particularly with regard to investigating the efficacy as well as the underlying mechanisms whereby certain dietary components are able to exert their cardioprotective effects.

Cultured neonatal cardiomyocytes that contract rhythmically and synchronously in a syncytium have been extensively used in the study of the cardioprotective effects of n-3 PUFAs and other compounds (Kang & Leaf, 1994; Kang *et al.*, 1995; Leaf, 1995; Kang & Leaf, 1995; Leaf & Kang, 1996; Kang & Leaf, 1996a; Kang & Leaf, 1996c). Neonatal cells display differences in intracellular morphology compared with cells derived from adult animals particularly with regard to the stage of development of the sarcoplasmic reticulum and the relative contributions of intracellular/extracellular calcium handling to the excitation-contraction cycle. In contrast to neonatal cardiomyocytes, cardiomyocytes isolated from adult animals are quiescent and remain so over periods of several days. Many cardiomyocytes can be obtained from the heart of an adult animal permitting numerous experimental manipulations to be performed using only small amounts of experimental compound(s). Furthermore, the actual concentration of such compounds at the cell surface can be accurately determined. Obvious disadvantages are the fact the cells are not under load when contracting (as in the whole heart), have been subject to the

calcium paradox (raising $[Ca^{2+}]_o$ from low to high concentrations quickly during cell isolation can cause hypercontracture) during isolation, and consequently, may have sustained damage to many regions including the gap junction area. However, isolated adult rat cardiomyocytes behave in a predictable manner with regard to mimicking the behaviour of the whole heart to an extremely wide range of pharmacological agents and changing physiological conditions. Adult cardiomyocytes display similar contractile properties to the intact tissue (Capogrossi *et al.*, 1986a; Capogrossi *et al.*, 1986b). Elevated $[Ca^{2+}]_i$ levels have been implicated to play a significant role in the progression of triggered cardiac arrhythmia in a variety of conditions such as those following myocardial ischaemia and reperfusion, as well as exposure to catecholamines and digitalis (cardiac glycosides) (Katz, 1977). Isolated cardiomyocytes respond to an increased $[Ca^{2+}]_o$ with the development of extra beats, tachyarrhythmias, chaotic beating activity, afterdepolarisations and triggered contractile activity (Marban *et al.*, 1986; Priori & Corr, 1990; Thandroyen *et al.*, 1991; Lakatta, 1992; Song *et al.*, 1992; Wu & Corr, 1992; Song & Belardinelli, 1994; Wu & Corr, 1994; Zeng & Rudy, 1995; De Ferrari *et al.*, 1995; Tweedie *et al.*, 1997); exhibiting an arrhythmia profile similar to that recorded from the isolated or *in vivo* heart (Hoffman & Rosen, 1981).

In order to make some extrapolation between isolated adult rat cardiomyocytes and the situation in the whole heart, the fact that the imposition of arrhythmic stimuli, which induce cells to beat in a manner out of synchrony with an applied electrical stimulus, indicates that an isolated cardiomyocyte has the potential to develop all the characteristics which would lead it, in association with neighbouring cells (were it within the heart itself), to give rise to a region of decremental conduction in the working myocardial fibres. Given the restriction that the whole heart cannot always provide an adequate

experimental model for many of the approaches required, and the situation that certain re-entrant pathways in the heart occur as a result of damage occurring to myocardial contractile tissue induced by arrhythmogenic agents or ischaemia, the isolated adult cardiomyocyte can offer advantages not available using the adult heart.

1.6.2 n-3 PUFAs and cardiomyocytes

The antiarrhythmic properties of (n-3) PUFAs have been studied acutely using both neonatal rat cardiomyocyte preparations (Kang & Leaf, 1994; Kang *et al.*, 1995; Leaf, 1995; Kang & Leaf, 1995; Leaf & Kang, 1996; Kang & Leaf, 1996a; Kang & Leaf, 1996c) and adult rat ventricular cardiomyocytes (Leifert *et al.*, 1997; Leifert, 1997). Malignant arrhythmias in cultured neonatal rat cardiomyocytes can be induced by a variety of chemical stimuli such as β-adrenergic receptor stimulation with isoproterenol, the membrane perturbent lysophosphatidylcholine, elevated extracellular calcium, or ouabain treatment (Kang & Leaf, 1996d). The n-3 PUFAs, and to a lesser extent, the n-6 PUFAs, but not saturated fatty acids, provide a protective effect against arrhythmias induced by exposure to the above arrhythmogens using cultured spontaneously contracting neonatal cardiomyocytes (Kang & Leaf, 1996d). It has been reported that acutely added PUFAs are required to be in their free acid form or in the form of a salt and are ineffective when added as ethyl esters. Furthermore, incorporation of fatty acids into the phospholipids of the sarcolemmal membrane was reported not to be required for the fatty acids to produce their antiarrhythmic effects in the neonatal cardiomyocyte model (Weylandt *et al.*, 1996). It has been suggested that n-3 PUFA enrichment of cardiomyocyte membrane lipids leads to a reduced degradation of membrane phospholipids via phospholipase action (Nalbone *et al.*, 1990), although this is in contrast to the results reported by Malis et al. (Malis *et al.*, 1990). If there is a reduced degradation of phospholipids this may benefit cell membrane stability and

provide protection during episodes of hypoxia and reoxygenation. Furthermore, the type of non-esterified fatty acid released following hydrolysis of membrane phospholipids may determine the nature of the arrhythmic response of the myocardium (Nair *et al.*, 1997). An enhanced release of n-3 PUFAs (Malis *et al.*, 1990) may result in a greater reserve of free fatty acids to act in an antiarrhythmic manner if indeed the antiarrhythmic effect requires the n-3 PUFAs to be in the non-esterified form. In neonatal cardiomyocytes, irregularities in the spontaneous contractile frequency were significantly reduced following 4 days of culture in medium supplemented with n-3 PUFAs (de Jonge *et al.*, 1996). An increase in membrane phospholipid n-3 PUFA content was also observed after this treatment. This is indicative that at least some components of the contractile cycle which rely on cell automaticity are altered following incorporation of n-3 PUFAs into cultured cardiomyocyte membrane phospholipids.

1.7 EICOSANOIDS

1.7.1 Eicosanoids and antiarrhythmic mechanisms

The 20 carbon PUFAs, AA and EPA which preferentially occupy the *sn*-2 position of cell membrane phospholipids can be released by the action of phospholipase A_2 and metabolised to form the eicosanoids (Leaf & Weber, 1988; Chin, 1994; Vandervusse *et al.*, 1997). AA is metabolised by cyclo-oxygenase to form the 2-series eicosanoids, thromboxane A_2 (TXA_2) and prostaglandin I_2 (PGI_2) (which are involved in inflammation and immune responses (Miles & Calder, 1998)), and the 4-series leukotrienes by lipoxygenase, while from EPA, the 3-series eicosanoids, TXA_3 and PGI_3 are synthesised. The types and amounts of eicosanoids synthesised are primarily determined by the availability of the respective precursors, by the activities of those enzymes which release the esterified PUFAs, by the activities of cyclooxygenase and lipoxygenases, and by the

nature of the stimulus (Miles & Calder, 1998). When the proportion of EPA and DHA in the membrane is increased, such as by dietary means, there is concomitantly less AA available to form the 2-series eicosanoids (Chin, 1994). EPA also competes with AA for the cyclooxygenase enzyme to synthesise the 3-series eicosanoids. In contrast to TXA_2 which has potent vasoconstriction and platelet aggregatory actions, TXA_3 possesses only weak biological properties (Chin, 1994). Conversely, PGI_3 has vasodilator properties similar to PGI_2. The net result of increased n-3 fatty acids in cell membranes (and their subsequent release) is a change in the haemostatic balance towards one of greater vasodilatation and less platelet aggregation (Leaf & Weber, 1988). Studies by Abeywardena (Abeywardena & Charnock, 1995) using rats demonstrated that dietary fish oil feeding reduced the incidence of ventricular arrhythmias which was attributed to a decreased myocardial TXA_2 synthesis and an elevated PGI_2/TXA_2 ratio. It is likely that the shift toward the synthesis of 3-series eicosanoids may be favourable for prevention of cardiovascular disease.

1.7.2 Eicosanoids and cardiomyocytes

During post-hypoxic reoxygenation of neonatal rat cardiomyocytes, the production of eicosanoids is in part dependent on the cell membrane phospholipid n-3 PUFA content (Oudot *et al.*, 1995). Both PGI_2 and TXA_2 synthesis is reported to be increased during myocardial ischaemia and reperfusion (Curtis *et al.*, 1993). Thromboxane A_2 has been shown to increase inositol(1,4,5)-trisphosphate (IP_3) production in neonatal rat cardiomyocytes by approximately 14-fold and this was likely due to activation of phospholipase C activity (Nakamura *et al.*, 1996). Dietary supplementation with n-3 PUFAs decreases the production of the biologically active thromboxane, TXA_2 in favour of TXA_3 (Sanigorski *et al.*, 1994), and therefore decreased thromboxane production by

fish oil supplementation may play a role in decreased IP_3 release during reperfusion. Under conditions of normoxia and reoxygenation conditions, the production of the 2-series prostaglandins was lower in n-3 PUFA supplemented cardiomyocytes compared with cells supplemented with n-6 PUFA medium (Oudot *et al.*, 1995). The effects of the cyclooxygenase and lipoxygenase metabolites of AA and EPA, on the activity of spontaneously contracting cultured neonatal rat cardiomyocytes were determined by Li (Li *et al.*, 1997) with changes in both the contraction amplitude and the beat rate of cultured cardiomyocytes being reported following acute addition of the AA metabolites PGD_2, PGE_2, $PGF_{2\alpha}$, or the compound U46619 (a thromboxane mimetic). Superfusion of neonatal cardiomyocytes with low concentrations of the above compounds resulted in the rapid development of tachyarrhythmias initially characterised by a regular fast rhythm with a reduction in contraction amplitude together with chaotic fibrillatory contractile activity occurring at higher concentrations. The 3-series cyclooxygenase metabolites of EPA (PGD_3 and PGE_3) were less potent than the 2-series cyclooxygenase products, PGE_2 and $PGF_{2\alpha}$. Furthermore, PGI_2 produced a marked reduction in the beat rate and terminated the tachyarrhythmias induced by $PGF_{2\alpha}$ or U46619. Eicosanoid-induced arrhythmias have been reported to be terminated by acute addition of EPA or AA plus the cyclooxygenase inhibitor indomethacin (Li *et al.*, 1997). These later results, while not directly related to dietary effects, may give insight as to how the balance of n-3/n-6 PUFAs could impact on the eicosanoid status of the cardiomyocyte and underlie the proarrhythmic or antiarrhythmic effects of these different families of polyunsaturated fatty acids.

1.8 EFFECTS OF FATTY ACIDS ON CARDIAC ELECTROPHYSIOLOGY

1.8.1 Na^+ and K^+ channels

Cardioprotective properties of dietary fatty acids may be due to modulation of the cardiac action potential (shown in Figure 1.3) with certain fatty acids acting as membrane stabilising agents to slow the rate of the upstroke velocity of the action potential (Kang *et al.*, 1995; Billman *et al.*, 1997). The degree of opening of the fast Na^+-channel and hence the initiation of the action potential is voltage dependent and influenced by the extent and rate of prior depolarisation (Katz, 1977). Certain antiarrhythmic drugs (e.g., quinidine) which cause partial depolarisation of the membrane, can slow the recovery of the ability of the Na^+-channels to reopen. Evidence obtained from measurement of Na^+ currents in patch-clamped neonatal cardiomyocytes (Kang *et al.*, 1995; Xiao *et al.*, 1995) indicate that the n-3 fatty acids may act in a similar manner. Increasing the extracellular K^+ concentration, $[K^+]_o$, lowers the resting membrane potential and causes the cardiomyocyte to be partially depolarised during diastole. Since EPA has been reported to alter the properties of neonatal cardiomyocytes in a similar manner (Kang *et al.*, 1995), n-3 PUFAs may modulate the membrane excitability by altering the $[K^+]_i/[K^+]_o$ ratio by direct interaction with certain cardiac K^+-channels such as the ATP-dependent K^+-channels (K_{ATP}) during ischaemia or, alternatively, indirectly via protein kinase C activation (Muller *et al.*, 1992). The underlying mechanism is possibly related to the inactivation of Na^+-channels and these effects of PUFAs on Na^+ current may be important with regard to the antiarrhythmic effects of the n-3 PUFAs.

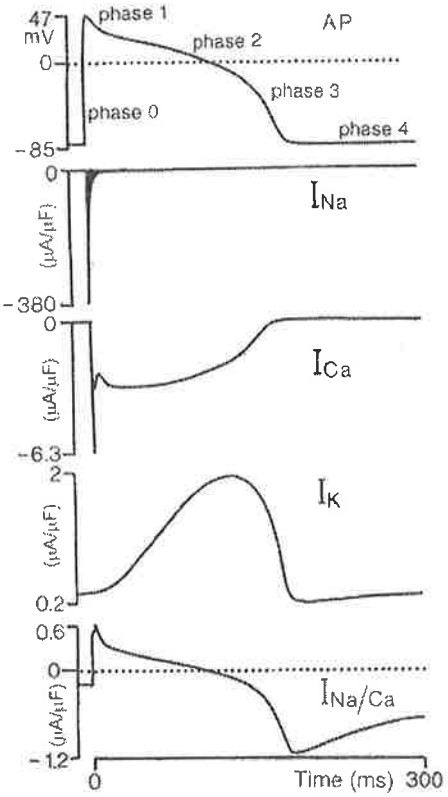


Figure 1.3 Computed currents and ventricular action potential.
The upper panel shows the phases of the action potential. Phase 0, initial rapid depolarisation; Phase 1, initial rapid repolarisation; Phase 2, plateau; Phase 3, repolarisation; Phase 4, resting membrane potential. Also shown are the contribution of various ion currents during the action potential, inlcuding the voltage-dependent Na^+, Ca^{2+} and K^+ currents and the Na^+/Ca^{2+} exchanger current. Taken from Opie (1998b).

1.8.2 Electrical excitability and n-3 PUFAs

A number of studies have demonstrated that acute addition of the n-3 PUFAs alters the automaticity of spontaneously-contracting, neonatal cardiomyocytes maintained in culture (Kang & Leaf, 1994; Kang *et al.*, 1995; Xiao *et al.*, 1995; Kang & Leaf, 1995; Kang & Leaf, 1996c). Application of 5-15 μM EPA or DHA during superfusion of neonatal cardiomyocytes markedly reduced the contraction rate of the rhythmically, spontaneously contracting syncytia. Furthermore, the n-3 PUFAs both prevented and terminated tachyarrhythmias induced by various arrhythmogens. Although a marked reduction in beat rate occurred, there was no significant change in systolic or diastolic $[Ca^{2+}]_i$. In contrast, verapamil (an L-type Ca^{2+}-channel blocker) did not slow the beat rate but induced a progressive decline in the amplitude of contractions and Ca^{2+} transients, both of which finally ceased. Togeteher, these data indicated that acute n-3 PUFA addition did

not mimic the action of Ca^{2+} channel blockers suggesting an inhibitory effect on the electrical automaticity of the cardiomyocytes (Kang & Leaf, 1996c). Similar results regarding the changes in cell excitability following treatment with n-3 PUFAs have been obtained using adult rat ventricular cardiomyocytes (Leifert *et al.*, 1997; Leifert, 1997).

Unlike neonatal cardiomyocytes, adult rat cardiomyocytes do not spontaneously contract but remain quiescent. Electrical field stimulation depolarises the cardiomyocyte sarcolemmal membrane initiating contractions in synchrony with the applied electrical stimulus. When arrhythmogenic agents such as isoproterenol (a β-adrenergic receptor agonist) are added to the superfusing buffer while electrical field stimulation is occurring, triggered activity (early and delayed afterdepolarisations), referred to here as "asynchronous" contractile activity develops, presumably as a result of Ca^{2+} overload (Priori & Corr, 1990; Zeng & Rudy, 1995; De Ferrari *et al.*, 1995; Tweedie *et al.*, 1997). Acute addition of micromolar concentrations of EPA or DHA (as the free acids) to the superfusing medium after the development of asynchronous contractile activity terminates the electrically-driven contractile activity. Upon increasing the applied voltage that was previously held just above the threshold level needed to initiate contractile activity, the cardiomyocytes recommence synchronous contractions even in the presence of the arrhythmogenic agent. This suggests that the addition of EPA or DHA had some influence on cardiomyocyte sarcolemmal membrane excitability. Similarly, when EPA and DHA were superfused over cardiomyocytes prior to the addition of isoproterenol, EPA and DHA also prevented such asynchronous contractile activity at a suprathreshold voltage (Leifert, 1997). The effect of the β-adrenergic receptor antagonist propranolol (a class II antiarrhythmic drug), was similar to that of the calcium channel antagonists (class IV antiarrhythmics) and quite distinct from that of the n-3 PUFAs.

EPA was also shown to protect neonatal cardiomyocytes against arrhythmias induced by a Ca^{2+} ionophore even when intracellular Ca^{2+} levels were maintained at relatively high levels by the experimental conditions (Kang & Leaf, 1996c). Modulation of cardiac contractility may be mediated via alterations in Ca^{2+} cycling within the cardiomyocyte or via changes in the sensitivity of the myofilaments (Damron & Summers, 1997). Alterations in contractile function may also be induced by altering cardiac ion channel activity. Arachidonic acid has been shown to increase the amplitude of the Ca^{2+} transient inducing a two-fold increase in cell shortening when added to spontaneously contracting neonatal cardiomyocytes (Damron & Summers, 1997). Therefore release of AA by phospholipase A_2 action in response to receptor activation by endogenous mediators or pathological stimuli, may be involved in mediating inotropic responses in the myocardium.

1.8.3 Electrical excitability and membrane fluidity

The electrophysiological mechanisms underlying the effects of EPA and DHA are likely to involve changes in automaticity or excitability of cardiomyocytes which may be induced by changes in the physical state of the sarcolemmal membrane lipids, thus affecting one or more of the five phases of the action potential. The mobility and conformation of intrinsic cell membrane proteins, and thus their function as receptors, enzymes and ion channels can be significantly influenced by the physical state of their surrounding membrane lipid environment (Katz & Messineo, 1981a; Katz & Messineo, 1981b; McMurchie *et al.*, 1988; McMurchie, 1988; Nalbone *et al.*, 1990; de Jonge *et al.*, 1996). Since fatty acids are able to quickly partition into the membrane bilayer lipids and likely change the threshold voltage for the gating of Na^+ channels which initiate the

action potential, it has been suggested that changes in membrane fluidity may be associated with the antiarrhythmic effects of the n-3 PUFAs (Leifert, 1997; McMurchie *et al.*, 1998). Indeed, at low concentrations, certain fatty acids display membrane stabilising effects (Katz & Messineo, 1981b) not unlike the effects of local anaesthetics and antiarrhythmic compounds such as lidocaine. Using the technique of steady-state fluorescence anisotropy (SSFA) with the fluidity probe N-((4-(6-phenyl-1,3,5-hexatrienyl)phenyl)propyl) trimethyl-ammonium p-toluenesulfonate (TMAP-DPH), changes in the membrane fluidity of adult rat cardiomyocytes were determined following acute addition of various fatty acids (Leifert, 1997; McMurchie *et al.*, 1998). SSFA values were significantly decreased by EPA and DHA, implying an increase in membrane fluidity and unaltered with either the saturated fatty acid, stearic acid (C18:0), docosanoic acid (C22:0) or the methyl ester form of DHA.

Fusion of DHA into mouse mitochondrial membranes by DHA incubation increased mitochondrial membrane fluidity as detected using the fluorescent membrane probe DPH (Stillwell *et al.*, 1997), which localises in the central hydrophobic region of the membrane bilayer (Lentz, 1989). Using this same system but with the fluidity probe TMA-DPH, which anchors near the bilayer surface due to its charged trimethylammonium head group (Beck *et al.*, 1993; Driessen *et al.*, 1995), it was reported that no change in mitochondrial membrane fluidity was apparent (Stillwell *et al.*, 1997). Interestingly, these same mitochondria infused with DHA were also shown to have decreased membrane potentials when measured using a membrane-potential sensitive fluorescent probe (Stillwell *et al.*, 1997).

Collectively, these data suggested that PUFAs directly alter the excitability of the cardiac sarcolemmal membrane. Thus the n-3 PUFAs may prevent asynchronous contractile activity in the isolated cell model and myocardial arrhythmias *in vivo*, by exerting effects on cell excitability thus preventing the generation of aberrant action potentials and re-entrant circuits.

1.9 EFFECTS OF FATTY ACIDS ON INTRACELLULAR Ca^{2+} MOBILISATION

Following depolarisation, the contractile activity of the myocardium is under the control of Ca^{2+}, which is controlled by extracellular Ca^{2+} influx into the cardiomyocyte as well as signalled release of Ca^{2+} from intracellular stores, notably the sarcoplasmic reticulum (SR). Relaxation is in part a reversal of these processes although different enzyme systems and sequestering mechanisms are involved. An enhanced release of the second messenger IP_3 from the SR has been reported to be associated with the development of ischaemic and reperfusion associated ventricular arrhythmias (Du *et al.*, 1995; Anderson *et al.*, 1995; Anderson *et al.*, 1996; Woodcock *et al.*, 1996). Inhibition of this IP_3 release has been suggested to exert an antiarrhythmic effect (Anderson *et al.*, 1996). Du (Du *et al.*, 1995) investigated the effect of fish oil supplementation administered by gavage on post-ischaemic reperfusion arrhythmias and reported that dietary n-3 PUFA supplementation significantly inhibited both increases in intracellular IP_3 levels and the incidence of reperfusion arrhythmias. Experiments using cultured neonatal cardiomyocytes exposed to DHA for 3 days also support the involvement of the IP_3 pathway in the antiarrhythmic action of DHA (Reithmann *et al.*, 1996). For example, the arrhythmias induced in cardiomyocytes by α_1-adrenoceptor stimulation (which utilises the phosphoinositide signalling system) were prevented following DHA incubation. In

addition, n-3 PUFA pretreatment has been reported to decrease the α_1-adrenoceptor-stimulated formation of IP_3 (de Jonge *et al.*, 1996; Reithmann *et al.*, 1996). Dietary EPA supplementation in a canine arrhythmia model has been reported to increase the (Ca^{2+}-Mg^{2+}) ATPase activity in a myocardial microsomal fraction (presumably enriched in sarcoplasmic reticulum membranes) (Kinoshita *et al.*, 1994). These effects were associated with an increased ratio of EPA to AA within this cellular fraction. Although feeding rats fish oil for 21 days has been shown to increase the n-3/n-6 fatty acid ratio in cardiac sarcoplasmic reticulum, the cardiac sarcoplasmic reticulum membrane associated Ca^{2+}-ATPase activity measured was reduced in animals fed the fish oil supplemented diet (Taffet *et al.*, 1993). It is therefore likely that the severity of ventricular arrhythmias may be reduced by inhibiting the accumulation of intracellular Ca^{2+} following ischaemia by modulating other mechanisms responsible for cellular Ca^{2+} extrusion such as the Na^{+}/Ca^{2+} exchanger. Preventing the frequency of spontaneous Ca^{2+} release from the sarcoplasmic reticulum may also be implicated (Negretti & O'Neill, 1997). Therefore, enrichment of the dietary n-3 PUFA supply which subsequently elevates the proportions of EPA and DHA in myocardial sarcolemmal and sarcoplasmic reticulum membrane phospholipids, may prevent IP_3-induced Ca^{2+} oscillations and the development of subsequent arrhythmias.

1.10 AIMS

Identify potential cardioprotective mechanisms of n-3 polyunsaturated fatty acid action at the level of the cardiomyocyte by comparing dietary and acute studies using adult rat cardiomyocytes.

1.11 HYPOTHESES

Dietary n-3 polyunsaturated fatty acids (from fish oil) provide antiarrhythmic effects by significantly changing the fatty acid profile of the cardiomyocyte lipids resulting in alterations of lipid-dependent processes that control membrane function such as the contractility and excitability of cardiac myocytes.

CHAPTER 2

2 "Acute effects of PUFAs on cardiomyocyte contractility"

2.1 INTRODUCTION

2.1.1 Antiarrhythmic effects of dietary n-3 PUFAs

Recent evidence suggests that dietary n-3 polyunsaturated fatty acids (PUFAs), in particular, docosahexaenoic acid (DHA; 22:6, n-3) which is abundant in fish oil, can reduce both CHD incidence and mortality (Siscovick *et al.*, 1995). Prominent amongst these studies are those of Kromhout (Kromhout *et al.*, 1985), Burr (Burr *et al.*, 1989) and Siscovick (Siscovick *et al.*, 1995). In the latter study it was demonstrated that an intake of 5.5 g of n-3 fatty acids per month (equivalent to one fatty fish meal per week) was associated with a 50% reduction in the risk of primary cardiac arrest. Taken together these results suggest that dietary n-3 PUFAs improve the chances of surviving an episode of myocardial ischaemia and prevent primary cardiac arrest, and they may do so by their intrinsic antiarrhythmic activity.

The above epidemiological studies augment experimental animal studies which have established that dietary supplementation with n-3 PUFAs in the form of fish oil, alters the membrane phospholipid fatty acid composition of cardiomyocytes primarily by an increase in the proportion of n-3 PUFAs, particularly DHA (McLennan *et al.*, 1988; Abeywardena & Charnock, 1995). Accompanying this, a decrease in the incidence of ischaemia-induced and reperfusion-induced ventricular arrhythmias (McLennan *et al.*, 1988; McLennan *et al.*, 1993), and an increase in the ventricular fibrillation threshold in both rats and marmosets have been reported (McLennan *et al.*, 1993; Pepe & McLennan, 1996).

2.1.2 *Cardiomyocyte automaticity*

Automaticity, or spontaneous electrical activity arising from a specific focus, is a normal property of sinus nodal, atrioventricular junctional, and His-Purkinje tissue. Under pathological conditions, automaticity can occur at accelerated rates in specialised fibres and in the working myocardium (Opie, 1998b). Therefore, the pathological process leads to partial depolarisation of the tissue, resulting in spontaneous impulses. Various tachyarrhythmias originating in infarcted myocardium are likely caused by such a mechanism (Katz, 1977).

Automaticity may also be caused by triggered activity. Triggered activity refers to arrhythmias arising as a consequence of depolarisations in membrane potential during the diastolic interval. Triggered activity requires an impulse from another site to first excite the focus, which then triggers repetitive action potentials by generating "afterdepolarisations". Early afterdepolarisations occur before the membrane has completely repolarised from a prior action potential. Membrane potential remains partially depolarised and is subsequently more likely to generate further action potentials. Delayed (or late) afterdepolarisations occur after the membrane has returned to the resting potential and are often caused by an increase in the intracellular $[Ca^{2+}]$ during diastole. Triggered arrhythmias resulting from afterdepolarisations include tachycardias arising from digitalis toxicity and ischaemia (Rosen & Bigger, 1991).

2.1.3 *Automaticity and n-3 PUFAs in cultured rat neonatal cardiomyocytes*

The antiarrhythmic properties of the n-3 PUFAs have been studied *in vitro* using isolated multicellular cardiomyocyte preparations from 2 day-old neonatal rats (Kang & Leaf, 1995;

Weylandt *et al.*, 1996; Leaf & Kang, 1996; Kang & Leaf, 1996c). In the abovementioned studies the neonatal cells were cultured and formed syncytia that spontaneously depolarise and contract. Following the introduction of various arrhythmogenic stimuli, the syncitia display tachyarrhythmia-type contractions (the rate of contractions are increased) and this is often followed by lethal ventricualr fibrillation. However, this type of aberrant contractility could be prevented or terminated by acute addition of free (non-esterified) n-3 PUFAs, and to a lesser extent, n-6 PUFAs, but not saturated fatty acids.

2.1.4 This study

The present study sought to determine whether the antiarrhythmic effects of the n-3 PUFA, DHA, were associated with changes in membrane fluidity using the adult rat ventricular myocyte as the experimental model. This cell preparation involves isolating viable ventricular cardiomyocytes from adult rat hearts. The preparation is not cultured and the cells remain quiescent (i.e., do not spontaneously depolarise and contract) under normal conditions. In contrast to previous results using electrically-field stimulated adult rat cardiomyocytes (McMurchie *et al.*, 1998), these studies involved establishing a model of predictable spontaneous contractile activity induced by isoproterenol or lysophosphatidylcholine as arrhythmogenic agents in non-stimulated adult rat cardiomyocytes. Whilst this model approximates the neonatal cardiomyocyte model developed by Leaf and colleagues (Kang & Leaf, 1995; Weylandt *et al.*, 1996; Leaf & Kang, 1996; Kang & Leaf, 1996c), importantly, it would allow a comparison to determine whether similarity exists between the freshly isolated adult rat cardiomyocytes and the cultured neonatal rat heart myocytes with regard to the antiarrhythmic effects of the n-3 PUFAs, particularly DHA. Additionally, it is necessary to determine whether the effects of the n-3

PUFAs are specific to the free fatty acids or whether other forms of lipids containing n-3 PUFAs, are also antiarrhythmic.

2.2 METHODS

2.2.1 Animals

Ethics approval (#555) was obtained from the CSIRO Health Sciences and Nutrition Animal Experimentation Ethics Committees. Male Sprague Dawley rats from the Adelaide University, Adelaide, SA, Australia, were used at 12 weeks of age. Animals were housed in groups of up to 5 per cage with food and water provided *ad libitum*. Room temperature was maintained at 23°C with constant 55% humidity, and lights were maintained on a 12 hour light (8am - 8pm)/dark cycle.

2.2.2 Perfusion and culture media

Calcium-free Tyrode perfusion media, contained (in mM) 137.7 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose, 10 (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (HEPES), pH 7.4. $CaCl_2$ was added prior to use to give the appropriate concentrations indicated. Tyrode solution was prepared using ultra-pure (Milli-Q) water, filtered through a 0.22 μm Millipore filter prior to use, and gassed with 100% O_2. DMEM culture medium was supplemented with 1 mM Ca^{2+}, 10 mM HEPES, 25 mM $NaHCO_3$, 100 U/ml Penicillin G, 100 μg/ml Streptomycin, 0.05 μg/ml Amphotericin B, 2 mM carnitine, 5 mM creatine, 5 mM taurine and 1 mg/ml BSA.

2.2.3 Preparation of adult rat ventricular cardiomyocytes

Enzymatic isolation of cardiac myocytes was performed by a method previously described by the author (Leifert, 1997). Briefly, male Sprague Dawley rats (300-350g) were injected with heparin (2000 units i.p.). After 30 min, pentobarbitone sodium

(60mg/kg) was injected intraperitoneally. The heart was removed, washed in an ice-cold, oxygenated, calcium-free Tyrode's solution for 1 min, and then perfused, via an aortic cannula. This facilitated the removal of blood from both the coronary vasculature and ventricular chambers. Hearts were perfused in a retrograde, non-recirculating Langendorff mode with Tyrode solution containing 1.5 mM Ca^{2+} for 4 min followed by nominally Ca^{2+}-free Tyrode solution (non-recirculating) for 2 min. The buffers were maintained at 37°C and gassed with 100% O_2 during perfusion. The heart was then perfused in a recirculating manner for 30 min with Tyrode solution supplemented with 20 μM Ca^{2+}, 300 U/ml collagenase (type 1A) and 0.1% (w/v) delipidated BSA (fraction V) at 37°C. Ventricles were teased apart and agitated in Tyrode solution containing 20 μM Ca^{2+}, 2% (w/v) BSA and 30 mM 2,3-butane-dione monoxime at 25°C. The suspension was filtered through a 250 μm nylon-mesh gauze. The concentration of Ca^{2+} was increased stepwise to 1 mM over 45 min (40, 100, 250, 500 and 1000 μM). Aliquots of cardiomyocyte suspension were added to petri dishes containing 12 mm (diameter) glass coverslips coated with laminin (25 μg/ml). This procedure allowed rod-shaped cardiomyocytes to adhere to coverslips within 60 min at room temperature (>90% rod-shaped and viable as determined using trypan blue staining). The glass cover slips with viable, adhering cardiomyocytes, were washed twice with DMEM culture medium containing 1 mM Ca^{2+}, pre-equilibrated with 5% CO_2:95% O_2. Cardiomyocytes were maintained in DMEM in a humidified incubator at 37°C and gassed with 5% CO_2 in air.

2.2.4 Induction of spontaneous and asynchronous contractile activity

Coverslips with adhering quiescent cardiomyocytes were placed in a custom-designed superfusion chamber (0.7 ml) mounted on the stage of an inverted Nikon microscope housed in a perspex chamber maintained at 37°C. Standard procedure included 3 min superfusion

(equilibration) of cardiomyocytes in Tyrode buffer containing 1 mM Ca^{2+} followed by a 4 min preincubation period with a fatty acid, phosphatidylcholine lipid, mono-, di- or tri-glyceride or a control as indicated in results (in the absence of electrical stimulation). Superfusion was at a rate of 2 ml/min.

Under these conditions quiescent cardiomyocytes (in the absence of electrical-field stimulation) when treated with the arrhythmogenic agents, isoproterenol (10 μM) or lysophosphatidylcholine (10 μM), begin to contract spontaneously. An example of a single cardiomyocyte displaying spontaneous contractile activity is shown in Figure 2.1. To test the effects of various agents on preventing spontaneous contractile activity, the agents were added to the superfusing solution 4 min prior to the addition of isoproterenol or lysophosphatidylcholine. The saturated fatty acids, stearic acid (18:0), docosanoic acid (22:0) or glycerol (30 μM) were used as controls. The percentage of cardiomyocytes spontaneously contracting was determined by "scoring" 20 - 40 cells in duplicate before and after induction of spontaneous contractile activity for the number of heart preparations shown in the figure legends. In some experiments, asynchronous contractile activity was scored following electrical-field stimulation (25 V, pulse duration of 5 ms at 1 Hz) using two platinum wire electrodes located at either side of the superfusion chamber connected to a Grass S4 stimulator. Asynchronously contracting cells (in the presence of electrical-field stimulation) were defined as those cells that exhibited a contraction rate exceeding the rate of applied electrical stimulation which was maintained at 1 Hz (i.e., cells not contracting in synchrony with the applied electrical stimulus). Asynchronous contracting cells usually contracted at 2-4 contractions per second as shown in Figure 2.2.

To determine changes in cell length (for the purpose of demonstrating spontaneous and asynchronous contractile activity in a single cardiomyocyte as in Figures 2.1 and 2.2) the following was carried out. Cardiomyocytes on coverslips were placed in a custom-designed superfusion chamber and superfused with Tyrode buffer containing 1mM Ca^{2+} at 37°C and allowed to equilibrate for 2 min. Cell length was determined as described previously (Leifert, 1997). Briefly, a CCD video camera mounted on an inverted Olympus microscope housed in a perspex chamber maintained at 37°C, transferred images at a frame rate of 25 sec^{-1} to a pentium computer connected between the camera and the monitor. An on-line, real-time computer program was developed using LabVIEW (National Instruments, Victoria, Australia) which allowed for continuous observation of changes in cell length during contractile activity (Leifert, 1997). Cellular arrhythmia, measured as spontaneous or asynchronous contractile activity, was induced as described above.

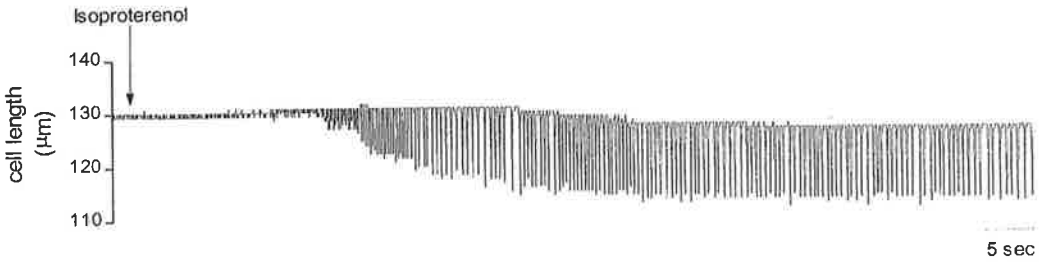


Figure 2.1 Spontaneous contractile activity in an adult rat cardiomyocyte.
Representative recording of the change in cell length of a single cardiomyocyte in response to 10 µM isoproterenol. The cardiomyocyte was continuously superfused with isoproterenol (added as indicated by the arrow) which resulted in the quiescent cell developing rapid spontaneous contractions after approximately 5 min. Similar spontaneous contractile activity to the above were obtained using lysophosphatidylcholine.

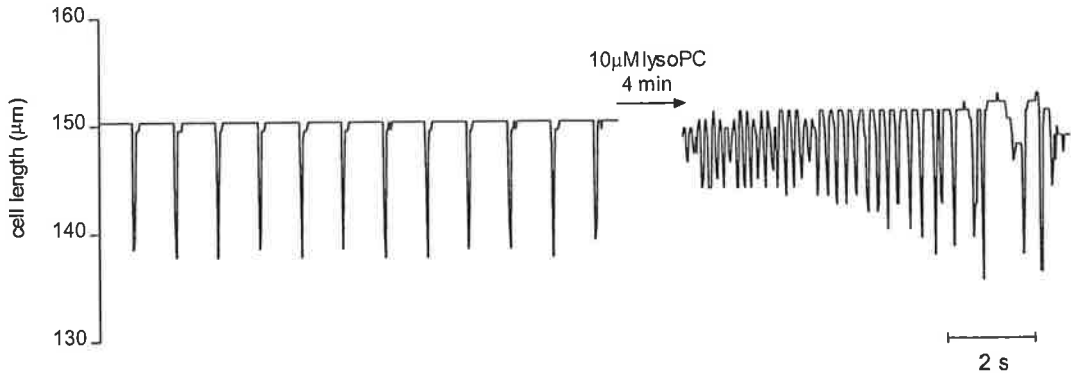


Figure 2.2 Asynchronous contractile activity in an adult rat cardiomyocyte.
Change in cell length of a single cardiomyocyte in response to 10 µM lysophosphatidylcholine. The cardiomyocyte was continuously electrically-field stimulated (1 Hz) and superfused with lysophosphatidylcholine for 4 min resulting in the development of rapid asynchronous contractile activity as shown on the right. Similar asynchronous contractile activity was also demonstrated with isoproterenol.

2.2.5 Cardiomyocyte membrane potential using DiBAC$_4$(3)

In experiments measuring membrane potential the anionic lipophilic potential-sensitive fluorescent dye bis(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC$_4$(3)] was used as described previously (Lakos *et al.*, 1990; Stevens *et al.*, 1994; Langheinrich & Daut, 1997). The partitioning of DiBAC$_4$(3) between the plasma membrane and cytosol is a function of the membrane potential and follows the Nernst equation (Langheinrich & Daut, 1997). With membrane depolarisation more dye enters the cell and this results in increased binding to proteins and enhanced fluorescence (Brauner *et al.*, 1984).

Freshly prepared cardiomyocytes on coverslips were incubated in the dark with Tyrode solution containing 50 nM DiBAC$_4$(3) for 10 min at 37°C and then continually superfused with this solution. Single cells were visualised using a 40x fluoresence objective mounted on an inverted Nikon (Tokyo, Japan) fluorescence microscope. Fluorescence was monitored at an excitation of 490 nm (175 W Xe lamp) and emission was set at 510 nm via a dichroic mirror and lightpath filter set at 535 nm and subsequently measured with a

photomultiplier tube. The fluorescence was only determined within the cell boundary by use of an adjustable diaphragm. The data acquisition rate was set at 5 samples·s^{-1}. The data are presented in arbitrary units of fluorescence (i.e., membrane potential was not determined by standard calibration procedures due to cell rounding and hypercontracture resulting in loss of dye when ionophores were used for calibration). However, relative membrane potential was estimated by considering K^+ only, using the Nernst equation:

$$E_m = -61.5 \log[K_i/K_o]$$

where E_m is the membrane potential, K_i and K_o are the concentrations of K^+ inside and outside the cell, respectively. K_i was taken as 80 mM (Opie, 1998b).

2.2.6 Cardiomyocyte sarcolemmal membrane fluidity measurements

Membrane fluidity was determined by measuring the steady-state fluorescence anisotropy (r_{ss}) of the probe N-((4-(6-phenyl-1,3,5-hexatrienyl)phenyl)propyl) trimethyl-ammonium p-toluenesulfonate (TMAP-DPH) (Molecular Probes, Eugene, OR) according to a modification of the method of de Jonge *et al.* (de Jonge *et al.*, 1996). This probe, which contains a three-carbon spacer between the fluorophore and the trimethylammonium substituent, readily partitions into the cell membrane (Lentz, 1989). Isolated ventricular cardiomyocytes attached to laminin-coated glass coverslips, were washed in Tyrode buffer. Cardiomyocytes (in triplicate) were then placed in glass cuvettes containing 1 μM TMAP-DPH and readings were taken at 3 min intervals. Constant r_{ss} values were obtained after 10 to 12 minutes and data were derived after a total incubation time of 15 minutes at 37°C according to the following formula as described by Lentz (Lentz, 1989):

$$r_{ss} = \frac{(I_{VV} - GI_{VH})}{(I_{VV} + 2GI_{VH})}$$

where I_{VV} and I_{VH} represent the fluorescence intensity parallel and perpendicular to the excitation plane (when set vertically), respectively. G is a correction factor for the difference in the transmission efficiency for vertically and horizontally polarised light, and is calculated by I_{HV}/I_{HH}. Measurements were obtained using a Hitachi 650-10S spectrofluorimeter (Tokyo, Japan) provided with vertical and horizontal polarisation filters (Polaroid, Australia). The excitation and emission monochromators were positioned at wavelengths of 350 nm and 430 nm respectively, with slitwidth set to 10 nm in both excitation and emission modes. Readings were corrected for both background fluorescence of TMAP-DPH and light scatter by the cardiomyocyte preparation itself.

2.2.7 Chemicals

DMEM culture medium, bovine serum albumin (BSA, fraction V), carnitine, creatine, taurine, 2,3-butane-dione monoxime, collagenase type 1A, laminin, isoproterenol and L-α-phosphatidyl choline were from Sigma Chemical Co. (Castle Hill, NSW, Australia). 50 mM stock solutions of fatty acids (and other lipids used) were prepared in ethanol containing 0.003% (w/v) 2[3]-t-butyl-4-hydroxyanisole and stored at -80°C under N_2. Fatty acids, phosphatidylcholine lipids, mono-, di- and tri-glycerides were from Nu-Chek-Prep-Inc. (Elysian, MN, USA). Solutions of isoproterenol (5 mM stock) were prepared daily in 10 mM ascorbic acid. Solutions of lysophosphatidylcholine were prepared in ethanol. Penicillin/Streptomycin was from GIBCO-BRL (Melbourne, Australia). BSA was delipidated by washing in acetone, petroleum spirit and diethyl ether. N-((4-(6-phenyl-

1,3,5-hexatrienyl)phenyl)propyl) trimethyl-ammonium p-toluene-sulfonate (TMAP-DPH) and bis-(1,3-dibutylbarbituric acid)trimethineoxonol ($DiBAC_4(3)$) was from Molecular Probes (Eugene, OR, USA). All other chemicals were of the highest grade available.

2.2.8 Statistics

Statistical analysis was performed using the computer software program Instat (GraphPad Software, CA, USA). Student's unpaired t-test, Welch's test or One-way or Two-way ANOVA with Bonferroni multiple comparison tests was used to compare differences between means. For each comparison the significance level was set at $P<0.05$. Data are expressed as mean ± SEM (n = the number of rat heart preparations).

2.3 RESULTS

Following the above isolation procedure, >90% of the isolated cardiomyocytes which adhered to the laminin-coated coverslips were quiescent and rod-shaped with clear cross-striations and no observable cell membrane blebbing.

2.3.1 Lysophosphatidylcholine-induced spontaneous and asynchronous contractile activity

Following 5 min treatment of cardiomyocytes with lysophosphatidylcholine, the number of cardiomyocytes spontaneously contracting (i.e., in the absence of electrical stimulation), or asynchronously contracting (during electrical stimulation), was dependent on the fatty acid preincubation. Figure 2.3 shows the percentage of cardiomyocytes spontaneously contracting was significantly lowered ($P<0.0001$) by preincubating cardiomyocytes with docosahexaenoic acid (DHA) compared with stearic acid (SA, 18:0). Furthermore, the antiasynchronous effect of DHA was also significantly lower than SA ($P<0.001$). Oleic acid (OA, 18:1, n-9) treatment resulted in a similar percentage of cardiomyocytes spontaneously or asynchronously contracting compared with that obtained with SA treatment of cardiomyocytes. However, the n-3 PUFA, α-linolenic acid (ALA, 18:3, n-3) significantly ($P<0.05$) prevented both spontaneous and asynchronous contractile activity compared with SA and OA.

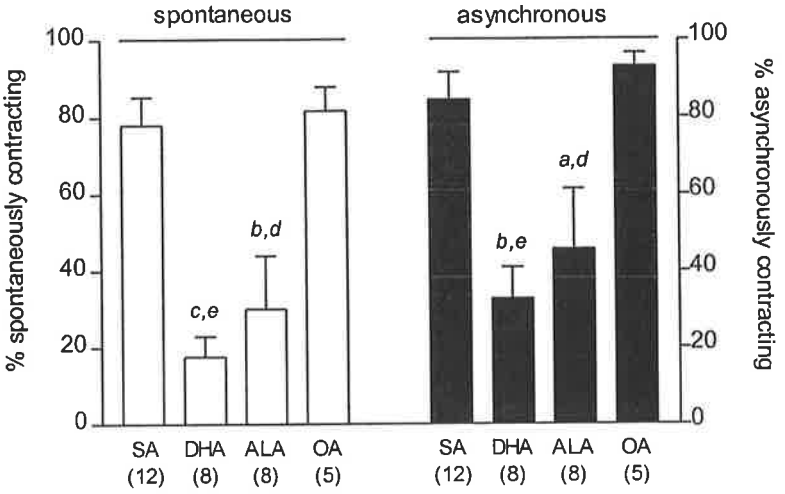


Figure 2.3 Effects of fatty acids on lysophosphatidylcholine-induced spontaneous and asynchronous contractile activity in adult rat ventricular cardiomyocytes.
Cardiomyocytes were preincubated for 5 min in 10 μM fatty acids as indicated. Following 5 min of lysophosphatidylcholine (10 μM) superfusion, the percentage of spontaneously-contracting cardiomyocytes (open bars) was determined as the number of cells exhibiting rapid spontaneous contractions (in the absence of electrical field stimulation, usually at a rate of 1-4 spontaneous contractions per second), or asynchronous contractions (filled bars) as contractions exceeding the rate of electrical-field stimulation of 1 Hz). Abbreviations; SA, stearic acid; DHA, docsoahexaenoic acid; ALA, α-linolenic acid; OA, oleic acid. Each value represents the mean ± SEM for the number of preparations indicated in parentheses. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ vs SA. $^{d}P<0.05$, $^{e}P<0.01$ vs OA.

To determine whether longer term treatment (20 h) of cardiomyocytes with DHA afforded protection from lysophosphatidylcholine-induced spontaneous contractile activity, cardiomyocytes were incubated with DMEM containing 0.1% BSA supplemented with 100 μM DHA (treatment) or an equivalent concentration of ethanol (control). Following a brief washout procedure to remove any unbound (non-esterified) DHA the cardiomyocytes were challenged with 1 μM lysophosphatidylcholine for 10 min. Following this procedure, there was no significant difference between the percentage of cardiomyocytes contracting spontaneously when comparing control and DHA (Figure 2.4). Although the majority of free (unesterified) DHA would have likely been washed away during this procedure, incorporation of DHA into membrane phospholipids following this 20 h incubation was not measured.

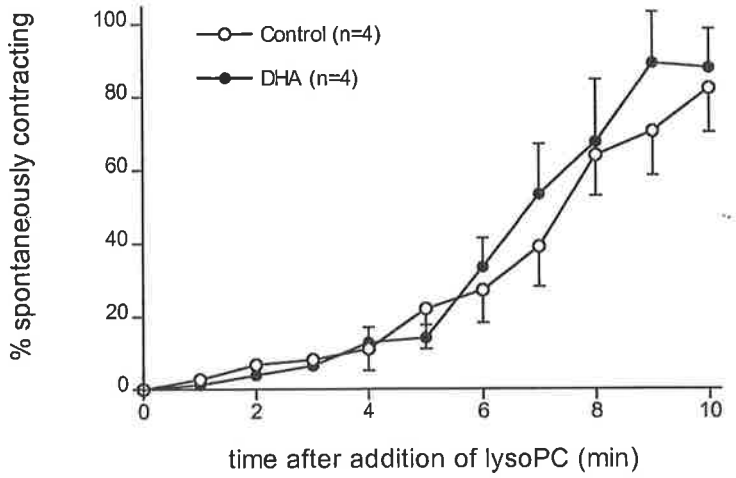


Figure 2.4 Effects of incubation with DHA on lysophosphatidylcholine-induced spontaneous contractile activity in adult rat ventricular cardiomyocytes.
Cardiomyocytes were incubated for 20 h in DMEM culture medium containing 0.1% (w/v) delipidated BSA (control, n=4) or BSA supplemented with 100 μM DHA (n=4). Coverslips containing adhering cardiomyocytes were washed and superfused for 5 min prior to addition of lysoPC (lysophosphatidylcholine). The percentage of spontaneously-contracting cardiomyocytes was determined as the number of cells exhibiting rapid spontaneous contractions (in the absence of electrical field stimulation). Each value represents the mean ± SEM for the number of preparations indicated in parentheses.

2.3.2 Effects of phospholipids and glyceride lipids on spontaneous and asynchronous contractility

To determine whether various forms of lipids could prevent spontaneous and asynchronous contractile activity, phosphatidylcholine lipids (all at 30 μM final concentration) with differing fatty acid groups were preincubtaed with cardiomyocytes prior to addition of lysophosphatidylcholine (n=3 cardiomyocyte preparations). The phosphatidylcholine lipids, phosphatidylcholine dimyristoyl (14:0/14:0), phosphatidylcholine β-linoleoyl-γ-stearoyl (18:2/18:0) and β-docosaheaxaenoyl-γ-stearoyl (22:6/18:0), all significantly prevented the development of spontaneous as well as asynchronous contractile activity induced by lysophosphatidylcholine as shown in Figure 2.5. Therefore, the protective effect against lysophosphatidylcholine was non-

specific and independent of the nature of the fatty acid class substituted into the phosphatidylcholine lipid. Similarly, Figure 2.6A demonstrates that there was a significant protective effect of all monoglycerides tested (containing the SA, OA, ALA or DHA fatty acid moieties) against lysophosphatidylcholine-induced spontaneous and asynchronous contractile activity, thus indicating non-specific effects. However, diglycerides and triglycerides of the same fatty acids failed to elicit protective effects against lysophosphatidylcholine-induced spontaneous and asynchronous contractile activity as shown in Figure 2.6 B and C.

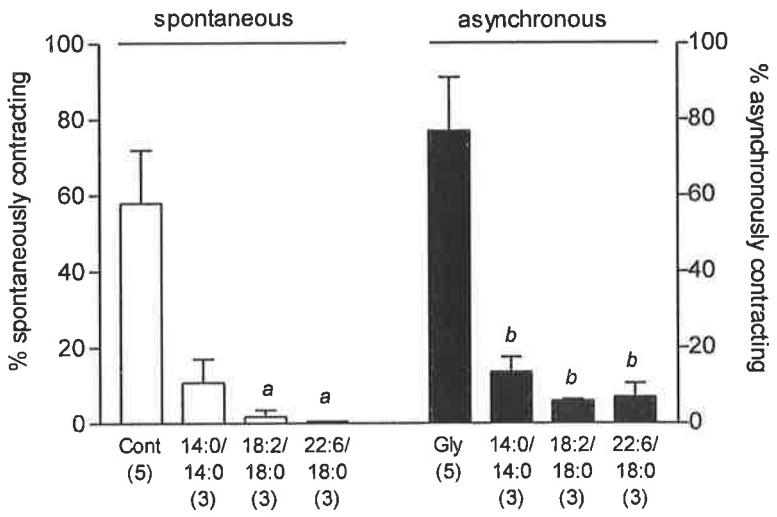


Figure 2.5 Effects of various phosphatidylcholine lipid species on lysophosphatidylcholine-induced spontaneous and asynchronous contractile activity in adult rat ventricular cardiomyocytes.
Cardiomyocytes were preincubated for 4 min in 30 μM phosphatidylcholine lipids as indicated. Following 5 min of lysophosphatidylcholine (10 μM) superfusion, the percentage of spontaneously-contracting cardiomyocytes (open bars) was determined as the number of cells exhibiting rapid spontaneous contractions (in the absence of electrical field stimulation, usually at a rate of 1 - 4 spontaneous contractions per second), or asynchronous contractions (filled bars) as contractions exceeding the rate of electrical-field stimulation of 1 Hz. Abbreviations; Cont, control; 14:0/14:0, phosphatidylcholine dimyristoyl; 18:2/18:0, phosphatidylcholine β-linoleoyl-γ-stearoyl; 22:6/18:0, β-docosaheaxaenoyl-γ-stearoyl. Each value represents the mean ± SEM for the number of preparations indicated in parentheses. $^{a}P<0.05$, $^{b}P<0.01$ vs Gly (control).

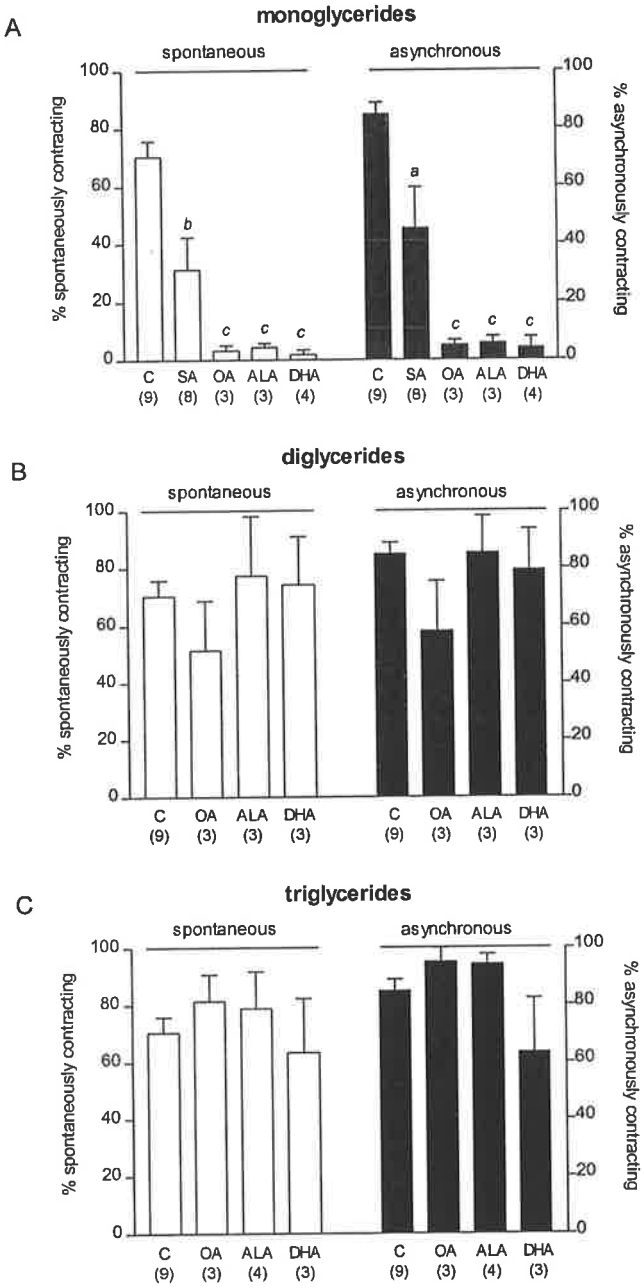


Figure 2.6 Effects of various glyceride lipids on lysophosphatidylcholine-induced spontaneous and asynchronous contractile activity in adult rat ventricular cardiomyocytes.
Cardiomyocytes were preincubated for 4 min in 30 μM of monoglyceride (*A*), diglyceride (*B*) or triglyceride (*C*) lipids as indicated. Following 5 min of lysophosphatidylcholine (10 μM) superfusion, the percentage of spontaneously-contracting cardiomyocytes (open bars) or asynchronously contracting cardiomyocytes (filled bars) was determined. Abbreviations; C, control (glycerol); OA, oleic acid, ALA, α-linolenic acid; DHA, docosahexaenoic acid. Each value represents the mean ± SEM for the number of preparations indicated in parentheses. $^{a}P<0.05$, $^{b}P<0.01$ vs control.

2.3.3 *Isoproterenol-induced spontaneous and asynchronous contractile activity*

Spontaneous contractile activity (in the absence of electrical field stimulation) was determined by comparing the contractility of cardiomyocytes before and after addition of 10 μM isoproterenol. As shown previously in Figure 2.1, the generation of spontaneous contractility in a single cardiomyocyte began after 5 min superfusion with Tyrode buffer containing isoproterenol. Under these conditions the population of quiescent cardiomyocytes began to progressively display rapid spontaneous contractions at a rate of usually 1-4 contractions per second. However, 5 min preincubation with 10 μM DHA significantly prevented the development of isoproterenol-induced spontaneous contractions (Figure 2.7). The time course for the percentage of cardiomyocytes spontaneously contracting is shown in Figure 2.7 which compares the effects of the saturated fatty acid (22:0), docosanoic acid (DA, n=5), OA (n=6), ALA (n=7), DHA (n=5) and the membrane fluidising agent, benzyl alcohol (n=3). The percentage of cardiomyocytes treated with the saturated fatty acid, DA, increased to 48.1 ± 7.7% after 10 min of isoproterenol incubation. However, treatment with DHA (7.1 ± 2.4%, $P<0.001$), ALA (10.6 ± 3.6, $P<0.001$) or benzyl alcohol significantly prevented isoproterenol-induced spontaneous contractile activity (Figure 2.7).

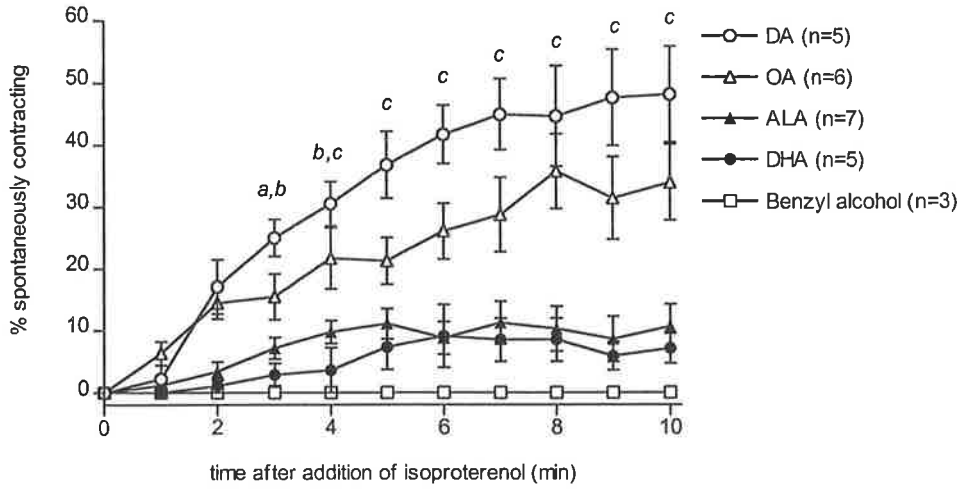


Figure 2.7 Time-course for the development of isoproterenol-induced spontaneous contractile activity in adult rat ventricular myocytes.

Cardiomyocytes were preincubated for 5 min in either 10 μM docosanoic acid (O), oleic acid (△), α-linolenic acid (σ) docosahexaenoic acid (DHA) (λ) or 10mM benzyl alcohol (□). Following addition of 10 μM isoproterenol to the superfusing medium, the number of spontaneously contracting cardiomyocytes was determined over 10 min as the percentage of cells exhibiting rapid spontaneous contractions. Each value represents the mean ± SEM. The number of preparations is indicated in parentheses. ^{a}P<0.05 ALA vs DA, ^{b}P<0.01, ^{c}P<0.001 for ALA, DHA and benzyl alcohol vs DA. Abbreviations, DA; docosanoic acid (22:0), OA; oleic acid (18:1, n-9), ALA; α-linolenic acid (18:3, n-3), DHA; docosahexaenoic acid (22:6, n-3).

2.3.4 Contractile responses of electrically-stimulated cardiomyocytes to KCl depolarisation.

To determine whether the n-3 PUFA, DHA exerts protective effects by a mechanism involving the prevention of depolarisation-induced contractile activity, cardiomyocytes were incubated in increasing concentrations of KCl. In the presence of electrical-field stimulation at 1 Hz, and using physiological concentrations of K^+ (6 mM), 100% of cardiomyocytes exhibited contractile responses. However, increasing the concentration of KCl in the superfusing buffer caused cardiomyocytes to progressively cease contracting in the presence of electrical-field stimulation as shown if Figure 2.8. At 45 mM $[K^+]_o$ (in the absence of DHA), 73.5 ± 5.4 % of cardiomyocytes were contracting, however, in the presence of 20 μM

DHA (and 45 mM KCl), the percentage of cardiomyocytes contracting was significantly reduced to 32.0 ± 6.0 % ($P<0.001$) as shown in Figure 2.8. This result suggests that the n-3 PUFA may be partially depolarising the cell, which together with possible inactivation of Na^+ channels, resulted in a reduction in the number of cardiomyocytes able to contract. In a separate experiment, the relative change in membrane potential was measured. Figure 2.9 demonstrates that there was an increase in the fluorescence of the membrane potential-sensitive dye $DiBAC_4(3)$ (50 nM), in the presence of DHA, suggesting that the n-3 PUFA was able to depolarise the cell. Accurate calibration of membrane potential was not possible due to the lytic effects of the ionophores required to be used, therefore a qualitative measure of membrane depolarisation could only be obtained. However, if the Nernst equation for the membrane potential (due to K^+ only) was applied, the estimated membrane potentials increased from –74 mV under control conditions to –26 mV with 30 mM KCl or to –30 mV with 15 μM DHA (once again, these "estimated" values should be viewed with caution, since the method did not allow for quantitative comparisons).

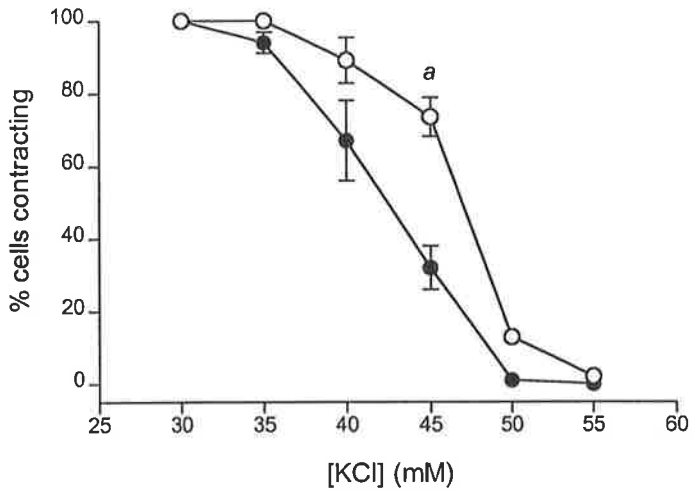


Figure 2.8 Contractile responses of electrically-stimulated cardiomyocytes to KCl depolarisation.
Cardiomyocytes were electrically stimulated to contract at 1 Hz in the presence of increasing concentrations of KCl. The percentage of cardiomyocytes contracting was determined in the absence (O) or presence (λ) of 20 μM DHA. [a]$P<0.001$. Each value represents the mean ± SEM (n=8 preparations).

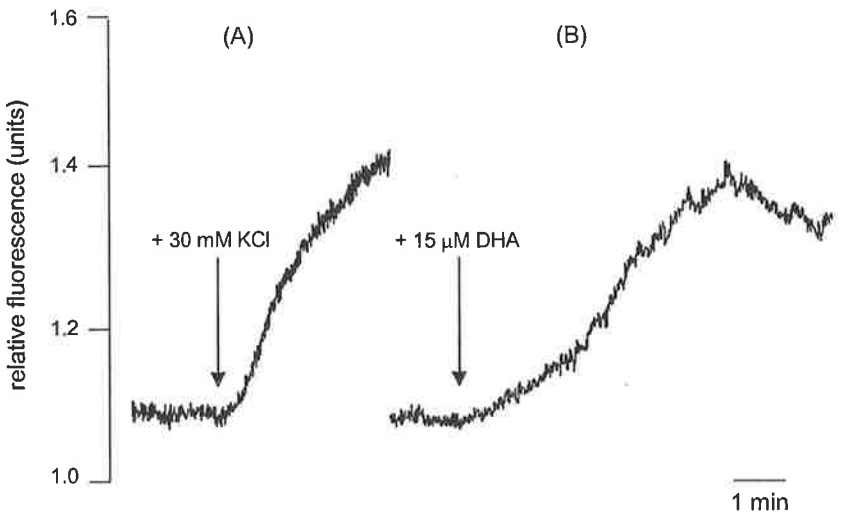


Figure 2.9 Changes in membrane potential in quiescent cardiomyocytes.
Fluorescence (arbitrary units) was determined using the membrane potential-sensitive dye $DiBAC_4(3)$ (50 nM). In the presence of $DiBAC_4(3)$ cardiomyocytes were superfused with Tyrode solution containing an extra 30 mM KCl (*A*) or 15 µM DHA with physiologic $[K^+]$ (*B*).

2.3.5 Steady-state fluorescence anisotropy of rat cardiomyocytes

To determine the effects of DHA and the membrane fluidising agent benzyl alcohol on sarcolemmal membrane fluidity, steady-state fluorescence anisotropy (r_{ss}) of TMAP-DPH was measured. This probe readily partitions at the lipid/water interface of the lipid bilayer (Beck *et al.*, 1993). Figure 2.10 shows the r_{ss} values of cardiomyocytes following treatment with DHA or benzyl alcohol incubation at several concentrations. DHA significantly decreased the fluorescence anisotropy value (r_{ss}) of TMAP-DPH (i.e., increased membrane fluidity) in cardiomyocytes from 0.260 ± 0.002 (no additions, n=6) to a value of 0.241 ± 0.003 with 10 µM DHA (n=6, $P<0.01$). Other fatty acids previously tested by the author previously (*in* McMurchie *et al.*, 1998), including the DHA methyl ester and the saturated fatty acids DA and SA, did not alter the membrane fluidity when compared with control. However, this study shows that benzyl alcohol at concentrations that provide protection

against spontaneous contractile activity (10 mM) significantly decreased the r_{ss} value from 0.259 ± 0.003 (no additions, n=6) to 0.232 ± 0.007 (n=6, $P<0.05$). The decreased value for r_{ss} indicates that both DHA and benzyl alcohol increased the fluidity (decreased anisotropy) in adult rat cardiomyocytes.

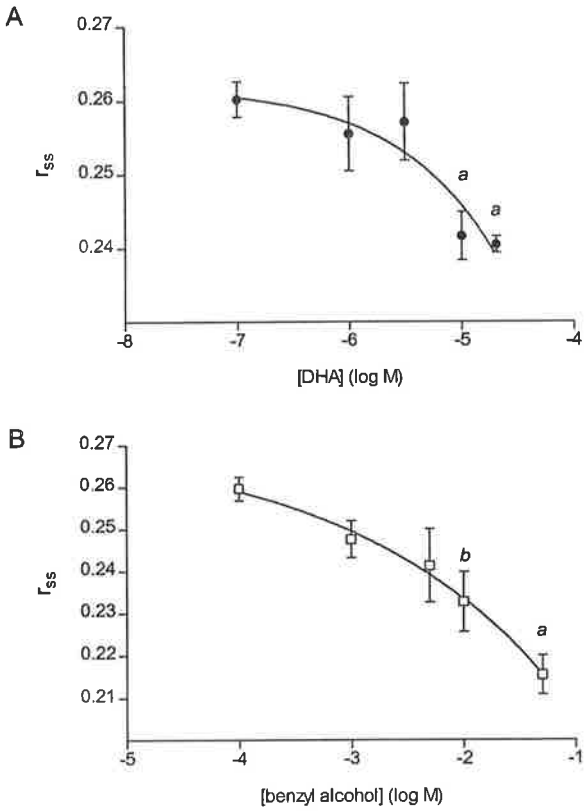


Figure 2.10 Adult rat ventricular cardiomyocyte sarcolemmal membrane fluidity.
Steady-state fluorescence anisotropy was determined as described in the methods using the probe TMAP-DPH following 10 min treatment in either (*A*) docosahexaenoic acid (DHA, n=6) or (*B*) benzyl alcohol (n=6). Each r_{ss} value represents the mean ± SEM. $^{a}P<0.001$, $^{b}P<0.05$.

2.4 DISCUSSION

This study demonstrated that in Ca^{2+}-tolerant ventricular cardiomyocytes isolated from adult rats, spontaneous contractile activity elicited in normally quiescent cardiomyocytes by isoproterenol or lysophosphatidylcholine could be prevented by acute addition of the n-3 PUFAs, ALA and DHA. DHA was also shown to increase cardiomyocyte sarcolemmal membrane fluidity in a dose-dependent manner as measured by steady-state fluorescence anisotropy using the fluorescent probe TMAP-DPH. As such, a mechanism for this effect on cardiomyocyte contractility may involve modulation of sarcolemmal membrane fluidity; a proposal supported by the similar effects observed using the membrane fluidising agent benzyl alcohol.

Many clinically encountered arrhythmias result from the phenomenon of re-entry and this can arise in many areas of the heart (Katz, 1977; Opie, 1998b). The normal propagation of the impulse conduction wave through areas of the functional syncytium can therefore be perturbed in such areas due to damage arising from, for example, heart failure or the imposition of ischaemia. Since the imposition of arrhythmic stimuli can induce normally quiescent cells to beat spontaneously, this indicates that single cardiomyocytes have the potential to develop automaticity in the whole heart. This may give rise to regions of abnormal excitability and conductivity in the working myocardium, thus generating arrhythmias. Therefore agents (such as n-3 PUFAs) that have been shown to protect isolated cells from displaying aberrant contractile behaviour may, in the above situation, work to protect these cells functioning abnormally in the working syncytia.

Elevated $[Ca^{2+}]_i$ levels play a significant role in the progression of triggered cardiac arrhythmia following ischaemia and reperfusion after exposure to catecholamines and

digitalis (Katz, 1977). Specifically, early afterdepolarisations elicited by β-adrenergic receptor stimulation are believed to be an underlying mechanism of ventricular tachycardia (De Ferrari *et al.*, 1995). Lysophosphatidylcholine has been reported to accumulate in ischaemic myocardium and exert deleterious effects on membrane integrity causing electrophysiological alterations and mechanical dysfunction leading to contracture (Sedlis *et al.*, 1997; Liu *et al.*, 1997). In addition, lysophosphatidylcholine induces Ca^{2+}-overload in isolated cardiomyocytes (Chen *et al.*, 1996), and may play an important role in ischaemia-reperfusion injury. Although this study demonstrates that the unesterified form of DHA (and ALA) could prevent spontaneous contractile activity induced by lysophosphatidylcholine, it was interesting to note that other forms of DHA, including phosphatidylcholine and monoglyceride could also prevent spontaneous contractile activity. However, other forms of phospholipids and monoglycerides containing saturated or unsaturated fatty acid constituents also had very similar protective effects. This suggests that lysophosphatidylcholine may have been damaging the cardiomyocyte sarcolemma and this could be prevented by incubating the cells in a solution containing a phospholipid or monoglyceride independent of the fatty acid class incorporated into the phospholipid or monoglyceride. Since lysophosphatidylcholine usually accumulates after the damaging events in myocardial ischaemia, this form of inducing arrhythmia may not be suitable for arrhythmia studies. As such, isoproterenol was considered to be a more appropriate pro-arrhythmogenic agent which lacks the membrane damaging effects observed with lysophosphatidylcholine.

The results of the present study are in general agreement with those reported by Leaf and colleagues using spontaneously contracting, cultured neonatal rat cardiomyocytes treated acutely with n-3 PUFAs (Kang & Leaf, 1994; Kang & Leaf, 1995; Leaf & Kang, 1996;

Kang & Leaf, 1996c). The results presented here confirm a previous study demonstrating that electrically-field stimulated adult rat cardiomyocytes are also protected from developing spontaneous or asynchronous contractile activity elicited by isoproterenol by acute addition of n-3 PUFAs (Leifert, 1997). This study further demonstrates that the membrane fluidising agent, benzyl alcohol also prevents spontaneous contractile activity. These "antiarrhythmic" effects may be due to changes in the excitability of the cells. In this regard it would seem that freshly isolated cardiomyocytes from the adult rat behave in a similar manner to cultured cardiomyocytes from neonatal rats with respect to the effect of acutely applied n-3 PUFAs. However, it should be noted that considerable differences exist between adult and neonatal cardiomyocytes, which include changes in excitation-contraction coupling (Klitzner, 1991), and considerable modulation of ion currents during development (Wetzel & Klitzner, 1996). It may well have been expected that the influence of n-3 PUFAs on the above parameters may have been different between these cell types. Therefore in light of these physiological differences between the cell types studied, it was considered important to document the above findings.

At the concentrations of n-3 PUFAs used in this study, rapid effects were observed inferring that transfer of the free fatty acids from the superfusion medium to the cardiomyocyte sarcolemmal membrane also occur in a very short time frame. Thus it is unlikely that covalent incorporation (esterification) of free fatty acids into membrane phospholipids occurred within this time frame; a notion supported by a previous study (Weylandt *et al.*, 1996). Indeed, this is also supported by other studies demonstrating that acutely administered n-3 PUFAs do not appear to require esterification into membrane phospholipids for their antiarrhythmic actions to be apparent (Kang & Leaf, 1995; Weylandt *et al.*, 1996; Leaf & Kang, 1996; Kang & Leaf, 1996c). However, some form of specific

membrane orientation may be required, possibly involving anchoring of the charged end of the molecule at the membrane/water interface, for these non-esterified fatty acids to elicit their effect. Furthermore, in this orientation, their effect appears to be one of reducing the excitability of the cardiomyocyte sarcolemmal membrane possibly preventing aberrant action potentials from occurring. In this study and a previous study, such effects were clearly evident by the need to increase the stimulating voltage following addition of the n-3 PUFAs (McMurchie *et al.*, 1998). Furthermore, DHA was shown here to potentiate the effects of KCl depolarisation, thus reducing the excitability of the cells resulting in less cells contracting in response to electrical stimulation.

In addition to the effects of DHA on suppressing spontaneous contractile activity in adult rat cardiomyocytes, DHA also influenced cardiomyocyte sarcolemmal membrane fluidity measured by SSFA using the fluorescent probe TMAP-DPH. A mechanism for the antiarrhythmic effect of the n-3 PUFAs that may involve effects on sarcolemmal membrane fluidity and is supported by the effects of the membrane fluidising agent benzyl alcohol. In this context, since ion channel activity is very much a membrane-associated, lipid-dependent process (McMurchie, 1988), the changes in membrane fluidity may have effects on ion channel activity.

The cell system utilised in this study using acutely applied n-3 PUFAs to mimic the action of antiarrhythmic agents, contrasts with other studies in which dietary n-3 PUFAs, incorporated into cell membrane phospholipids, exhibit antiarrhythmic effects in experimental animal models (McLennan *et al.*, 1988; McLennan *et al.*, 1993; Pepe & McLennan, 1996). However, the two systems are not mutually exclusive and the antiarrhythmic action of dietary n-3 PUFAs may proceed via similar mechanisms in both situations. As pointed out

by Nair (Nair *et al.*, 1997) incorporation of dietary n-3 PUFAs and their longer chain metabolites into cell membrane phospholipids, may provide a pool of n-3 PUFAs, which can be liberated by phospholipase action following the imposition of arrhythmogenic stimuli. The resulting non-esterified fatty acids could then exert antiarrhythmic effects in a manner similar to that described in this study, and in the studies using neonatal rat cardiomyocytes (Kang & Leaf, 1995; Weylandt *et al.*, 1996; Leaf & Kang, 1996; Kang & Leaf, 1996c).

2.4.1 Conclusions

The results of the present study which show a potent effect of the n-3 PUFAs on cardiac contractility at the cellular level possibly mediated by effects on membrane fluidity, provide the beginnings of a mechanistic explanation for the antiarrhythmic effects of n-3 PUFAs reported from various epidemiological and clinical studies, and studies with experimental animals (Kromhout *et al.*, 1985; Burr *et al.*, 1989; de Lorgeril *et al.*, 1994; Siscovick *et al.*, 1995).

CHAPTER 3

3 "Effects of acute n-3 polyunsaturated fatty acids on whole-cell Na^+ currents"

3.1 INTRODUCTION

A number of epidemiological studies have shown that the consumption of a diet high in n-3 polyunsaturated fatty acids (PUFAs) can confer protection from coronary heart disease (Kromhout *et al.*, 1985; Burr *et al.*, 1989; Siscovick *et al.*, 1995; Kromhout *et al.*, 1995). In animal studies dietary PUFAs have a similar protective action particularly in relation to cardiac arrhythmias (McLennan *et al.*, 1988; Hock *et al.*, 1990; Pepe & McLennan, 1996; McLennan *et al.*, 1996). Studies have also demonstrated an antiarrhythmic effect of acutely added PUFAs using isolated neonatal cultured myocyte preparations (Kang & Leaf, 1994; Kang *et al.*, 1995; Kang & Leaf, 1996c) or as an intravenous infusion in surgically manipulated dogs (Billman *et al.*, 1994; Billman *et al.*, 1997). The arrhythmias generated in single cell preparations as well as the whole heart can originate from abnormal electrical impulses. Fast inward Na^+ currents are involved in the initiation of cardiac action potentials (and therefore excitation-contraction coupling) and class I antiarrhythmic agents such as lidocaine (lignocaine) are used clinically to block these channels. Since results from the previous chapter suggested that cardiomyocytes were less excitable in the presence of n-3 PUFAs, there remains the possibility that n-3 PUFAs applied acutely to isolated adult rat cardiomyocytes could block the voltage-dependent Na^+ channels as previously shown in neonatal cardiomyocytes (Xiao *et al.*, 1995).

3.1.1 Na^+ channels – voltage-dependence and states

One of the first events in response to the onset of depolarisation of phase 0 of the action potential is opening of sodium ion (Na^+) channels when the voltage approaches –70 to –60 mV, the threshold for activation. Therefore the opening of the Na^+ channels are "voltage-dependent". The Na^+ current flows inward rapidly during the first millisecond of depolarisation with subsequent inactivation of the Na^+ channels resulting in a slower Na^+ current during the inactivation phase. Cardiac Na^+ channels are believed to exist in one of three states: resting (closed), activated (open) and inactivated. Largely the membrane potential determines the states in which Na^+ channels may exist. Depolarisation changes the resting state to the activated state and upon transition back to the resting state, the Na^+ channels enter the inactivated state (Opie, 1998b) according to the following scheme:

resting (closed) ↔ activated (open) ↔ inactivated

The steep voltage-dependence of the Na^+ channels requires that the channels have charged amino acid residues or strongly oriented dipoles within the electric field of the phospholipid bilayer (Catterall, 1988). The steepness of the voltage-dependence of activation of Na^+ channels requires the movement of protein-bound positive charges from the inner surface of the membrane bilayer to the outer surface during activation. Movement of the gating charges (or "voltage sensors") under the force of the electric field is believed to initiate a conformational change in the Na^+ channel proteins resulting in activation. The movement of gating charge is blocked if the Na^+ channel is first inactivated before depolarisation. The activation process resulting in Na^+ channel conductivity is transient because prolonged activation causes transition of the channel to an inactivated state that cannot be activated by further depolaristaion (Catterall, 1988). Since this process can be slowed by neurotoxins or anaesthetics acting at the extracellular surface (Catterall, 1988; Pugsley & Goldin, 1998; Opie, 1998b), which essentially

"blocks" the Na^+ current, it is conceivable that n-3 PUFAs (either acutely applied, or esterified into membrane phospholipids by dietary means) alter this process. Indeed, the Na^+ channels' conformation and voltage sensing is likely to be highly dependent on the immediate lipid environment in which it resides.

The electrophysiological mechanism underlying the acute effect of fatty acids, at least in isolated cardiomyocytes, may involve an increase in the threshold for the generation of the action potential (Kang *et al.*, 1995; McMurchie *et al.*, 1998), suggesting that PUFAs may mediate their effects by interaction with Na^+ currents. In this context, n-3 PUFAs have been shown to have potent effects on Na^+ currents evoked in neurons (Vreugdenhil *et al.*, 1996), in HEK293t cells (human embryonic kidney cells) transfected with the alpha subunit of the human cardiac Na^+ channel (Xiao *et al.*, 1998) and in neonatal cardiac myocytes (Xiao *et al.*, 1995). However, difficulties arise in the extrapolation of results obtained with cloned channels expressed in transfected cells to adult tissues, since the results are always subject to reservations as to whether channel properties have been modified by the expression system, and whether crucial subunits are missing or modified. Similarly, cultured neonatal cardiomyocytes exhibit important differences from the adult myocardium since many ionic currents are subject to considerable modulation during development (Klitzner, 1991; Wetzel *et al.*, 1993; Matsubara *et al.*, 1993; Nuss & Marban, 1994; Touyz *et al.*, 1996; Wetzel & Klitzner, 1996; Roden & George, 1997). An additional consideration with neonatal cells is that Na^+ channels undergo substantial changes in their properties during development (Sada *et al.*, 1995) which include changes in the effect that class I antiarrhythmic agents have on such channels (Xu *et al.*, 1991; Xu *et al.*, 1992). Because of these developmental changes neonatal cardiomyocytes have a

fundamentally different electrophysiological profile from adult cells and should be considered in the context of arrhythmias.

Dietary n-3 PUFA supplementation has been shown to significantly decrease the spontaneous beat rate in isolated, working hearts from rats under normoxic conditions and normalise the heart rate during ischaemia (Demaison & Grynberg, 1991; Pepe & McLennan, 1996). Other studies have used surgically-prepared dogs that were susceptible to ventricular fibrillation (VF) following coronary artery occlusion and exercise (Billman *et al.*, 1997; Billman *et al.*, 1999). Following acute intravenous administration of the n-3 non-esterified fatty acid (NEFA), DHA, final plasma concentrations of DHA increased to 546 μM from a basline of 0 μM DHA (Billman *et al.*, 1999). In separate studies the acute administration of n-3 NEFAs resulted in a significant slowing of the heart rate both before and during exercise (Billman *et al.*, 1997). Since n-3 PUFAs are known to inhibit Na^+ channels in various cell types as discussed above and can slow the contraction of spontaneously-contracting neonatal cardiomyocytes (Kang & Leaf, 1994), it is therefore possible that n-3 PUFAs may alter and even block the voltage-dependence of Na^+ channels from adult rat cardiomyocytes, providing a possible mechanism of antiarrhythmic action. Furthermore, several studies have suggested that modulation of various ion channels by PUFAs may be related to changes in the physical nature of the membrane bilayer, in particular, membrane fluidity (Haydon & Urban, 1983; Bregestovski & Bolotina, 1989; Gasser *et al.*, 1990).

3.1.2 This study

Most class I antiarrhythmic agents cause a use-dependent inhibition, a negative shift of the voltage-dependence of inactivation curve and a slower recovery of Na^+ channels from

their inactivated state (Su *et al.*, 1997). This study therefore investigated the potential for a variety of n-3 polyunsaturated fatty acids, differing in their degree of unsaturation and chain length, to modify the activation, inactivation and kinetic properties of Na^+ currents in isolated adult rat cardiac myocytes using whole-cell patch-clamp recording techniques. In addition, the effects of the n-3 PUFAs and benzyl alcohol on sarcolemmal membrane fluidity was determined by steady-state fluorescence anisotropy. This was investigated to determine whether changes in cell membrane physical properties correlate with the effects of PUFAs on Na^+ current activity.

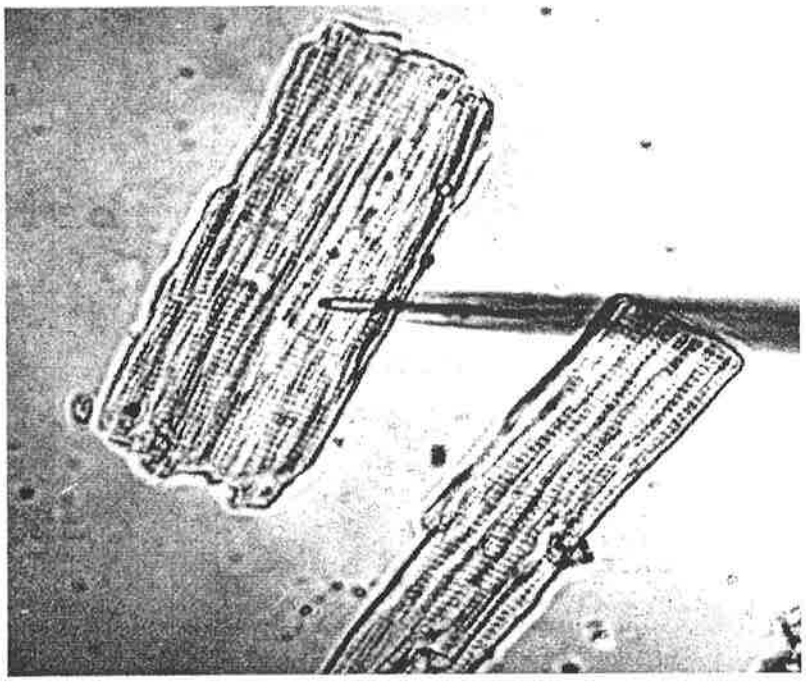
3.2 METHODS

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

3.2.1 Electrophysiological recording of cardiac Na^+-current.

Cardiomyocytes were isolated as described in Chapter 2. Electrodes were prepared from borosilicate glass using a two-stage puller (Narishige Scientific Instruments, Tokyo, Japan) and resistances were typically between 1-3 MΩ when containing the pipette solution. Whole cell currents were recorded 5 min after achievement of a whole-cell patch clamp configuration (see panel 3.1). Current recording was performed using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA). Whole cell capacitance and series resistance compensation was achieved using the controls on the amplifier; recording was only performed if series resistance compensation of at least 90% could be achieved. Satisfactory voltage control was indicated by the following criteria: 1) The negative limb of the current-voltage curve spanned at least 25 mV; 2) There were no abnormal notches in the current-voltage curve; 3) There was no crossover between recordings at different voltages of the inactivation curve. Whole cell Na^+ currents were evoked by voltage steps generated by a computer program that outputs the waveform via a digital to analogue converter connected to the command input of the amplifier. The resulting currents were filtered at 5 kHz and recorded through an analogue to digital converter operating at 20 kHz. Glass coverslips with adhering cardiomyocytes were transferred to a superfusion chamber (mounted on an inverted microscope) containing 0.5

ml of bath solution. Cardiomyocyte currents were recorded 5 min after achieving a whole-cell patch clamp configuration and the effect of fatty acids was determined after 5 min incubation.



Panel 3.1 Photograph of a patch-clamped cardiomyocyte.
Single cardiomyocytes were patch-clamped by directing a borosilicate glass microelectrode (using a micromanipulator) against the surface membrane of a cardiomyocyte as shown, and then applying gentle suction to form a 1-3 MΩ seal. The microelectrode was filled with a solution as described in methods (section 3.2.2). The microelectrode solution and superfusion chamber solution were both designed to to block all ionic currents other than Na^+ currents. The microelectrode was connected to an amplifier that was programmed to stimulate the cardiomyocytes at appropriate voltages in order to elicit a Na^+ current, for current-voltage (I-V) relationship analyses.

3.2.2 *Solutions and fatty acids.*

The standard external (bath) solution used for Na^+ current measurements contained (mM): NaCl, 20; TES (N-tris-(hydroxy-methyl)-methyl-2-aminoethanesulphonic acid), 10; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; $CoCl_2$, 5; CsCl, 5; glucose, 10; cholineCl, 110, pH adjusted to 7.4 with 5.0 M NaOH. The pipette solution for all experiments contained (mM): CsF, 120; TES, 10; $MgCl_2$, 2; EGTA-free acid, 20; $CaCl_2$, 2; pH adjusted to 7.4 with 5.0 M KOH.

These solutions are designed to block all ionic currents other than Na^+ currents. In addition, the low extracellular Na^+ concentration (20 mM) is designed to reduce the peak Na^+ current and hence minimise series resistance errors in the clamp potential. Fatty acids (Sigma) were dissolved in ethanol at a final concentration of 50 mM containing 0.003% (w/v) butylated-hydroxyanisole and stored at -80°C.

3.2.3 *Membrane fluidity measurements.*

Cardiomyocytes were loaded with 1 μM TMAP-DPH for 15 min at 37°C. The coverslips were placed in a glass cuvette containing 20 μM test fatty acids or 10 mM benzyl alcohol and r_{ss} values (inversely related to membrane fluidity) were determined as described in Chapter 2.

3.2.4 Statistical analysis.

Appropriate equations were fitted to individual data sets using the algorithm built into the graphics program GraphPad Prizm version 2.00 (Graphpad Software Inc., San Diego, CA, USA). Data are presented as mean ± standard error (S.E.M.) Significance between means was tested using either the two-tailed Student's paired *t* test or One-Way ANOVA with Dunnett's multiple comparisons test. The significance level was set at $P<0.05$.

3.3 RESULTS

3.3.1 Block of voltage-dependent Na^+ currents in adult rat cardiomyocytes.

The average whole-cell capacitance of cardiomyocytes was 120.3 ± 5.2 pF (n=51). Na^+ currents in control cells were activated at approximately -55 mV and reached a maximum current at approximately -25 mV. The maximum Na^+ current densities elicited by a voltage step from -90 mV to -30 mV for extracellular Na^+ concentrations of 20 mM and 70 mM were 8.9 ± 0.4 pA/pF (n=51) and 13.8 ± 2.9 pA/pF (n=7), respectively, demonstrating the Na^+-dependence of the currents recorded. Figure 3.1A shows an example of typical Na^+ currents evoked in a single myocyte by a step in membrane potential to –30 mV from a holding potential of -90 mV, and the effect of 25 μM DHA on this current. In this cell, 25 μM DHA applied to the extracellular solution blocked the peak current amplitude by 42%, after approximately 4 min of incubation. The time course of this block is shown in Figure 3.1B. The block by DHA reached a plateau after approximately 3 min exposure and elimination of the effect of DHA could be achieved by a washout procedure by adding delipidated bovine serum albumin (BSA) to the extracellular solution for 2 minutes (in the absence of fatty acid).

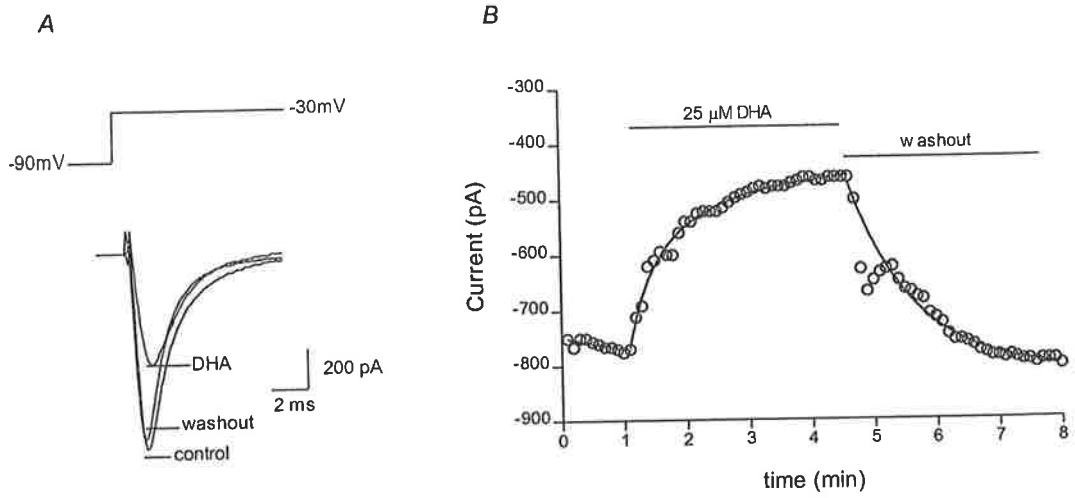


Figure 3.1 Time course of Na^+ current inhibition by DHA in an adult rat ventricular myocyte.
A, depiction of the voltage step (upper panel) and the current evoked by a step in membrane potential to -30 mV from a holding potential of -90 mV (lower panel) and following incubation in DHA (25 µM) and subsequent washout. *B*, time course of the inhibitory effect of DHA on the peak Na^+ current recorded from an adult rat ventricular myocyte. Na^+ currents were evoked in whole cell recording mode once every 6 s with 300 ms duration pulses to -30 mV from a holding potential of -90 mV. The horizontal bars indicate the period during which DHA was applied to the cell or the period of washout with a solution containing 1 mg ml^{-1} delipidated BSA.

3.3.2 Effect of fatty acids on Na^+ current activation.

The effect of fatty acids on the voltage-dependence of activation was determined by evoking currents voltage steps to various potentials between -90 mV and +30 mV from a holding potential of -140 mV (as depicted in upper panel of Figure 3.2A). Representative Na^+ currents are shown in the lower panel of Figure 3.2A. The maximum amplitude of the currents evoked was plotted against the test potential, as shown in Figure 3.2B for control cells and cells in the presence of 25 µM DHA. The data points were fitted by the equation

$$I = [G_{max} \cdot (V-E_{rev})] \cdot [1 / 1+ e^{(V-V')/k}] \quad \text{.....1}$$

using a least squares fitting algorithm, where G_{max} is the maximum conductance, V' is the membrane potential for 1/2 activation of the channels, V is the test membrane potential, E_{rev} is the reversal potential for the current and k is a slope factor. The control data were best fit by G_{max} = 19.3 nS, V' = -37.2 mV, k = 4.17 mV^{-1} and E_{rev} = 25.9 mV (Figure 3.2B). In the presence of 25 μM DHA the maximum conductance, G_{max}, was reduced by 50%, to 9.6 nS, k = 6.43 mV^{-1} and V' was -26.2 mV (i.e., shifted to more positive potentials by 11.0 mV) and E_{rev} was 29.0 mV (not significantly different from control). The means of all the above parameters for the least squares fit of equation 1 for the n-3 PUFAs DHA, EPA and ALA, all at 25 μM final concentration, are given in Table 3.1. Following treatment with DHA (n=5), EPA (n=10) or ALA (n=6), G_{max} values were significantly lower than controls ($P<0.01$) and the I-V relation was significantly shifted to more positive potentials. This shift in membrane potential was similar for all n-3 PUFAs tested (9.2 ± 2.0 mV, 10.0 ± 1.1 mV and 8.3 ± 0.9 mV for DHA, EPA or ALA-treated cardiomyocytes, respectively). However, DHA was more potent than either EPA or ALA at inhibiting G_{max} in these cells. Figure 3.3 shows that linoleic acid (18:2 n-6), oleic acid (18:1 n-9) and the saturated fatty acid, stearic acid (18:0), did not significantly alter any of the Na^+ current activation parameters measured (Table 3.1). However, the membrane fluidising agent, benzyl alcohol at 10 mM (n=3) significantly ($P<0.05$) reduced the G_{max} as shown in Table 3.1 and Figure 3.3.

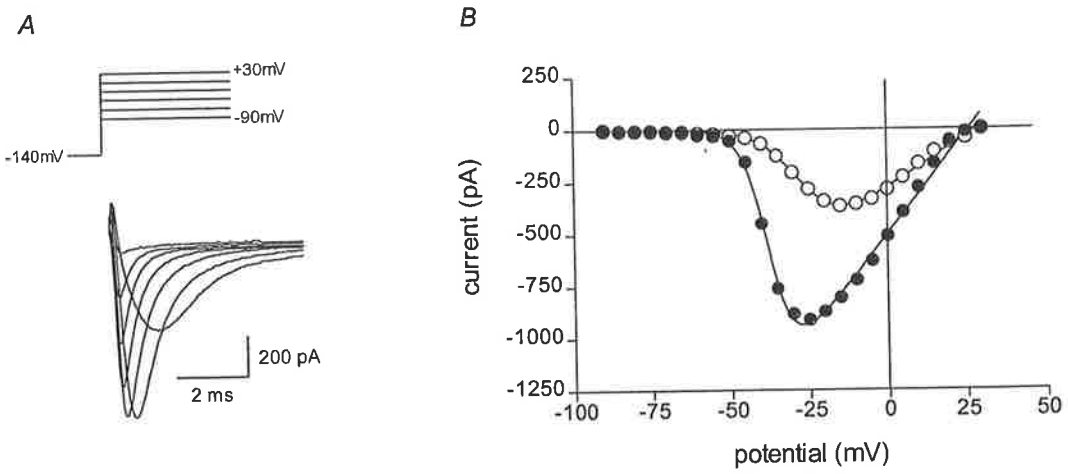


Figure 3.2 Effect of DHA on the voltage-dependence of activation in an adult rat ventricular myocyte. *A*, Na^+ currents were evoked by voltage steps from a holding potential of -140 mV to various voltages between -90 mV and +30 mV as depicted (upper left). Plotted below are superimposed currents evoked at -40, -30, -20, -10, 0, 10 and 20 mV. *B*, the peak current amplitude was plotted against the pulse potential. Typical data are shown as points representing control data (O) or data in the presence of 25 μM DHA (λ). The solid line shows the least squares best fit of equation 1. The parameters for the best fit in each case were control, G_{max} = 19.3 nS, V' = -37.2 mV, k = 4.17 mV^{-1} and E_{rev} = 25.9 mV. In the presence of 25 μM DHA, G_{max} = 9.6 nS, V' = -26.2 mV, k = 6.43 mV^{-1} and E_{rev} = 29.0 mV, where G_{max} is the maximum conductance, V' is the voltage at which 50% of the channels are activated, k is the slope factor for the voltage-dependence of activation and E_{rev} is the reversal potential.

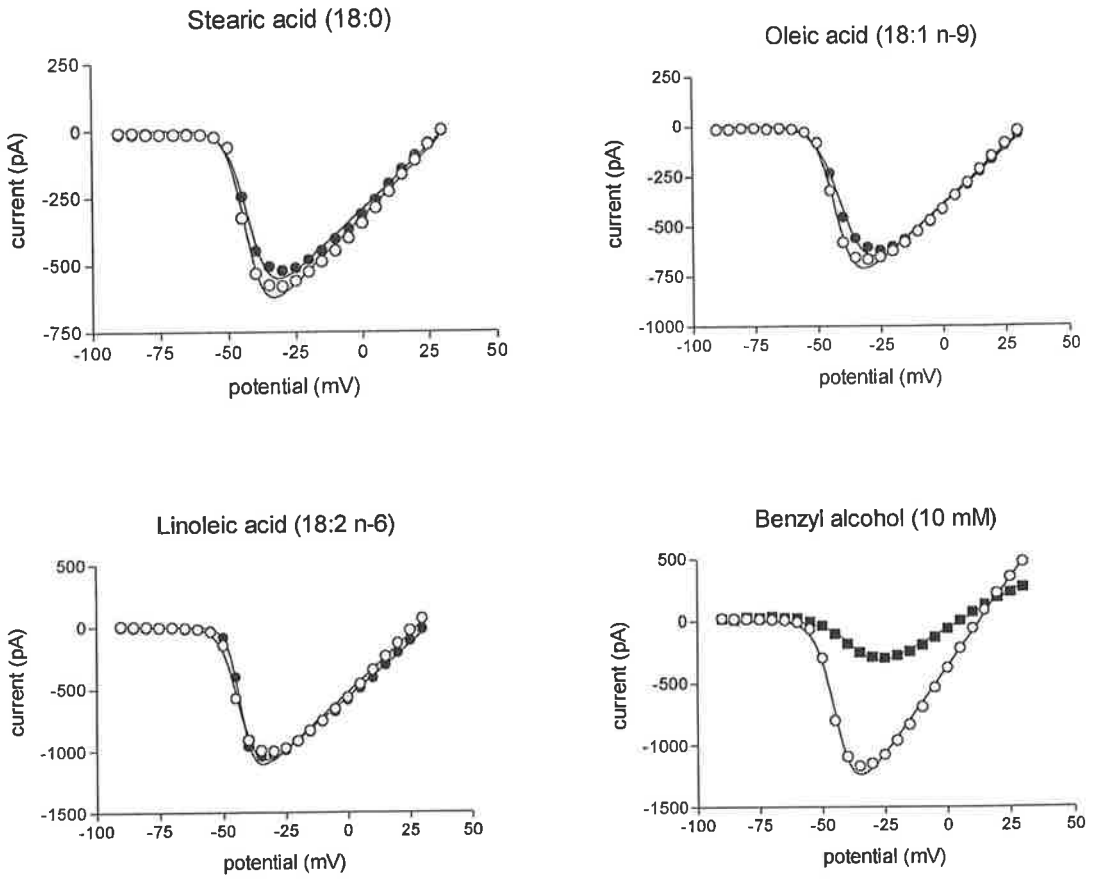


Figure 3.3 Effect of various fatty acids on the voltage-dependence of activation in adult rat ventricular myocytes.
Na^+ currents were evoked by voltage steps to various potentials from a holding potential of -140 mV to various voltages between -90 mV and +30 mV as depicted in Figure 3.2*A* (upper left). The peak current amplitude was plotted against the pulse potential. Points represent typical control data (O) or data in the presence of 25 μM fatty acid (λ) or benzyl alcohol (ν) as indicated.

Table 3.1 Activation parameters from least squares fit of equation 1

	Structure	G_{max} (nS)	V' (mV)	k (mV^{-1})	V_{rev} (mV)
Control		20.5 ± 1.7	-42.9 ± 2.9	2.7 ± 0.5	23.9 ± 1.1
DHA 25 μM	(22:6 n-3)	13.4 ± 1.4†	-33.7 ± 2.1†	5.0 ± 0.5†	25.7 ± 2.0
Control		22.2 ± 1.3	-39.2 ± 2.1	3.2 ± 0.4	37.8 ± 2.3
EPA 25 μM	(20:5 n-3)	15.6 ± 1.0†	-29.2 ± 2.0‡	4.8 ± 0.4‡	39.8 ± 2.2
Control		22.1 ± 1.7	-33.6 ± 2.5	4.1 ± 0.3	31.9 ± 2.2
ALA 25 μM	(18:3 n-3)	18.2 ± 1.2†	-25.3 ± 2.1‡	5.3 ± 0.4†	40.9 ± 3.7*
Control		18.4 ± 2.1	-41.7 ± 1.6	2.9 ± 0.8	27.4 ± 0.4
LA 25 μM	(18:2 n-6)	17.4 ± 2.0	-39.9 ± 1.5	2.5 ± 0.9	32.9 ± 2.6
Control		16.6 ± 2.7	-46.6 ± 2.9	2.4 ± 0.5	32.6 ± 1.9
OA 25 μM	(18:1 n-9)	16.2 ± 2.5	-46.2 ± 2.3	2.3 ± 0.7	34.1 ± 1.4
Control		17.9 ± 2.2	-47.8 ± 2.8	2.2 ± 0.4	32.2 ± 1.9
SA 25 μM	(18:0)	17.9 ± 2.1	-49.0 ± 2.8	1.4 ± 0.2*	30.3 ± 3.0
Control		23.7 ± 0.5	-46.0 ± 1.5	5.1 ± 0.9	10.5 ± 1.4
Benzyl alcohol	(10 mM)	7.7 ± 3.0*	-38.2 ± 1.8	4.8 ± 0.3	9.1 ± 1.1

*P<0.05, †P<0.01, ‡P<0.001, significantly different from control (two tailed Student's paired *t* test). Abbreviations; DHA, docosahexaenoic acid (n=5); EPA, eicosapentaenoic acid (n=10); ALA, α-linolenic acid (n=6); LA, linoleic acid (n=5); OA, oleic acid (n=7); SA, stearic acid (n=8), benzyl alcohol (n=3).

3.3.3 Effect of fatty acids on Na^+ current inactivation.

The effect of the fatty acids on the voltage dependence of inactivation of the Na^+ current was investigated by stepping the membrane potential to a test potential of -30 mV from holding potentials which varied between -140 and -30 mV (Figure 3.4A). The peak amplitude of the evoked current was plotted (Figure 3.4B) against the holding potential for both control data and data obtained in the presence of 25 μM DHA, and the data points fitted with the Boltzmann equation:

$$I = I_{max} \cdot [1 / 1 + e^{(V-V')/k}] \quad \text{.....2}$$

where I_{max} is the maximum current, V is the test membrane potential, V' is the membrane potential at which half of the channels are inactivated, and k is a slope factor. In the cell from which data is shown in Figure 3.4, 25 μM DHA induced a shift in the voltage-dependence of inactivation to more hyperpolarised potentials by 24.7 mV and I_{max} was reduced from a control value of -746 pA to -320 pA in the presence of DHA. In separate experiments, the inactivation curves in the presence of various concentrations of EPA were obtained by normalising the test current amplitudes to the maximum current (I/I_{max}) as shown in Figure 3.5. Currents were elicited by the voltage protocol as shown in the upper panel of Figure 3.5. The curves generated were also fitted to equation 2. In the presence of increasing concentrations of EPA, the normalised inactivation curves were shifted to the left to more negative potentials in a concentration-dependent manner. In this cell, the V' was -69.8 mV for control, -72.1 mV, -89.6 mV, -97.9 mV and -110.3 mV, for 1 μM, 10 μM, 25 μM and 50 μM EPA, respectively.

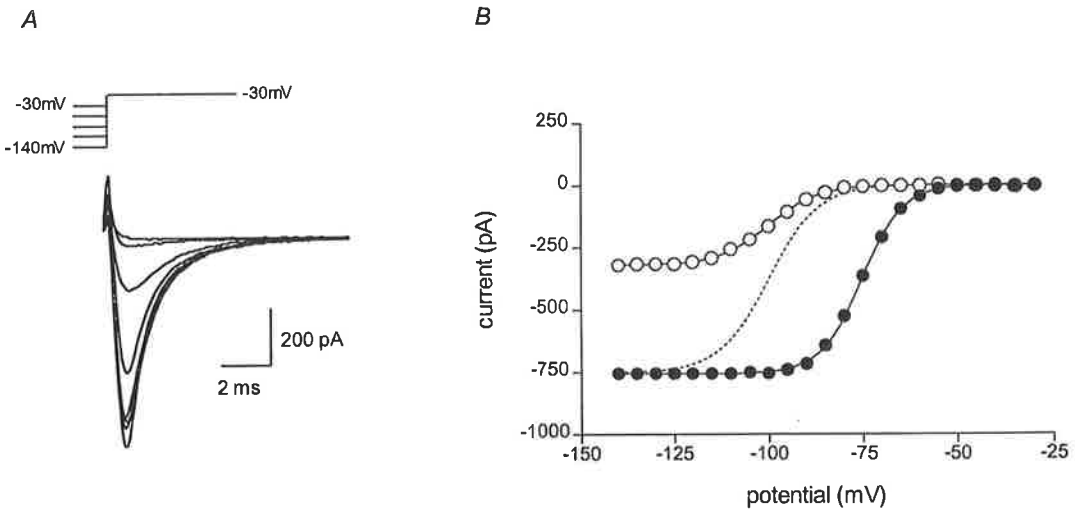


Figure 3.4 Effect of DHA on the voltage-dependence of inactivation in adult rat ventricular myocytes. *A*, Na^+ currents were evoked by voltage steps to -30 mV from various holding potentials, as depicted (upper left). Plotted below are currents evoked at -130, -120, -110, -100, -90, -80, -70, -60 and -50 mV, shown superimposed. *B*, the peak current amplitude is shown plotted against the holding potential. Points represent typical control data (O) or data in the presence of 25 μM DHA (λ). The solid line shows the least squares best fit of equation 2 for control or DHA. The parameters for the best fit in each case were, control; I_{max} = -746 pA, V′ = -75.2 mV and k = 5.28 mV^{-1}, and in the presence of 25 μM DHA; I_{max} = -320 pA, V′ = -99.9 mV and k = 6.60 mV^{-1}. The dotted line shows the data points for DHA scaled to the same maximum Na^+ current as the control data.

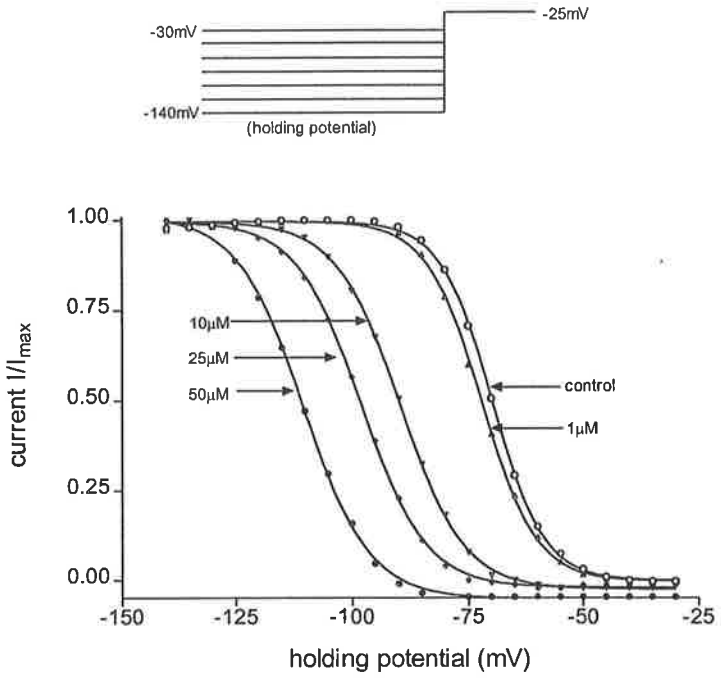


Figure 3.5 Concentration-dependent shift in inactivation of Na^+ currents by EPA.
Upper panel shows the inactivation-voltage protocol used. Lower panel shows the normalised steady-state inactivation of the Na^+ current following incubation with various concentrations of EPA (indicated). Na^+ currents (I) were normalised as a fraction of the control (maximum) current (I_{max}). The solid line shows the least squares best fit of equation 2.

The mean values for the parameters I_{max} , V' and k, for the least squares fit of equation 2 for the n-3 polyunsaturated fatty acids DHA (n=6), EPA (n=10) and ALA (n=6) all at 25 μM final concentration, as well as the membrane fluidising agent, benzyl alcohol (n=3) at 10 mM, are given in Table 3.2. In addition to the n-3 PUFAs, the following fatty acids were also tested: linoleic acid (n=5), oleic acid (n=7) and the saturated fatty acid, stearic acid (n=8) (Table 3.2). A significant reduction in I_{max} occurred following treatment with either DHA, EPA or ALA by 36% ($P<0.05$), 35% ($P<0.01$) and 25% ($P<0.01$), respectively. Benzyl alcohol also significantly reduced I_{max} (n=3, $P<0.01$). The voltage-dependence of inactivation (V') was shifted to more hyperpolarised potentials by 22.3 ± 0.9 mV ($P<0.001$), 17.1 ± 3.7 mV ($P<0.01$) and 20.5 ± 1.0 mV ($P<0.001$) in the presence

of DHA, EPA and ALA, respectively. Benzyl alcohol also shifted the voltage-dependence of inactivation of Na^+ currents to more negative potentials by approximately 24 mV (a change of 29%). The fatty acids linoleic acid, oleic acid and stearic acid did not significantly alter I_{max}, although addition of linoleic acid and oleic acid was associated with a small shift of V' to more hyperpolarised potentials (6.3 ± 0.6 mV and 3.5 ± 1.2 mV, respectively). Nevertheless, the shift in voltage dependence was considerably less with these three fatty acids than that observed for any of the n-3 PUFAs (Table 3.2).

Table 3.2 Inactivation parameters from least squares fit of equation 2

	Structure	I_{max} (pA)	V' (mV)	k (mV^{-1})
Control		-850 ± 92	-74.3 ± 1.5	5.2 ± 0.1
DHA 25 μM	(22:6 n-3)	-544 ± 88*	-96.6 ± 1.3‡	6.5 ± 0.2‡
Control		-1258 ± 107	-69.3 ± 2.1	6.5 ± 0.2
EPA 25 μM	(20:5 n-3)	-816 ± 106†	-86.5 ± 3.5†	7.4 ± 0.3‡
Control		-988 ± 103	-68.4 ± 2.0	5.8 ± 0.3
ALA 25 μM	(18:3 n-3)	-740 ± 83†	-88.9 ± 2.0‡	7.0 ± 0.2†
Control		-869 ± 105	-71.0 ± 2.8	6.3 ± 0.4
LA 25 μM	(18:2 n-6)	-898 ± 119	-77.3 ± 3.0‡	6.9 ± 0.4
Control		-901 ± 128	-76.0 ± 2.9	6.2 ± 0.3
OA 25 μM	(18:1 n-9)	-902 ± 156	-79.5 ± 2.3*	6.4 ± 0.1
Control		-974 ± 121	-73.7 ± 1.7	6.2 ± 0.2
SA 25 μM	(18:0)	-919 ± 105	-73.6 ± 2.2	6.9 ± 0.4
Control		-716 ± 63	-86.0 ± 1.8	8.4 ± 0.4
Benzyl alcohol (10 mM)		-140 ± 77†	-110.6 ± 3.8*	6.8 ± 1.4

*P<0.05, †P<0.01, ‡P<0.001, significantly different from control (two tailed Student's paired *t* test). Abbreviations; DHA, docosahexaenoic acid (n=5); EPA, eicosapentaenoic acid (n=10); ALA, α-linolenic acid (n=6); LA, linoleic acid (n=5); OA, oleic acid (n=7); SA, stearic acid (n=8), benzyl alcohol (n=3).

3.3.4 *Concentration-dependence of Na^+ current block by n-3 polyunsaturated fatty acids.*

When whole cell Na^+ currents were evoked from a holding potential of -90 mV (close to the resting potential of the cells *in vivo*), DHA, EPA and ALA produced a concentration-dependent block of peak Na^+ current amplitude over the concentration range 1 - 50 μM (Figure 3.6A). The degrees of block by DHA, EPA and ALA over this concentration range in several cells are shown in Figure 3.6B. The EC_{50} for each of the fatty acids was determined from the least squares fit of the Hill equation

$$y = 1 / 1 + (K_A / [A])^{\eta} \quad \text{.....3}$$

where y is the fractional block, K_A is the apparent affinity constant, [A] is the concentration of fatty acid and η is the Hill coefficient. The most potent polyunsaturated fatty acid was DHA (EC_{50} = 6.0 ± 1.2 μM, n=5), followed by EPA (EC_{50} = 16.2 ± 1.3 μM, n=5), and ALA (EC_{50} = 26.6 ± 1.3 μM, n=6). The potency for each of the fatty acids DHA, EPA and ALA to shift the voltage dependence of inactivation was also determined by subtracting the V' under control conditions from the V' following incubation with DHA, EPA or ALA. Figure 3.6C shows the dose-response relationship for the shift in inactivation ($\Delta V'$) plotted against log concentration for each of the fatty acids. In this case there was no obvious difference in potency between DHA, EPA and ALA. The other fatty acids examined, linoleic acid, oleic acid and stearic acid, did not produce a measurable shift in voltage dependence up to concentrations of 25 μM.

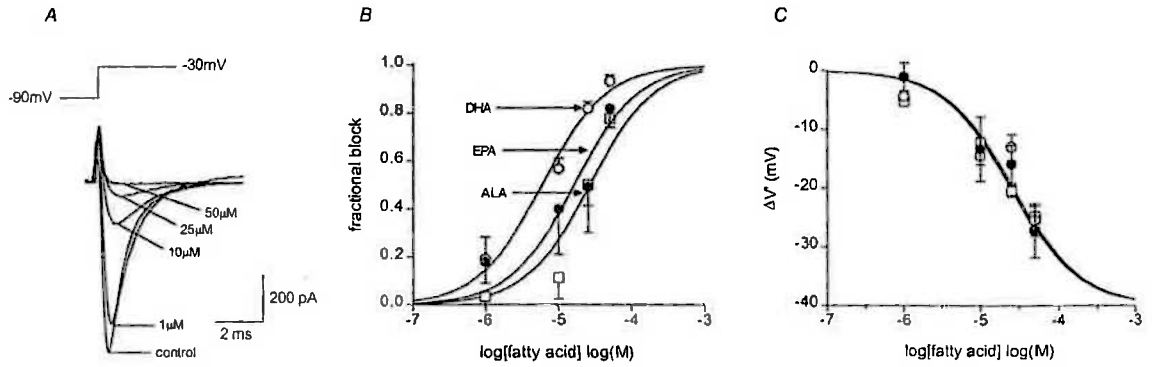


Figure 3.6 Concentration-dependence block of Na^+ currents by n-3 polyunsaturated fatty acids in adult rat ventricular myocytes.
A, Na^+ currents were evoked by voltage steps to -30 mV from a holding potential of -90 mV, in the presence of increasing concentrations of DHA (as indicated). Currents are shown superimposed. *B*, Concentration-response curve for the n-3 PUFAs, DHA, EPA and ALA. The degree of block of the Na^+ current for each cell was measured as a fraction of the control current, to compensate for differences in peak current amplitude due to differences in cell size. The lines show the least squares fit using the Hill equation, which gave EC_{50} values of 6.0 ± 1.2 μM (n=5), 16.2 ± 1.3 μM (n=5) and 26.6 ± 1.3 μM (n=6) for DHA (O), EPA (λ) and ALA (□), respectively. *C*, dose-response relationship for shift in voltage-dependence of inactivation (ΔV'). For each concentration of n-3 fatty acid used, the change in the V_{50} (V') for inactivation was determined and plotted against fatty acid concentration.

3.3.5 Effects of n-3 PUFAs on kinetics of inactivation

In addition to the effects on voltage-dependence of steady-state inactivation, the effect of EPA and DHA on the kinetics of activation and inactivation of Na^+ currents as well as recovery from inactivation (frequency-dependence) were investigated. The time course of the incline and decline in the Na^+ current was taken as an indication of the rate of Na^+ current activation and inactivation, respectively, as shown previously (Saint, 1998). Na^+ currents were evoked from a holding potential of -140 mV, a membrane potential that would allow all available Na^+ channels to reside in a "resting" state and therefore be available for activation. Figure 3.7A shows an example of Na^+ currents evoked in one cell by a voltage step to -30 mV from a holding potential of -140 mV in control solution or in the presence of 25 μM DHA. The data points were fitted to the following equation:

$$I = I_{max}.[1-e^{(-t/\tau 1)}]^3.[e^{(-t/\tau 2)}] \quad \text{.....4}$$

which is essentially the Hodgkin-Huxley equation described previously (Hodgkin & Huxley, 1952; Saint, 1998). $\tau 1$ is the time constant of the activation process and $\tau 2$ is the time constant of inactivation and was derived from the exponential phase of the Na^+ current during its inactivation phase. In these experiments there was no significant difference in $\tau 1$ when comparing control and 25 μM EPA or DHA addition (the $\tau 1$ value was approximately 0.2 ms following all treatments). However, as shown in Figure 3.7A, the time constant of Na^+ current inactivation ($\tau 2$) in a single cell in control solution was 0.814 ms and this was increased to 1.444 ms in the presence of 25 μM DHA. The average $\tau 2$ of five experiments comparing the effects of EPA and DHA is shown in Figure 3.7B. Both EPA and DHA significantly increased the time constants for Na^+ current inactivation under the conditions tested.

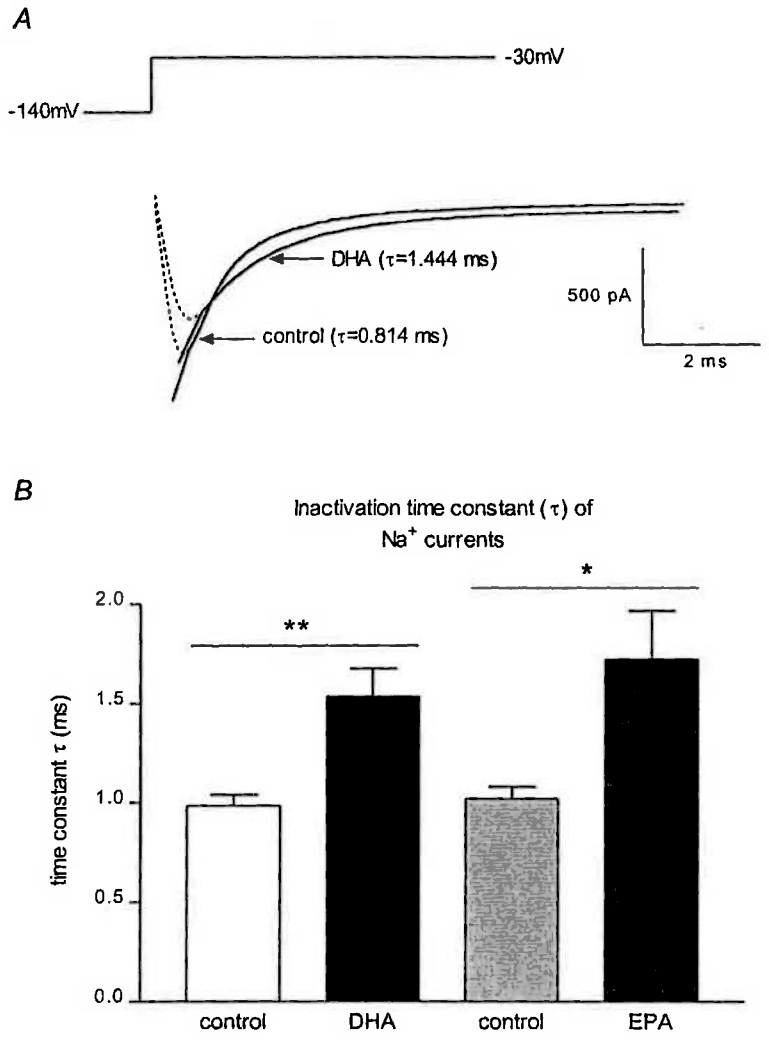


Figure 3.7 Inactivation time constants.
A, Na^+ currents (dotted line) were evoked in a single cell by a voltage step to -30 mV from a holding potential of -140 mV (above panel) in the absence (control) or presence of 25 μM DHA as indicated. The time-constant of inactivation of the Na^+ current was calculated from the exponential fit which is shown superimposed, and was increased from 0.814 ms (control) to 1.444 ms following 5 min incubation with 25 μM DHA. Time constants for inactivation (τ2) were calculated from the exponential fit shown superimposed. *B*, data were averaged as shown for both DHA (n=5) or EPA (n=5) incubation as indicated. Data represent mean ± SEM. *P<0.05, **P<0.01.

The effect of EPA and DHA on the rate of recovery of the Na^+ current from inactivation was investigated by use of a two-pulse protocol as previously described (Park & Ahmed, 1992; Su *et al.*, 1997; Saint, 1998). The first current was evoked by a pulse from a holding potential of -120 mV and stepped to – 20 mV. The cell was then held at –120 mV and subsequent voltage steps to -20 mV were given with a variable interval, t, between

pulses as shown in Figure 3.8A (upper panel). The lower panel in Figure 3.8A shows the resulting Na^+ current profile initially generated followed by a second Na^+ current. The second Na^+ current (I_2) was reduced to approximately 50 % of the amplitude of the first current (I_1), since there was insufficient time for complete recovery of all Na^+ channels to the resting (closed) state. The effect of the n-3 PUFAs DHA or EPA on the amplitude of the second current, as a fraction of the first, was plotted against t. At short intervals, the second pulse failed to evoke a Na^+ current since the channels had insufficient time to recover from the "inactivated state" induced by the first pulse. As the time interval between pulses was progressively increased, the Na^+ channels recovered from inactivation and the second pulse therefore elicited a measurable current. When the size of the peak inward Na^+ current evoked by the second pulse (and normalised as a fraction of pulse 1 as described above) was plotted against t, the recovery process could be fitted well with a single exponential:

$$I_2/I_1 = (I_{min}/e^{(t/\tau)})$$

where I_1 is the peak Na^+ current of the first pulse, I_2 is the peak Na^+ current of the second pulse, t is the time between pulses and τ is the time constant. The time constant for recovery in control solution (i.e., no additions) was 2.52 ± 0.28 ms and this value was significantly increased in the presence of 25 μM DHA to 5.37 ± 0.39 ms (n=4, $P<0.01$) as shown in Figure 3.8B. DHA at 50 μM also increased the time constant for recovery as shown in Figure 3.8C (τ=2.74 ms vs 7.56 ms, n=2 for control and DHA, respectively). In separate experiments the effects of 25 μM EPA was tested on the recovery of Na^+ current inactivation. Under control conditions (no additions) the value of τ for rate of recovery from inactivation was 2.63 ± 0.91 ms. However, in the presence of EPA the value for τ was significantly increased to 9.50 ± 1.01 ms (n=3, $P<0.05$). Following EPA washout, the

value for τ was restored back to a similar value compared with control conditions (3.82 ± 0.30 ms) as shown in Figure 3.8D.

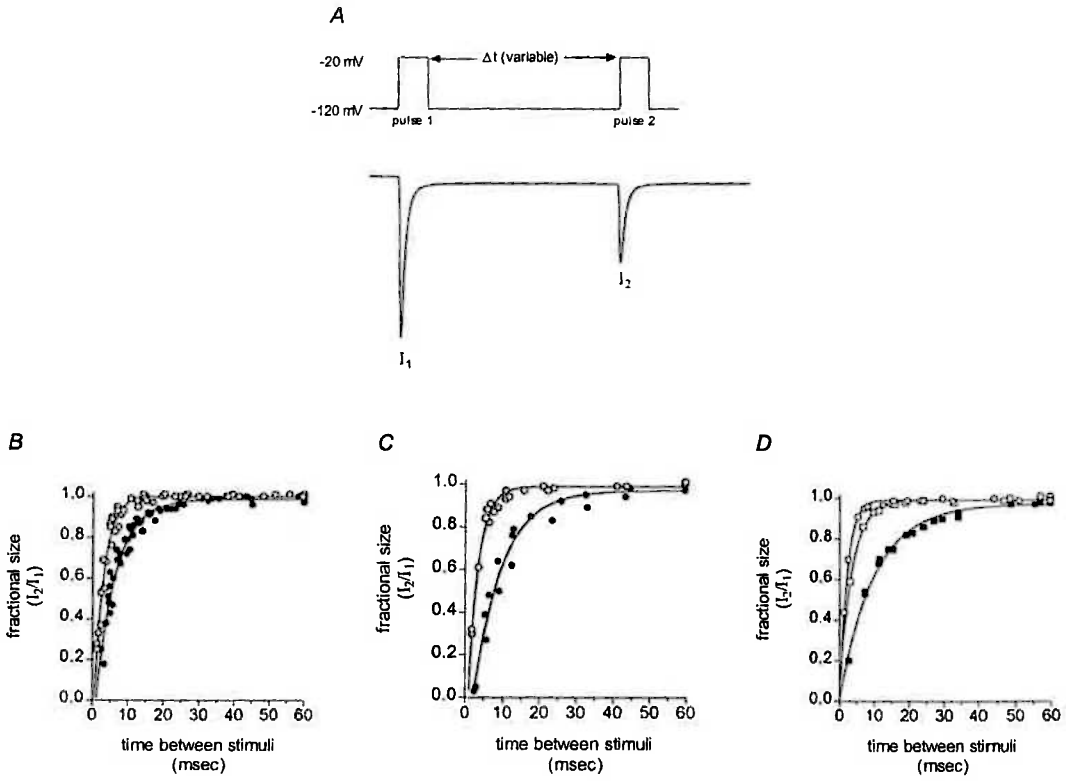


Figure 3.8. Slowing of the rate of recovery from inactivation by EPA and DHA.
A two-pulse protocol was applied to determine the rate of recovery of Na^+ currents from inactivation by n-3 PUFAs. The first current was evoked by a pulse from a holding potential -120 mV. Subsequent voltage steps to -20 mV were given with a variable interval, t, between pulses as shown in *A* (upper panel). The lower panel in (*A*) shows an initial Na^+ current followed by a second Na^+ current. The second Na^+ current was reduced to approximately 50 % of the amplitude of the first current, since there was insufficient time for complete recovery of all Na^+ channels to the resting state. The effect of 25 μM DHA (*B*), 50 μM DHA (*C*) or 25 μM EPA (*D*) on the amplitude of the second current, as a fraction of the first, is plotted against t. The points show data obtained in control solution (○) or in the presence of DHA (λ), EPA (ν), or EPA washout (□). The data were well fitted by a single exponential function (as shown by the lines of best fit on each plot). Time constants (τ) for (*B*) control = 2.52 ± 0.28 ms, 25 μM DHA = 5.37 ± 0.39 ms (n=4, P<0.01), (*C*) control = 2.74 ms, 50 μM DHA = 7.56 ms (n=2), (*D*) control = 2.63 ± 0.91 ms, 25 μM EPA = 9.50 ± 1.01 ms (n=3, P<0.05), washout = 3.82 ± 0.30 ms.

3.3.6 Steady-state fluorescence anisotropy of rat cardiomyocytes.

The effect of acute addition of fatty acids on cardiomyocyte membrane fluidity was determined by steady-state fluorescence anisotropy using the probe TMAP-DPH. The

fluorescence anisotropy (r_{ss}) following incubation in (20 μM) stearic acid (n=6), oleic acid (n=10) or linoleic acid (n=7) was not significantly different from control (no fatty acid additions, n=23, Figure 3.9). However, r_{ss} was significantly decreased following incubation with (20 μM) DHA (0.199 ± 0.004, n=21, P<0.01), EPA (0.204 ± 0.006, n=9, P<0.01), or ALA (0.213 ± 0.005, n=11, P<0.01) compared with control (0.239 ± 0.003). These data indicate that cardiomyocyte membrane fluidity was increased following acute addition of DHA, EPA and ALA. In addition, the fluorescence anisotropy of TMAP-DPH was significantly decreased to 0.214 ± 0.009 (indicative of an increase in membrane fluidity) following addition of the membrane fluidising agent benzyl alcohol (10 mM).

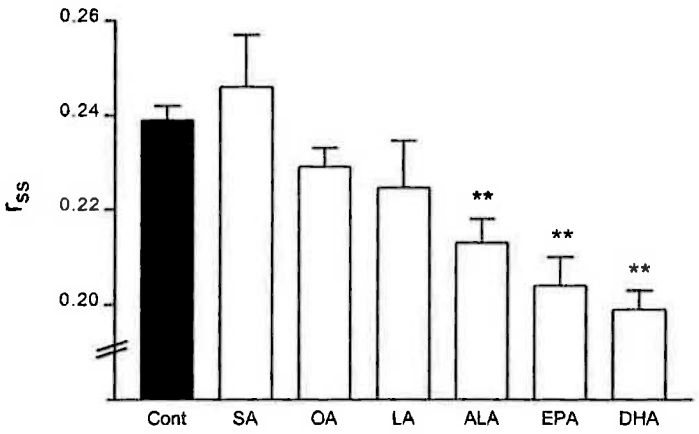


Figure 3.9 Effect of fatty acids on adult rat ventricular myocyte sarcolemmal membrane fluidity.
Ventricular cardiomyocytes plated on coverslips were incubated for 15 min with 1μM TMAP-DPH at 37°C. Cardiomyocytes were then transferred to a glass cuvette containing 20 μM fatty acids or 10 mM benzyl alcohol and the steady-state fluorescence anisotropy of TMAP-DPH was measured (as described in "methods"). Data are presented as mean ± SEM. **P<0.01 compared with control. Abbreviations: Cont, control (no additions); SA, stearic acid (18:0); OA, oleic acid (18:1 n-9); LA, linoleic acid (18:2 n-6); ALA, α-linolenic acid (18:3 n-3); EPA, eicosapentaenoic acid (20:5 n-3); DHA, docosahexaenoic acid (22:6 n-3); B Alc, benzyl alcohol.

3.4 DISCUSSION

Polyunsaturated fatty acids, particularly of the omega-3 class, are known to exert an antiarrhythmic effect in a variety of *in vitro* and *in vivo* preparations. This may in part be a consequence of an alteration in the electrophysiology of the myocardium, since a reduction in excitability of the myocardium (either in the intact heart or in single cardiac myocytes) is the most often observed effect. While it has been shown that PUFAs can influence a range of ionic currents in cardiac myocytes, for example, Ca^{2+} currents (Pepe *et al.*, 1994) and transient outward K^+ currents (Bogdanov *et al.*, 1998), one of the most powerful means of producing a reduction in cell excitability is by block of voltage dependent Na^+ channels, which produces an increase in threshold for action potential generation. Indeed, voltage-dependent Na^+ channel blockade is the principal mechanism of action of the class I antiarrhythmic agents such as lidocaine.

This study shows that acute addition of micromolar concentrations of n-3 PUFAs to adult rat ventricular myocytes rapidly blocks the Na^+ current and shifts the voltage dependence of inactivation to more hyperpolarised potentials. In the presence of DHA, the most potent of the n-3 PUFAs tested, the maximum conductance was reduced by 50%, and the voltage dependence of inactivation shifted by about 20 mV (when tested at a final concentration of 25 μM). These effects were also apparent for the other n-3 PUFAs tested, EPA and ALA. This observation indicates that at the resting membrane potential (approximately –90 mV) more Na^+ channels will exist in the inactivated state and less in the "resting" (or ready) state and they are likely to inactivate more slowly in the presence of n-3 PUFAs. Furthermore, since the rate of recovery from inactivation was also decreased, the membrane is less likely to undergo spontaneous depolarisations that are associated with aftercontractions and arrhythmias. The non n-3 fatty acids stearic, linoleic

and oleic acid were without effect on most of the activation and inactivation parameters associated with the Na^+ current. Furthermore, this latter group of fatty acids did not significantly modulate sarcolemmal membrane lipid fluidity. The fatty acids presumably exert their effects on membrane fluidity by dissolving in the cell membrane, since washing with control solution failed to reverse their effects, but perfusion with medium containing de-lipidated BSA did reverse the effects (presumably by removing membrane-associated PUFAs because of the high affinity of BSA for unesterified fatty acids).

The mechanism(s) by which n-3 PUFAs may effect cardiac Na^+ currents are unclear. The spectrum of changes produced by the n-3 PUFAs (a reduction in maximum current, a slight shift in the voltage dependence of activation, and a substantial shift in the voltage dependence of inactivation), is similar to that observed with certain general anaesthetic agents (Saint, 1998). It has been suggested that the effects of general anaesthetics on ion channel activity may be induced by alterations in membrane physical properties, particularly fluidity (Haydon & Urban, 1983), leading to the hypothesis that fatty acids may similarly affect Na^+ channels via a change in membrane fluidity. Certain alcohols have also been shown to inhibit Na^+ currents in the squid giant axon (Haydon & Urban, 1983). In the abovementioned study with squid giant axons, the orientation of the alcohols within the membrane appeared to be an important prerequisite to inhibit Na^+ currents (probably as a result of the hydroxyl groups anchoring the alcohols at the bilayer interface). As a result of such an orientation, the authors suggested that increases in membrane fluidity are likely to have occurred. Similarly, free n-3 PUFAs (NEFAs) may also require such a specific membrane orientation and this would be provided by their carboxyl groups localising at the bilayer:water interface, allowing the long fatty acyl chain to penetrate the bilayer. Consistent with this notion, the fatty acids increased

cardiomyocyte membrane fluidity in this study, as measured by steady-state fluorescence anisotropy, in a similar rank order potency to their effect on the cardiomyocyte Na^+ currents. The n-3 PUFAs were most potent with respect to both parameters while fatty acids of other classes were much less potent at both. Within the n-3 PUFA class examined, DHA was the most potent and ALA the least potent with regards to effects on both membrane fluidity and Na^+ current block. The results therefore suggest that the n-3 PUFAs induce a block of ventricular myocyte Na^+ currents which may in part be associated with changes in membrane lipid physical properties, since one would anticipate that protein molecules embedded in the membrane would be influenced by the properties of the membrane lipids which surround them (McMurchie, 1988; McMurchie *et al.*, 1997). This conclusion was supported by the data on the well known membrane fluidising agent benzyl alcohol (Gordon *et al.*, 1980), a molecule unrelated in structure to fatty acids, which also increased membrane fluidity and blocked cardiomyocyte Na^+ currents. This is in agreement with results that have been reported in which changing membrane lipid content, and thus membrane fluidity, modified the function of ion channels such as Ca^{2+}-activated K^+-channels in smooth muscle cells (Bregestovski & Bolotina, 1989).

The shift in the membrane potential following n-3 PUFA addition which results in a stronger electrical stimulus being required for eliciting an action potential and subsequent contraction is consistent with earlier findings using electrical-field stimulated cardiomyocytes from adult rats (Leifert, 1997; McMurchie *et al.*, 1998). These earlier studies demonstrated that an increased electrical stimulus was required to maintain electrically-induced contractions following acute incubation with the n-3 PUFAs. The present study showed that during the inactivation of whole-cell Na^+ currents, more Na^+

channels are probably recruited into the "inactivated state" at more negative membrane potentials when n-3 PUFAs were added compared with control, thus producing less whole-cell currents. For example, at -90 mV, the current is still at its maximum in the control situation, however in the presence of DHA, the Na^+ current is almost entirely abolished (as shown in Figure 3.4 and Figure 3.6B). This may be partly related to the effects of n-3 PUFAs on the inactivation phase of the Na^+ currents as shown in Figure 3.7A and B, thus prolonging the time taken for the Na^+ current to return to zero following a stimulus. Additionally, the data from Figure 3.8 demonstrates that Na^+ channels may be partially inactivated in the presence of n-3 PUFAs, thus preventing a second electrical pulse (originating within a very short time interval from the first) from generating full current flow. Such an effect has antiarrhythmic implications. It is interesting to note that a recent study investigating the effects of halothane, an anaesthetic that depresses cardiac conduction by a mechanism of Na^+ channel blockade, also has more potent effects when the Na^+ channels are in the inactivated state (Weigt *et al.*, 1997). It is possible that n-3 PUFAs have a higher affinity to inactivated channels than to channels residing in a resting state, such as the situation following halothane treatment (Weigt *et al.*, 1997).

Recent studies reported that inhibition of cardiac Na^+ channels, for example following spiradoline treatment, protects the heart from arrhythmias by a mechanism of Na^+ channel blockade and subsequent slowing of the heart rate (Eng *et al.*, 1998; Pugsley *et al.*, 1998). Furthermore, the extent of heart rate suppression has been reported to be correlated with protection from arrhythmias (Bernier *et al.*, 1989) and elevated heart rate has been reported as a predictor of increased cardiovascular morbidity (Palatini, 1999). Therefore, results from this study may be interpreted to indicate that the n-3 NEFAs could potentially induce antiarrhythmic effects by a mechanism mediated by Na^+ channel

blockade, which in the whole heart, may secondarily involve a significant reduction in heart rate. However, cardiomyocytes in normally functioning myocardium charactersied by normal values for the resting membrane potential are probably not affected by the n-3 PUFAs to a large degree. Therefore, cardiomyocytes that are partially depolarised which may occur for example under conditions of myocardial ischaemia or increased β-adrenergic activation, the presence of n-3 PUFAs may cause a voltage-dependent shift to more hyperpolarised potentials (as shown by the voltage-dependence of inactivation curves). In essence, this would result in the membrane becoming less excitable to incoming, potentially arrhythmic depolarising stimuli in ischaemic (or partially depolarised) myocardium. Following dietary supplementation with n-3 PUFAs, this protection would also be enhanced due to the increased pool of available n-3 NEFAs (Nair *et al.*, 1999).

As noted above, the original impetus for work on effects of fatty acids in the heart came from epidemiological and dietary studies. Dietary fatty acids are incorporated into the phospholipid structure of the membrane, but nevertheless appear to exert an antiarrhythmic effect. However, following dietary supplementation of pigs with n-3 PUFAs, DHA concentrations in the NEFA fraction were found to be 246 nmol/g heart in normoxic myocardium of the NEFA fraction. The nonesterified DHA was further selectively increased to 447 nmol/g heart (in pigs) following hypoxia (Nair *et al.*, 1999). Similar increases of the NEFAs, EPA and docospentaenoic acid (DPA, 22:5, n-3) in the myocardium was observed. In beef tallow fed pigs, the NEFA concentrations of DHA under normoxic conditions was only 45 nmol/g heart and increased to 55 nmol/g heart following hypoxia (Nair *et al.*, 1999). It is possible that non esterified n-3 PUFAs may be selectively taken up from plasma and stored within the myocardial cells as free fatty

acids, triglycerides, phospholipids or in other storage forms to act as a reserve of n-3 PUFAs. It is known that the accumulation of free fatty acids which occurs early during myocardial ischaemia depends upon activation of phospholipase A_2, and it has been estimated that the free fatty acid concentration following release of fatty acids from membrane phospholipids may result in a concentration of 20 μM in the rat heart after 1 h of ischaemia (Vandervusse *et al.*, 1997). Hence, the concentrations of PUFAs used in this study (1 - 50 μM) are similar to those found in the ischaemic myocardium. Therefore, the release of fatty acids from the membrane by phospholipases or their availability in the NEFA fraction following the consumption of a fish oil supplemented diet may play an important role in the prevention of cardiac arrhythmias.

CHAPTER 4

4 "Effects of dietary fish oil on contractile activity of cardiomyocytes"

4.1 INTRODUCTION

Since most of the previous studies investigating n-3 PUFAs have used acutely applied n-3 PUFAs to cells, the present study sought to determine whether the antiarrhythmic effects of dietary n-3 PUFAs could be demonstrated in isolated adult rat cardiomyocytes maintained on a diet supplemented with either fish oil as the source of n-3 PUFAs (containing EPA and DHA), canola oil containing a source of "essential" n-3 PUFA (ALA) or olive oil as a control (containing mostly oleic acid, 18:1, n-9 and lacking n-3 PUFAs). Importantly, this would allow for determination of changes in sarcolemmal membrane fluidity as a result of n-3 PUFA incorporation into the membrane phospholipids by dietary means as well as determining cardiomyocyte contractile sensitivity to KCl depolarisation. This study was also designed as a prelude to larger more involved studies on the effects and mechanisms of dietary n-3 PUFA action at the level of the cardiomyocyte.

4.2 METHODS

4.2.1 Animals and dietary oils

Ethics approval for this study was obtained from the CSIRO Health Sciences and Nutrition Animal Ethics and Experimentation Committee. Male Sprague Dawley rats were obtained from the Adelaide University, Central Animal House (Adelaide, SA, Australia) at 4 weeks of age. All animals were housed in the CSIRO small animal colony in groups of no more than 5 per cage with food (standard laboratory rat chow) and water provided ad libitum. Room temperature was maintained at 23°C with constant 55% humidity, and lights were maintained on a 12 hour light (8am - 8pm)/dark cycle. Animals at 12 weeks of age were gavaged with either 2 mL of olive oil (OO), canola oil (CO) or fish oil (FO) once daily for 7 days. This volume of lipid supplement was chosen as it represented an (approximately) additional 10% dietary supplement and therefore it was known that all rats were obtaining the same volume of lipid supplement daily. OO and CO was obtained from Meadow Lea Foods Ltd. (Ryde, NSW, Australia) and FO was from Nippon Suisin Kaisho Ltd. (Japan). The fatty acid composition of the basal rat diet and the dietary oils are given in Table 4.1.

4.2.2 Induction of spontaneous or asynchronous contractions

Cardiomyocytes were prepared as described in Chapter 2 and superfused with Tyrode solution gassed with 100% O_2 containing 10 μM isoproterenol for 10 min at 37°C (in the absence of electrical stimulation). Quiescent cardiomyocytes, in the absence of electrical-field stimulation, when treated with isoproterenol are induced to contract spontaneously. The percentage of cardiomyocytes spontaneously contracting at the end of the 10 minute phase was determined. At least 20 cardiomyocytes were observed in triplicate

experiments for data analysis. Thereafter, asynchronous contractile activity (i.e., contractions not in synchrony with the electrical-field stimulation), was determined by applying a suprathreshold electrical stimulation for 1 min (at 1 Hz). This was achieved using platinum electrodes spaced 10 mm apart in the superfusing chamber.

4.2.3 Extraction of total lipids

Total lipids were extracted from ventricular tissue, the standard colony diet and dietary oils using a slight modification of the method of Bligh and Dyer (Bligh & Dyer, 1959). For analysis of ventricular lipid composition, tissue was homogenised in 1 mL water using a Tenbroeck hand-held homogeniser. 4 mL of 2-propanol was added and the mixture boiled for 30 s. After cooling, 8 mL of chloroform was added, the mixture shaken, and the organic phase collected (when >300 mg tissue was used, the above volumes were doubled to allow adequate extraction of total lipids). The organic phase was evaporated to dryness under N_2. The phospholipids were separated from the other lipid classes by thin layer chromatography (TLC) on silica gel 150A-LK5D plates (Whatman, Clifton, NJ, USA), and developed in a solvent system of petroleum ether:acetone (3:1 [v/v]). The phospholipids remaining at the origin were scraped from the plate.

4.2.4 Preparation of fatty acid methyl esters for GC analysis

Phospholipid fatty acid methyl esters (FAMEs) were prepared as described previously (McMurchie *et al.*, 1983) by heating the samples (total phospholipids, phospholipid classes or non-esterified fatty acids) at 50°C overnight in 2ml 1% (v/v) H_2SO_4 in dried methanol. 200 µl water was added and FAMEs were extracted using hexane.

Contaminants were removed using a Florisil column (activated magnesium silicate). All solvents used for lipid extraction, TLC and preparation of FAMEs contained the antioxidant butylated hydroxytoluene (0.05% w/v). FAMEs from lipid extracts of the lipid supplemented diets, and the myocardial phospholipid extracts, were analysed by GLC. GLC was performed using a Hewlett Packard HP 5710 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) fitted with a 50 m BPX70 capillary column (Scientific Glass Engineering, Melbourne, Victoria, 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/min. A cold on-column injector was used with the flame ionisation detector temperature set at 250°C. FAMEs were identified using authentic lipid standards (Nu-Chek-Prep Inc., Elysian, MN, USA) by GLC and using Delta Chromatography software (Delta chromatography data systems version 5.0, DataworX Pty. Ltd., Scarborough, QLD, Australia). The proportions of the total fatty acids (wt/wt) were normalised to a value of 100%.

4.2.5 Statistics

Statistical analysis was performed using the computer software program Instat (GraphPad Software, CA, USA). One-way ANOVA with Dunnett's multiple comparison test (data were $\log_{10}$ transformed to generate Gaussian distributed values if required) or Bonferroni multiple comparison test were used to compare differences between the effects of dietary treatment. Results are expressed as the mean ± SEM. For each comparison the level of significance was set at $P<0.05$ for the indicated number of animals per dietary group.

4.3 RESULTS

4.3.1 Fatty acid composition of diets and ventricular phospholipids

The fatty acid composition of the olive oil (OO), canola oil (CO) and fish oil (FO) used in this study are given in Table 4.1. There was no detectable levels of EPA or DHA in the olive oil or canola oil as demonstrated by GLC. However, the fish oil contained approximately 37% EPA and 14% DHA. Following 7 days gavage the fatty acid composition of the total ventricular phospholipids isolated from young adult rats fed the 3 dietary oil supplements OO, CO, or FO are shown in Table 4.2. It should be noted that changes in total cellular phospholipid composition are likely to reflect changes in the membrane phospholipids. No attempt was made in these studies to fractionate the various membranes associated with rat cardiomyocytes. Therefore it is not possible to identify the changes in total cellular phospholipid fatty acids with any one particular membrane, such as that from the mitochondria, sarcoplasmic reticulum, T-tubules or sarcolemmal membranes. However, it is assumed that there would be no *a prior* reason not to exclude the fact that changes in the sarcolemmal membrane phospholipid fatty acids would not have occurred as a result of the dietary lipid treatments which changed the total ventricular phospholipids. Therefore, when further reference is made to changes in "ventricular phospholipid fatty acid composition" in this thesis, it is assumed that these changes reflect in part changes in the sarcolemmal membrane phospholipid fatty acid composition, as described previously (Shinitzky, 1984; McMurchie, 1988). The major fatty acids, palmitic (16:0), stearic (18:0), oleic (18:1; both n-9 and n-7 isomers), linoleic (18:2 n-6), arachidonic (20:4 n-6), docosapentaenoic (22:5 n-3) and docosahexaenoic (22:6 n-3) represented in total, greater than 96% of the proportion of fatty acids present in samples from each dietary group. The n-3 fatty acid, eicosapentaenoic acid (20:5 n-3), was substantially elevated in the myocardial membrane phospholipids in the FO group

compared with the OO group (2.05 ± 0.15% and 0.04 ± 0.03%, respectively, P<0.001). Significant increases in the proportion of 22:5 n-3 were apparent in the CO (1.93 ± 0.11%, P<0.01) and FO groups (2.74 ± 0.08%, P<0.001) in comparison with the OO group (1.38 ± 0.08%), however, 22:6 n-3 was only significantly increased in the FO group (13.4 ± 0.49%, P<0.001) compared with the OO group (8.93 ± 0.25%). The major effect of dietary FO supplementation was apparent in the ratio of the total n-6 to n-3 polyunsaturated fatty acids (PUFAs) which was significantly increased in the FO group compared with the OO group (18.27 ± 0.52% vs 11.80 ± 0.80%, respectively, P<0.05). This was mainly due to the increase in the proportion of the total n-3 PUFAs.

The various dietary treatments did not alter the proportions of 20:4 n-6 between groups. Dietary lipids did induce changes in the proportions of some of the major fatty acids. Compared with the OO group, 18:0 was significantly higher in the CO and FO groups (P<0.05), although the total proportion of saturated fatty acids between groups was not significantly different (approximately 42%). In the CO group 18:1 n-9 was significantly decreased (P<0.05) while 18:1 n-7 was significantly increased (P<0.05). The proportion of 18:2 n-6 of total phospholipid fatty acids in the CO group was significantly increased from 10.72 ± 0.62% (OO) to 12.99 ± 0.54% (P<0.05). In the FO group 18:2 n-6 was significantly decreased to 8.66 ± 0.36% compared with the OO group (P<0.05).

Table 4.1 Fatty acid composition (wt%) of standard diet and dietary oils[a]

FAME[b]	Ref diet	Olive Oil	Canola Oil	Fish Oil
14:0	1.79	n/d	0.07	6.03
14:1	0.40	n/d	n/d	0.21
16:0	18.73	11.86	4.67	8.93
16:1	1.37	0.39	0.19	11.10
18:0	8.08	1.78	1.80	0.97
18:1(n-9)	31.44	75.30	58.64	8.33
18:1(n-7)	n/d	n/d	n/d	2.66
18:2(n-6)	33.27	9.86	21.66	1.25
18:3(n-3)	2.21	0.61	11.57	0.90
20:0	0.46	0.60	0.31	0.61
20:1	0.43	n/d	1.05	0.55
20:2	n/d	n/d	0.05	0.07
20:3(n-6)	n/d	n/d	n/d	0.30
20:4(n-6)	n/d	n/d	n/d	1.68
20:3(n-3)	n/d	n/d	n/d	n/d
22:0	n/d	n/d	0.15	0.18
22:1	n/d	n/d	0.24	0.57
20:5(n-3)	1.03	n/d	n/d	37.51
24:0	n/d	n/d	n/d	0.49
22:5(n-3)	n/d	n/d	n/d	3.21
22:6(n-3)	1.11	n/d	n/d	14.46
Σ Sat.	29.06	14.24	7.00	17.20
Σ Mono.	33.64	75.69	60.12	23.42
Σ Poly.	37.61	10.47	33.27	59.38
Σ n-6	33.27	9.86	21.66	3.23
Σ n-3	4.35	0.61	11.57	56.07
n-6/n-3	7.65	16.08	1.87	0.06

[a]Data shown are means (n=2). [b]FAME, fatty acid methyl esters. n/d not detectable.

Table 4.2 Fatty acid composition (wt%) of ventricular phospholipids[a]

FAME[b]	Olive Oil	Canola Oil	Fish Oil
14:0	0.24 ± 0.06	0.16 ± 0.02	0.35 ± 0.07
16:0	21.35 ± 1.20	18.64 ± 0.32	17.42 ± 2.53
18:0	21.69 ± 0.62	23.31 ± 0.15[c]	23.39 ± 0.44[c]
18:1(n-9)	10.42 ± 0.95	7.13 ± 0.19[c]	7.53 ± 1.17
18:1(n-7)	3.50 ± 0.26	4.32 ± 0.18[c]	3.50 ± 0.10
18:2(n-6)	10.72 ± 0.62	12.99 ± 0.54[c]	8.66 ± 0.36[c]
18:3(n-3)	1.40 ± 0.92	0.18 ± 0.03	0.09 ± 0.05
20:0	0.00 ± 0.00	0.27 ± 0.07	0.22 ± 0.04
20:1	0.00 ± 0.00	0.10 ± 0.04	0.03 ± 0.02
20:2	0.00 ± 0.00	0.16 ± 0.06	0.11 ± 0.03
20:3(n-6)	0.16 ± 0.06	0.38 ± 0.09	0.18 ± 0.04
20:4(n-6)	19.93 ± 0.68	20.71 ± 0.37	19.83 ± 0.83
20:3(n-3)	0.05 ± 0.05	0.04 ± 0.04	0.00 ± 0.00
22:0	0.02 ± 0.02	0.15 ± 0.06	0.16 ± 0.05
22:1	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01
20:5(n-3)	0.04 ± 0.03	0.20 ± 0.02	2.05 ± 0.15[e]
24:0	0.10 ± 0.07	0.29 ± 0.03	0.25 ± 0.07
24:1	0.05 ± 0.05	0.09 ± 0.02	0.09 ± 0.04
22:5(n-3)	1.38 ± 0.08	1.93 ± 0.11[d]	2.74 ± 0.08[e]
22:6(n-3)	8.93 ± 0.25	8.95 ± 0.46	13.40 ± 0.49[e]
Σ Sat.	43.41 ± 1.07	42.81 ± 0.20	41.80 ± 2.20
Σ Mono.	13.97 ± 0.72	11.65 ± 0.35	11.16 ± 1.19
Σ Poly.	42.61 ± 1.63	45.54 ± 0.43	47.04 ± 1.52
Σ n-6	30.81 ± 1.24	34.08 ± 0.33	28.66 ± 1.08
Σ n-3	11.80 ± 0.80	11.30 ± 0.54	18.27 ± 0.52[d]
n-6/n-3	2.66 ± 0.16	3.07 ± 0.16	1.57 ± 0.04[d]

[a]Data shown are mean ± SEM (n=7-8). [b]FAME, fatty acid methyl esters. [c]$P<0.05$, [d]$P<0.01$, [e]$P<0.001$ vs olive oil.

4.3.2 Cardiomyocyte parameters following dietary lipid supplementation

Table 4.3 shows the body weights of rats following 7 days gavaging of OO, CO or FO and basal contractile parameters of isolated ventricular cardiomyocytes to determine whether any lipid-induced changes occurred following the short-term supplementation protocol. No significant differences were noted between body weights, ventricular

cardiomyocyte length at rest or change in cardiomyocyte length (dL%) under electrical-field stimulated conditions (when measured in standard Tyrode buffer). Since acute application of n-3 PUFAs results in the reduction of electrical excitability (evidenced by the necessity to increase the stimulating voltage to maintain cardiomyocyte contractions), the voltage required (this was determined from the Grass stimulator) to elicit contractile activity was determined at two levels. The voltage required to induce 50% of cardiomyocytes to contract ($V_{50\%}$) was highest in the FO group (6.3 ± 0.2 V) compared with the OO group (5.8 ± 0.2 V) and the CO group (6.1 ± 0.2 V), although this effect was not significantly different compared with the OO or CO groups. While this result was not statistically significant, the increase in voltage required in the FO group is consistent with the effects of acute application of n-3 PUFAs on electrical excitability discussed earlier. However, the voltage required to stimulate 100% of cardiomyocytes to contract was similar in all dietary groups as shown in Table 4.3.

Table 4.3 Body weights and cardiomyocyte parameters of rats gavaged 7 days with various dietary lipid supplements

	Olive Oil	Canola Oil	Fish Oil	P-value
Body weight (g)	446.4 ± 18.6	438.1 ± 10.4	436.6 ± 21.5	ns
Cell length (μm)	150.3 ± 3.4	145.1 ± 2.2	150.7 ± 3.4	ns
dL (%)[a]	6.8 ± 1.4	5.0 ± 1.2	6.5 ± 1.2	ns
$V_{50\%}$[b]	5.8 ± 0.2	6.1 ± 0.2	6.3 ± 0.2	ns
$V_{100\%}$[c]	10.4 ± 0.5	10.2 ± 0.5	10.5 ± 0.6	ns

[a]Change in cell length during electrical-field stimulation. [b]The voltage at which 50% of cardiomyocytes are driven to contraction during electrical-field stimulation. [c]The voltage at which 100% of cardiomyocytes are driven to contraction during electrical-field stimulation.change. ns; not significantly different by ANOVA (n=8 per dietary group).

4.3.3 *Isoproterenol-induced spontaneous contractions*

In a similar manner to that reported in Chapter 2, incubation of cardiomyocytes for 10 min with 10 μM isoproterenol resulted in the development of spontaneous contractions. Figure 4.1 demonstrates that the percentage of cardiomyocytes spontaneously contracting after 10 min of isoproterenol treatment was not significantly different between the OO and CO groups (34.6 ± 8.1% and 27.2 ± 3.4%, respectively). However, the percentage of cardiomyocytes spontaneously contracting from the FO group was significantly lower (16.1 ± 1.9%, $P<0.05$). The percentage of cardiomyocytes which were found to hypercontract following this procedure was lowest in the FO group but this was not significantly different compared with the CO or OO dietary groups (6.3 ± 1.8%, 10.7 ± 4.6% and 15.2 ± 5.3%; and for FO, CO and OO, respectively). During conditions of electrical-field stimulation the fish oil group had the lowest percentage of asynchronous contracting cardiomyocytes, although this was not significantly different to the canola oil or olive oil groups (61.7 ± 4.6%, 68.4 ± 5.5% and 72.7 ± 4.3%, for FO, OO and CO, respectively).

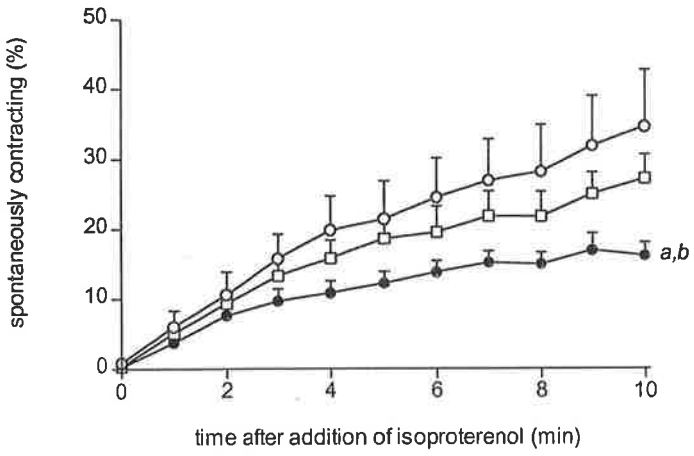


Figure 4.1 Time-course for the development of isoproterenol-induced spontaneous contractile activity in adult rat ventricular myocytes following dietary lipid supplementation for 7 days.
Cardiomyocytes were incubated with 10 μM isoproterenol added to the superfusing medium. The number of spontaneously contracting cardiomyocytes was determined over 10 min as the percentage of cells exhibiting rapid spontaneous contractions. Data represent mean ± SEM from animals supplemented with olive oil (O), canola oil (□), or fish oil (λ) from n=8 rats per dietary group. $^{a}P<0.05$ FO vs OO, $^{b}P<0.05$ FO vs CO.

4.3.4 *Contractile responses of electrically-stimulated cardiomyocytes to KCl depolarisation.*

To determine whether the dietary lipid supplements altered the depolarisation-induced contractile activity as was found following acute application of DHA in Chapter 2, the cardiomyocytes were incubated in increasing concentrations of KCl (as shown in Figure 4.2). In the presence of electrical-field stimulation at 1 Hz, and using physiological concentrations of KCl, 100% of cardiomyocytes exhibited synchronous contractile responses. However, increasing the concentration of KCl in the superfusing buffer caused cardiomyocytes from all dietary groups to progressively cease contracting in the presence of electrical-field stimulation as shown if Figure 4.2. At 45 mM $[K^+]_o$ 62.9 ± 4.1 % of cardiomyocytes were contracting from the OO group. The percentage of cardiomyocytes contracting in both the CO and FO groups was significantly less (49.1 ± 6.9 % and 46.6 ± 6.9 %, respectively) compared to the OO group as shown in Figure 4.2. This result suggests that the CO and FO lipid supplements may be partially depolarising the ventricular cardiomyocytes thus inactivating Na^+ channels, resulting in a reduction in the number of cardiomyocytes able to contract.

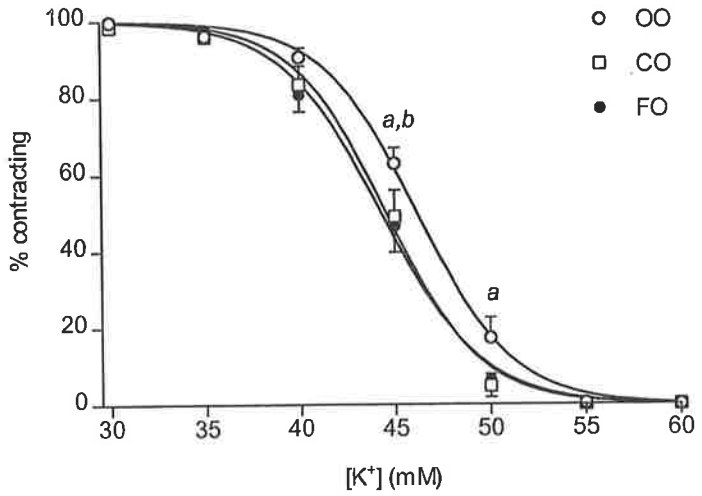


Figure 4.2 Contractile responses of electrically-stimulated cardiomyocytes to KCl depolarisation following 7 days dietary lipid supplementation.
Cardiomyocytes were electrically stimulated to contract at 1 Hz in the presence of increasing concentrations of KCl. The percentage of cardiomyocytes contracting was determined. ^{a}P<0.05 CO vs OO, ^{b}P<0.01 FO vs OO. Each value represents the mean ± SEM, n=8.

4.3.5 Dietary lipid effects on membrane fluidity measured by fluorescence anisotropy

Cardiomyocyte cell membrane fluidity was measured by steady-state fluorescence anisotropy (r_{ss}) using the probe TMAP-DPH as described in earlier sections. For these studies, 3 coverslips containing cardiomyocytes were analysed per rat and then these values were subsequently averaged to obtain a mean r_{ss} value "per rat". A total of six rats per dietary group was used to determine the r_{ss} values. Figure 4.3 demonstrates that there were no significant differences in the r_{ss} values in non-isoproterenol treated cardiomyocytes following the dietary lipid treatments. This would indicate that under the conditions employed using TMAP-DPH, that no significant effect on cardiomyocyte sarcolemmal membrane fluidity was evident. Furthermore, no significant difference in r_{ss} was evident following the addition of 10 μM isoproterenol to the cardiomyocytes when comparisons were made between the three dietary groups. Additionally, the r_{ss} values and

hence the relative membrane fluidity of isoproterenol treated cardiomyocytes was not significantly different from control (non-isoproterenol treated) cardiomyocytes.

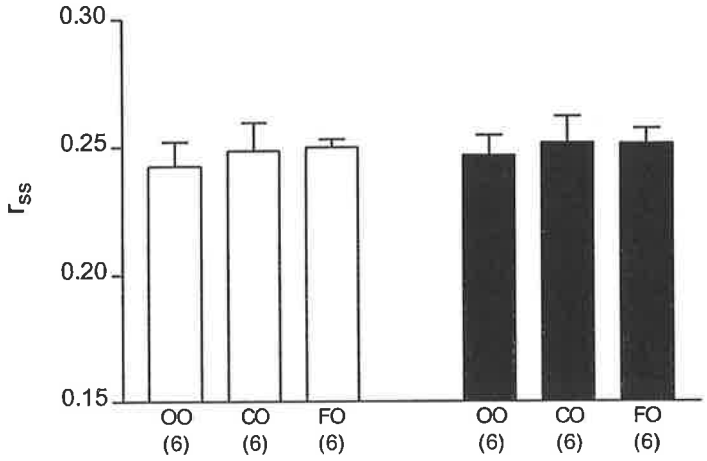


Figure 4.3 Steady-state fluorescence anisotropy value (r_{ss}) obtained using the probe TMAP-DPH in cardiomyocytes.
Cardiomyocytes were isolated from rats gavaged for 7 days with either OO, CO, or FO, and r_{ss} values measured (as described in Chapter 2) in the absence (open bars) or presence (filled bars) of 10 μM isoproterenol. Each value represents the mean ± SEM, n=6.

4.4 DISCUSSION

This study was aimed at reconciling whether dietary incorporated n-3 PUFAs act in a similar manner to that observed following acute addition. The study system made use of cardiomyocytes isolated from young adult rats fed a diet supplemented with olive oil (OO), canola oil (CO) or fish oil (FO) by gavage for 7 days, and examined the incorporation of these dietary oils into cardiac (membrane) phospholipids, cardiomyocyte membrane lipid fluidity, and cardioprotective effects against isoproterenol and KCl depolarisation.

This study shows that only 7 days of dietary lipid supplementation with canola oil or fish oil was required to observe significant increases in the incorporation of n-3 PUFAs (in particular, DPA, 22:5, n-3 in the CO group and DHA in the FO group) into membrane phospholipids compared with an olive oil supplement. However, the changes in DHA composition of membrane phospholipids in this study using FO were somewhat small compared to a previous study supplementing rat diets with fish oil for 5 days (Yang *et al.*, 1993). In that study, the rat myocardium from the butter-enriched diet group contained 10 % DHA compared to 20 % DHA in the fish oil group. In a longer term feeding study comparing OO and CO as the lipid supplements for 12 weeks (McLennan & Dallimore, 1995), rat myocardial phospholipids from the CO group had significantly higher levels of DHA in the membrane phospholipids compared to OO (11.6 ± 0.2 % and 8.0 ± 0.2 %, for CO and OO, respectively). However, in this study there was no significant difference in DHA composition (%) of myocardial membrane phospholipids comparing the OO and CO groups (both groups had approximately 9 % of total phospholipid fatty acids as DHA).

This brief dietary supplementation protocol did not result in changes of any of the basal cardiomyocyte contractile parameters measured, including cell length, % change in cell length and the voltage required to elicit contractile activity. However, the physiological response to high concentrations of the β-adrenergic agonist, isoproterenol, i.e., spontaneous contractile activity was significantly different between the dietary groups. Cardiomyocytes from the FO group were less prone to develop spontaneous contractile activity when compared to both OO or CO supplementation. Therefore, the "antiarrhythmic" effect observed following FO supplementation might be related to the total level of incorporation of n-3 PUFAs into the membrane phospholipids, which was significantly higher in the FO group compared with the OO and CO groups. Interestingly, dietary supplementation with CO and FO resulted in a small but significant potentiation of the contractile response to KCl depolarisation. Therefore, it is feasible to consider that following dietary CO or FO supplementation and under conditions that may partially depolarise myocardial tissue, such as during ischaemia (when K^+ leaks out of the cardiomyocytes), the myocardium may be protected from aberrant and potentially arrhythmic electrical stimuli. Normally, Na^+ channels become inactivated when the cell depolarises, thus preventing further action potentials and contractions (Opie, 1998b). It may be possible that dietary CO and FO feeding may contribute to the number of Na^+ channels becoming inactivated, thus providing an antiarrhythmic effect under conditions of partial membrane depolarisation. Indirectly, this notion supports some of the whole heart, ischaemic arrhythmia experiments that demonstrated that CO and FO supplementation prevented ischaemic hearts from developing cardiac arrhythmias (Yang *et al.*, 1993; McLennan & Dallimore, 1995). Therefore, these effects may be partly responsible for the antiarrhythmic mechanisms during ischaemia-reperfusion in the whole heart.

Earlier chapters demonstrated that acute addition of n-3 PUFAs to rat cardiomyocytes prevented spontaneous contractile activity and was associated with an increase in cardiomyocyte sarcolemmal membrane fluidity (decrease in anisotropy) measured by the membrane fluidity sensitive probe TMA-P-DPH. The rapid action of acute addition of n-3 PUFAs on asynchronous contractile activity and on membrane fluidity would suggest that infusion of these lipids into the sarcolemmal membrane, as opposed to their incorporation into membrane phospholipids, is sufficient for them to exert their antiarrhythmic/antiasynchronous action. Given the above observation, and the finding from previous studies in which antiarrhythmic actions of n-3 PUFAs were reported following dietary supplementation and concomitant incorporation into cardiac membrane phospholipids, it was necessary to determine whether incorporation of n-3 PUFAs into membrane phospholipids was associated with changes in membrane fluidity. Initially, it was anticipated that treatment of cardiomyocytes with isoproterenol would increase intracellular [Ca^{2+}], thus activating phospholipase A_2 activity and thereby liberating non-esterified (n-3) PUFAs indirectly (Hashizume *et al.*, 1997), thus modulating membrane fluidity. However, under basal conditions as well as in the presence of isoproterenol, there were no changes in cardiomyocyte membrane fluidity observed. This suggests that either the probe was not suitable to measure membrane fluidity changes in the experimental situation in which n-3 PUFAs were incorporated (esterified) into the phospholipids, or that changes in membrane fluidity are secondary to the antiarrhythmic effects and only occur following release of high concentrations of n-3 PUFAs from pools of cellular fatty acids (such as the membrane phospholipids). Alternatively, it may be a requirement that a higher percentage of incorporation of n-3 PUFAs into membrane phospholipids is needed in order to be able to detect changes in membrane fluidity with the probe TMAP-DPH.

CHAPTER 5

5 Study 1 "Effects of dietary fish oil on whole-cell Na^+ and K^+ currents"

5.1 INTRODUCTION

Experimental animal and human studies have shown that consumption of dietary n-3 PUFAs lead to changes in myocardial phospholipid fatty acid composition (McLennan *et al.*, 1992; Pepe & McLennan, 1996; Blake *et al.*, 2000). It is conceivable that changes in the degree and nature of unsaturation of membrane phospholipids could lead to changes in the function of various lipid-associated proteins, including messenger systems, receptors, ion transporters and ion channels (McMurchie *et al.*, 1988; McMurchie, 1988; Bregestovski & Bolotina, 1989; Bendahhou *et al.*, 1997). Of particular interest, in terms of cardiac arrhythmias where there are known disturbances in ion channel properties, is the possibility that the antiarrhythmic effects of dietary n-3 PUFAs (in the form of fish oil supplementation) may alter various ion channels known to result in abnormal action potentials and triggered activity. Chapter 3 demonstrated that acute addition of n-3 PUFAs to adult rat cardiomyocytes could block and inactivate Na^+ currents and such changes may have been associated with an increase in membrane fluidity. However, it is not known whether supplementation of the rat diet with n-3 PUFAs in the form of a concentrated fish oil preparation can elicit similar effects on cardiac Na^+ and K^+ currents, which are both important in the regulation and development of the cardiac action potential and the process of excitation-contraction coupling.

5.1.1 *Modification of Na^+ channel function by lipid environment*

There has been limited research on determining the effects of modifying the lipid composition of cell membranes on Na^+ channels and currents. One such study used rat diencephalic neurons grown in a defined medium supplemented with the essential fatty acids, ALA (n-3) or LA (n-6), which were subsequently washed to remove free fatty acids prior to experiments (Park & Ahmed, 1992). The kinetic parameters of the Na^+ currents were modified and the steady-state inactivation curve was shifted to the right. As a consequence, the neurons would be able to generate action potentials at a higher frequency. In studies investigating hypercholesterolaemia in adult rabbits, altered functional properties of the Na^+ channels were reported, including a reduction in the Na^+ current density compared with normolipidaemic rabbits (Wu *et al.*, 1995). Interestingly, in the abovementioned study, the whole-cell capacitance was increased by approximately 33% in the hypercholesterolaemic group, indicating an increase in cell surface area possibly mediated by cell hypertrophy.

At the cellular level, evidence for the antiarrhythmic action of acutely applied n-3 PUFAs was discussed in Chapter 3. In a manner similar to that reported by Xiao et al., (Xiao *et al.*, 1995) results from Chapter 3 demonstrate that whole cell Na^+ currents in adult rat cardiomyocytes could be inhibited by the acute addition of n-3 PUFAs, and this was associated with changes in the voltage-dependence of inactivation of Na^+ channels. Acute addition of n-3 PUFAs to adult rat cardiomyocytes have also been reported to inhibit K^+ currents (Bogdanov *et al.*, 1998), and collectively, these results support the notion that the n-3 PUFAs exert potent antiarrhythmic effects and they may do so via modulating membrane ion channel activity.

5.1.2 K^+ channels

Following action potential initiation by the opening of voltage-dependent Na^+ channels, the action potential is terminated by opening of voltage-dependent K^+ channels that mediate the outward movement of K^+ ions (the transient outward current, I_{to}), which repolarises the cell. Voltage-sensitive K^+ channels also set the resting membrane potential of the cardiac cells and modulate the action potential frequency and threshold. Voltage-gated K^+ channels can exist in three basic conformations (similar to the Na^+ channels): closed (or resting), open and inactivated, of which only the open state can conduct K^+ ions. The conformations are voltage-dependent with negative voltage tending to favour closure of the channels, and positive voltage favouring the formation of open and inactivated states:

$$\textit{closed} \leftrightarrow \textit{open} \leftrightarrow \textit{inactivated}$$

Conventionally, the I_{to} is often studied by holding the membrane at a highly negative voltage, inducing closure of all channels, and then following the application of a depolarising (positive) voltage, K^+ channels open in response to the test pulse and an outward current arises which is then followed by an inactivating phase, and the current subsequently falls. The detailed shape of the K^+ current time course reflects the balance between the forward rates which require opening and inactivation of channels, and the backward rates which requires closing and recovery of channels from inactivation. Abnormalities in I_{to} can lead to aberrant action potentials which in turn are increasingly recognised as being associated with a variety of acquired cardiac diseases associated with arrhythmias, including long Q-T syndrome (Miller, 1996; Roden & George, 1997; Knopp *et al.*, 1999).

5.1.3 K^+ channels and PUFAs

Previous studies have shown that the physiological and pharmacological properties of K^+ channels can be modulated by their lipid environment (Kim & Clapham, 1989; Kirber *et al.*, 1992; Honore *et al.*, 1994; Crumb *et al.*, 1999). Acute application of DHA was reported to block K^+ channels in dissociated cells from the pineal gland and isolated neocortical neurons (Poling *et al.*, 1995; Poling *et al.*, 1996). In adult rat ventricular cardiomyocytes I_{to} was inhibited by EPA and DHA resulting in a prolongation of the action potential (Bogdanov *et al.*, 1998; Macleod *et al.*, 1998). Therefore, inhibition of the I_{to} would reduce the current that normally repolarises the adult rat membrane potential, thus prolonging the action potential. The ability of the n-3 PUFAs to inhibit the I_{to} suggests an antiarrhythmic mechanism similar to that of the class III antiarrhythmic agents that work by prolonging the cardiac action potential duration and thereby increasing the refractory period. These results suggest a potential mechanism of antiarrhythmic action of free n-3 PUFAs involving the I_{to}. It should be noted however, that the authors of both these studies recognise that their significance remains unclear since PUFAs incorporated into phospholipids of the membrane bilayer may have different effects on the ion channels than do free fatty acids. Futhermore, the effects of dietary PUFA incorporation into membrane phospholipids on I_{to} have not been reported previously.

5.1.4 Study 1

This first study therefore aimed to establish whether dietary fish oil and saturated fat could alter the activity of whole-cell Na^+ and K^+ currents. Since ion channel function plays a central role in arrhythmogenesis, this study was designed to establish whether

dietary lipid supplementation of fish oil (containing n-3 PUFAs) influenced cellular Na^+ and K^+ ion current activity. This has been shown to occur with respect to the acute addition of n-3 PUFAs to cardiomyocytes derived from both adult (Chapter 3) and neonatal rats (Xiao *et al.*, 1995).

5.2 METHODS

5.2.1 *Animals and dietary oils*

Ethics approval for this study was obtained from the CSIRO Health Sciences and Nutrition Animal Ethics Committee and Adelaide University Animal Ethics Committee. Male Sprague Dawley rats were obtained from the University of Adelaide, Central Animal House (Adelaide, Australia) at 4 weeks of age. All animals were housed in the CSIRO small animal colony in groups of no more than 5 per cage with food (standard laboratory rat chow) and water provided *ad libitum*. Room temperature was maintained at 23°C with constant (55 %) humidity, and lights were maintained on a 12 hour light (8am - 8pm)/dark cycle. Animals were maintained on the rat chow from 4 weeks of age and for the duration of the experiment.

At 9 weeks of age the rats were gavaged with either 2 mL of saturated fat (SF) warmed to 30 °C, canola oil (CO) or fish oil (FO). The SF was beef and mutton fat from Metro Quality Foods, Greenacres, NSW, Australia. The CO was obtained from Meadow Lea Foods Ltd, Ryde, NSW, Australia. The FO was a n-3 PUFA concentrate in the ethyl ester form obtained from the National Institute of Health, Bethesda, MD, USA, as part of that Institute's Biomedical Test Materials Program. Rats were gavaged daily usually after 4 pm with 2 mL of one of the above supplements for a period of 3 weeks. The fatty acid composition of the colony rat diet and the dietary oils administered by gavage is given in Table 5.1.

5.2.2 *Electrophysiological recording of cardiac Na^+ current.*

As described in Chapter 3. Up to 6 cardiomyocyte recordings per rat heart were carried out in duplicate. All values obtained were then averaged for each rat heart.

5.2.3 *Solutions used for Na^+ current measurements*

The standard external (bath) solution used for Na^+ current measurements contained (mM): NaCl, 20; TES (N-tris-(hydroxy-methyl)-methyl-2-aminoethanesulphonic acid), 10; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; $CoCl_2$, 5; CsCl, 5; glucose, 10; cholineCl, 110, pH adjusted to 7.4 with 5.0 M NaOH. The pipette solution for all experiments contained (mM): CsF, 120; TES, 10; $MgCl_2$, 2; Na_2-EGTA, 20; $CaCl_2$, 2; pH adjusted to 7.4 with 5.0 M KOH. These solutions are designed to block all ionic currents other than Na^+ currents. In addition, the low extracellular Na^+ concentration (20 mM) is designed to reduce the peak Na^+ current and hence minimise series resistance errors in the clamp potential.

5.2.4 *Electrophysiological recording of cardiac K^+ current*

The peak transient outward current (I_{to}) amplitude was measured as the difference between the peak current and the steady state current at the end of a 300 ms voltage step. For the steady state activation protocol, the cells were prepulsed at -90mV for 50 ms then held at -120 mV for 300 ms and stepped to various potentials between -90 and +50 mV for 300 ms. For the inactivation protocol, the cells were prepulsed at -90 mV for 50 ms and stepped from various potentials between -120 mV and -90 mV (held for 300 ms) to a holding potential of +30 mV for 300 ms. Up to 6 cardiomyocyte recordings per rat heart were carried out in duplicate. All values obtained were then averaged for each rat heart.

5.2.5 *Solutions used for K^+ current measurements*

All experiments were conducted at room temperature (21-23°C). The bath solution contained (in mM): 5, KCl; 1, $MgCl_2$; 1, $CaCl_2$; 5, $CoCl_2$; 10, TES; 130, choline chloride; with pH adjusted to 7.35 using KOH. The solution in the recording pipette contained (in

mM): 115, KCl; 10, KF; 5, $MgCl_2$; 20, EGTA; 10, HEPES; with pH adjusted to 7.35 with KOH. These solutions are designed to block all ionic currents other than K^+ currents.

5.2.6 Total phospholipid analysis

As described in Chapter 4.

5.2.7 Statistics

Where necessary, statistical analysis was performed using the computer software program Instat version 3.0 (GraphPad Software, CA, USA). One-way ANOVA with Bonferroni multiple comparison test was used to compare differences between effects of the dietary treatment. Results are expressed as the mean ± SEM. For each comparison, the level of significance was set at $P<0.05$ for the indicated number of animals per dietary group. Where data appear as n=x,y; n= number of rat hearts and y=number of cells studied.

5.3 RESULTS

Body weights of the rats were weighed at the end of the experimental protocol and were not significantly different between the SF (455 ± 19 g), CO (444 ± 10 g) and FO (465 ± 16 g) groups.

5.3.1 Fatty acid composition of dietary lipid supplements

Fatty acid analysis of the standard colony diet used as well as the dietary lipid supplements is shown in Table 5.1. SF contained approximately 58% saturated fatty acids, mainly as 16:0 and 18:0. Apart from the presence of approximately 0.7% 18:3n-3, no other n-3 PUFAs were present in this supplement. CO contained approximately 6% saturated fatty acids mainly in the form of 16:0, while 18:1n-9 comprised about 59% of the total fatty acids. Of the PUFAs present, 18:2n-6 comprised approximately 22% while 18:3n-3 was about 12% of the total fatty acids. The major fatty acids of the FO supplement were 20:5n-3 (50%) and 22:6n-3 (27%).

Table 5.1 Fatty acid composition (wt%) of the reference (colony diet) and dietary oils[a]

FAME[b]	Reference Diet	Saturated Fat (SF)	Canola Oil (CO)	Fish Oil (FO)
14:0	0.82	8.36	n/d	0.19
14:1	0.07	1.56	n/d	n/d
16:0	15.32	32.00	4.55	0.17
16:1	0.75	2.81	0.18	0.32
18:0	4.40	16.89	1.51	0.41
18:1(n-9)	23.60	35.71	58.8	4.87
18:1(n-7)	n/d	n/d	n/d	n/d
18:2(n-6)	46.20	1.34	21.76	n/d
18:3(n-3)	4.57	0.68	12.06	0.50
20:0	0.47	0.65	0.17	0.16
20:1	0.39	n/d	1.06	10.70
20:2	0.16	n/d	n/d	n/d
20:3(n-6)	0.04	n/d	n/d	0.15
20:4(n-6)	0.02	n/d	n/d	1.71
20:3(n-3)	n/d	n/d	n/d	n/d
22:0	0.30	n/d	n/d	n/d
22:1	0.12	n/d	n/d	0.73
20:5(n-3)	0.96	n/d	n/d	50.47
24:0	0.22	n/d	n/d	0.88
24:1	0.06	n/d	n/d	n/d
22:5(n-3)	0.15	n/d	n/d	1.28
22:6(n-3)	1.18	n/d	n/d	27.54
Σ Sat.	21.53	57.89	6.23	1.81
Σ Mono.	24.99	40.08	60.04	16.61
Σ Poly.	53.48	2.02	33.82	81.65
Σ n-6	46.46	1.34	21.76	1.86
Σ n-3	6.86	0.68	12.06	79.79
n-6/n-3	6.77	1.97	1.81	0.02

[a]Data shown are means (n=2). [b]FAME, fatty acid methyl esters. n/d not detectable.

5.3.2 Fatty acid composition of rat ventricular phospholipids

The fatty acid composition of rat ventricular phospholipids after dietary lipid supplementation is shown in Table 5.2. Small changes were observed in the n-6 and n-3 classes of PUFAs mainly as a result of the FO supplementation. In comparison to the SF and CO supplemented diets, FO supplementation led to a decreased proportion of n-6

PUFAs, mainly with regard to the proportion of 18:2n-6, and a significantly higher proportion of 20:5n-3 and 22:6n-3.

Table 5.2 Fatty acid composition (wt%) of ventricular cardiomyocyte phospholipids after dietary lipid supplementation[a]

FAME[b]	Saturated Fat (SF)	Canola Oil (CO)	Fish Oil (FO)
14:0	0.18 ± 0.01	0.09 ± 0.02	0.11 ± 0.01
16:0	16.52 ± 0.36	14.81 ± 0.98	14.61 ± 0.77
17:0	0.41 ± 0.07	0.34 ± 0.06	0.44 ± 0.01
18:0	27.17 ± 0.43	26.13 ± 0.63	28.56 ± 0.47[f]
18:1(n-9)	2.85 ± 0.27	3.70 ± 0.25[c]	2.09 ± 0.08[h]
18:1(n-7)	3.05 ± 0.25	4.34 ± 0.29[d]	2.63 ± 0.13[h]
18:2(n-6)	11.21 ± 0.23	12.11 ± 0.48[c]	9.94 ± 0.70[f]
18:3(n-3)	0.07 ± 0.00	0.13 ± 0.01	0.06 ± 0.00
20:0	0.14 ± 0.00	0.43 ± 0.21	0.15 ± 0.01
20:1	0.41 ± 0.00	0.14 ± 0.03	0.08 ± 0.01
20:2	0.18 ± 0.02	0.17 ± 0.01	0.14 ± 0.01
20:3(n-6)	0.34 ± 0.02	0.40 ± 0.02	0.30 ± 0.02
20:4(n-6)	20.59 ± 0.45	21.12 ± 0.63	19.73 ± 0.34
20:5(n-3)	0.43 ± 0.04	0.57 ± 0.04	1.91 ± 0.14[e,h]
24:0	0.29 ± 0.02	0.22 ± 0.02	0.26 ± 0.02
22:5(n-3)	2.51 ± 0.06	2.35 ± 0.06	2.50 ± 0.08[h]
22:6(n-3)	14.13 ± 0.61	13.03 ± 0.58	16.56 ± 0.71[c,g]
Σ Sat.	47.31 ± 0.25	45.64 ± 0.69	46.12 ± 0.48
Σ Mono.	3.11 ± 0.26	4.39 ± 0.30[d]	2.65 ± 0.12[h]
Σ Poly.	49.37 ± 0.46	49.87 ± 0.77	51.14 ± 0.56
Σ n-6	32.15 ± 0.29	33.63 ± 0.60	29.28 ± 0.41[c,h]
Σ n-3	17.11 ± 0.60	16.08 ± 0.60	21.03 ± 0.85[d,h]
n-6/n-3	1.89 ± 0.08	2.11 ± 0.09	1.44 ± 0.08[d,h]

[a]Data shown are mean ± SEM for 6 animals per dietary group. [b]FAME, fatty acid methyl esters. [c]$P<0.05$, [d]$P<0.01$, [e]$P<0.001$ vs SF and [f]$P<0.05$, [g]$P<0.01$, [h]$P<0.001$ vs Canola oil (by ANOVA with Bonferonni multiple comparison test).

It must be noted that the n-3 PUFA content of the ventricular phospholipids of the SF and CO dietary supplemented animals was very similar. This is in contrast to the very different fatty acid profiles of these two supplements. Furthermore, the extent of increase in the n-3 PUFA content of ventricular phospholipids in comparison to other dietary supplements was well below that expected (for example, see chapter 4). However, it is important to note from this data, the ventricular phospholipid 22:6n-3 content was relatively high in all dietary groups. This point together with the high ALA content of the REF diet, will be discussed later and will form the basis of undertaking a second study.

5.3.3 Effect of dietary lipid supplementation on rat cardiomyocyte Na^+ currents

5.3.3.1 Voltage-dependence of activation of Na^+ currents

The average whole-cell capacitance of all cardiomyocytes from dietary supplemented rats was 132.0 ± 9.4 pF (n=34), 137.1 ± 5.1 pF (n=33) and 135.1 ± 5.8 pF (n=42) for SF, CO and FO supplemented rats, respectively. Na^+ currents in all cells were activated at approximately -60 mV and reached a maximum current at approximately -30 mV. The effect of the diets on the voltage-dependence of activation was determined by evoking currents by voltage steps to various potentials between -90 mV and +30 mV from a holding potential of -140 mV (as depicted in upper panel of Figure 5.1A). Figure 5.1A (bottom panel) shows an example of typical Na^+ currents evoked in a single cardiomyocyte by a step in membrane potential from a holding potential of -140 mV to potentials of 0, -10, -20, -30, -40 and -50 mV. The current density (maximum amplitude of the Na^+ currents/whole-cell capacitance) was plotted against the holding potential, as shown in Figure 5.1B for cardiomyocytes isolated from rats supplemented for 3 weeks

with either SF, CO or FO. The maximum Na^+ current densities elicited by a voltage step from -140 mV to -30 mV for SF, CO and FO supplemented rats were -17.8 ± 1.0 (n=6), -17.2 ± 1.6 (n=7) and -18.3 ± 0.9 (n=7) pA/pF, respectively (Figure 5.1B). The data points were fitted by the equation:

$$I = [G_{max} \cdot (V-E_{rev})] \cdot [1 / 1 + e^{(V-V')/k}] \quad \ldots\ldots 1$$

using a least squares fitting algorithm, where G_{max} is the maximum conductance, V' is the membrane potential for half activation of the channels, V is the test membrane potential, E_{rev} is the reversal potential for the current, and k is a slope factor. The means of all the above parameters for the least squares fit of equation 1 are given in Table 5.3. There was no significant difference between any of the dietary supplemented groups for any of the parameters examined.

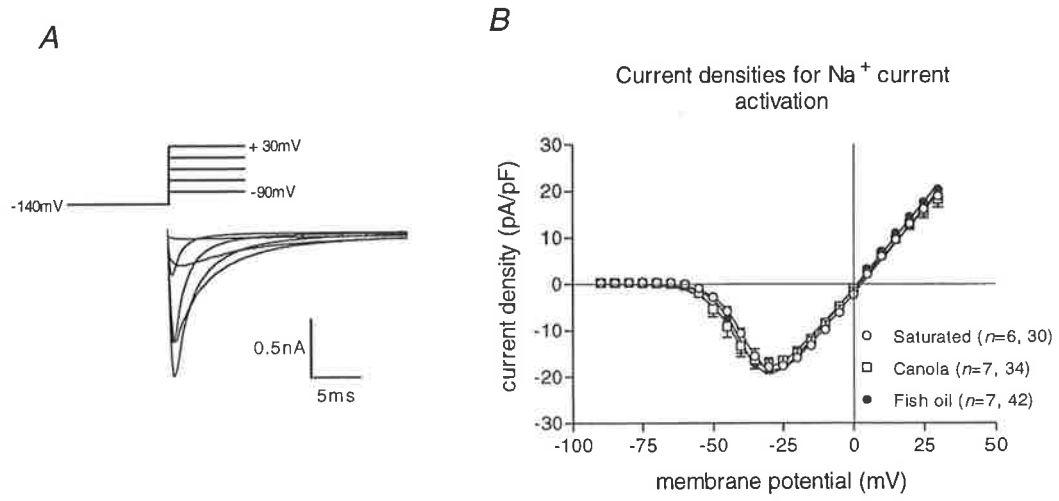


Figure 5.1 Effect of dietary supplementation of either saturated fat, canola oil or fish oil on the voltage-dependence of activation of Na^+ currents in adult rat ventricular myocytes.
A, Na^+ currents were evoked by voltage steps from a holding potential of -140 mV to various voltages between -90 mV and +30 mV as depicted (upper panel). Plotted below are superimposed currents evoked at 0, -10, -20, -30, -40 and -50 mV. *B*, The peak current density (peak current amplitude/cell capacitance (pA/pF) was plotted against the pulse potential. Data represent mean ± SEM from animals supplemented with saturated fat (○), canola oil (□), or fish oil (●). The solid lines show the least squares best fit of equation 1. The parameters for the best fit in each case are shown in Table 5.3. n=x, y refers to the number of dietary animals per group (x) and number of cardiomyocytes studied (y), repectively.

Table 5.3 Activation parameters from least squares fit of equation 1

Dietary group	G_{max} (pS/pF)	V_{50} (mV)	E_{rev} (mV)	k (mV^{-1})
SF (*n*=7)	0.75 ± 0.05	-37.2 ± 1.0	2.06 ± 1.56	4.47 ± 0.13
CO (*n*=7)	0.72 ± 0.06	-38.0 ± 1.9	3.07 ± 1.15	4.25 ± 0.08
FO (*n*=7)	0.75 ± 0.04	-38.0 ± 0.9	1.44 ± 0.77	4.44 ± 0.28

Abbreviations: SF, saturated fat diet; CO, canola oil diet; FO, fish oil diet. Data represent mean ± SEM (numbers in parentheses represent the number of hearts, *n*=30-42 cells examined for each dietary group).

5.3.3.2 Voltage-dependence of inactivation of Na^+ currents

The effect of the dietary supplementation on the voltage dependence of inactivation of the Na^+ current was investigated by stepping the membrane potential to a test potential of -30 mV from holding potentials which varied between -140 and -35 mV (Figure 5.2A, upper panel). The current density (maximum amplitude of the Na^+ currents/whole-cell capacitance) was plotted against the holding potential, as shown in Figure 5.2B for cardiomyocytes isolated from rats supplemented for 3 weeks with either SF, CO or FO. The data points were fitted using the Boltzmann equation:

$$I = I_{max} \cdot [1 / 1 + e^{(V-V')/k}] \quad \text{.....2}$$

where I_{max} is the maximum current, V is the test membrane potential, V' is the membrane potential at which half of the channels are inactivated, and k is a slope factor. The mean values for the parameters I_{max}, V' and k, for the least squares fit of equation 2 are given in Table 5.4. There was no significant difference between any of the dietary supplemented groups for any of the parameters examined.

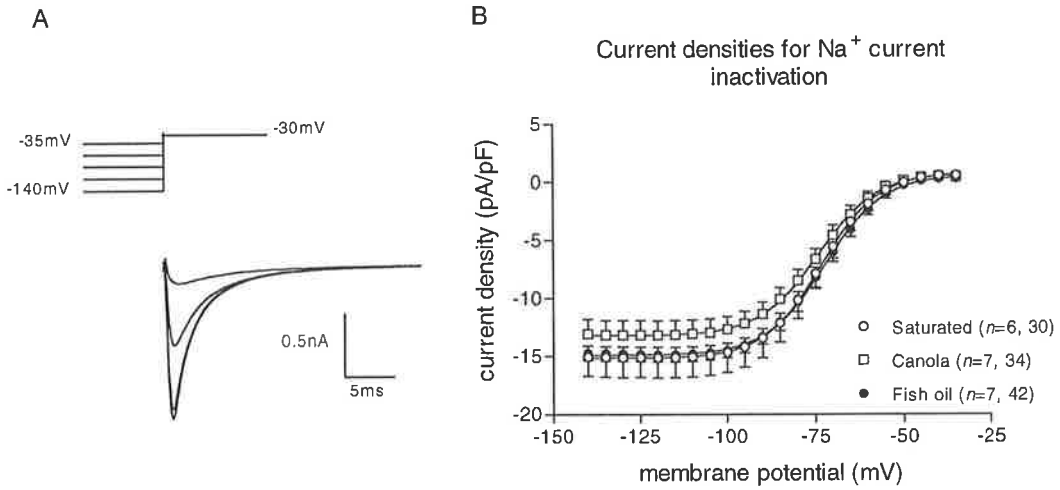


Figure 5.2 Effect of dietary supplementation of saturated fat, canola oil or fish oil on the voltage-dependence of inactivation of Na^+ currents in adult rat ventricular myocytes.
A, Na^+ currents were evoked by voltage steps to -30 mV from various holding potentials between -140 and -35 mV, as depicted (upper panel). Plotted below are currents evoked at -130, -110, -90, -70, and -50 mV, shown superimposed. *B*, The peak current density (peak current amplitude/cell capacitance ($pA \cdot pF^{-1}$)) was plotted against the pulse potential. Data represent mean ± SEM from animals supplemented with saturated fat (○), canola oil (□), or fish oil (●). The solid line shows the least squares best fit of equation 2. The parameters for the best fit in each case are shown in Table 5.4.

Table 5.4 Inactivation parameters from least squares fit of equation 2

Dietary group	I_{max} (pA/pF)	V_{50} (mV)	k (mV^{-1})
SF (*n*=7)	-15.0 ± 1.6	-73.9 ± 1.0	7.3 ± 0.17
CO (*n*=7)	-14.4 ± 1.5	-73.1 ± 2.0	6.9 ± 0.19
FO (*n*=7)	-14.9 ± 0.6	-72.8 ± 1.6	7.3 ± 0.22

Abbreviations: SF, saturated fat diet; CO, canola oil diet; FO, fish oil diet. Data represent mean ± SEM (numbers in parentheses represent the number of hearts, *n*=30-42 cells examined for each dietary group).

5.3.3.3 Kinetics of activation and inactivation

The effect of the diets on the kinetics of activation and inactivation of the Na^+ current was investigated. The time course for the increase in the Na^+ current was taken as the (macroscopic) rate of activation (τ_1) of the currents and the time course for the decline in

the Na^+ current was taken as an indication of the rate of inactivation (τ_2). Figure 5.3 shows a representative trace of the Na^+ current in a single cell elicited by a voltage step to -30 mV from a holding potential of -90 mV. The data points shown were fitted with a modified Hodgkin-Huxley equation (Hodgkin & Huxley, 1952):

$$I=I_{max}[1-e^{(-t/\tau 1)}]^3.[e^{(-t/\tau 2)}]\ ]..3$$

τ_1 is the time constant of the activation process and τ_2 the time constant of inactivation. The time constant for activation (τ_1) was similar for all dietary groups (Figure 5.3). However, both CO and FO groups had slower activation rates. Similarly, the time constants for inactivation (τ_2) were not significantly different between the dietary groups, although both CO and FO had faster inactivation rates (Figure 5.4).

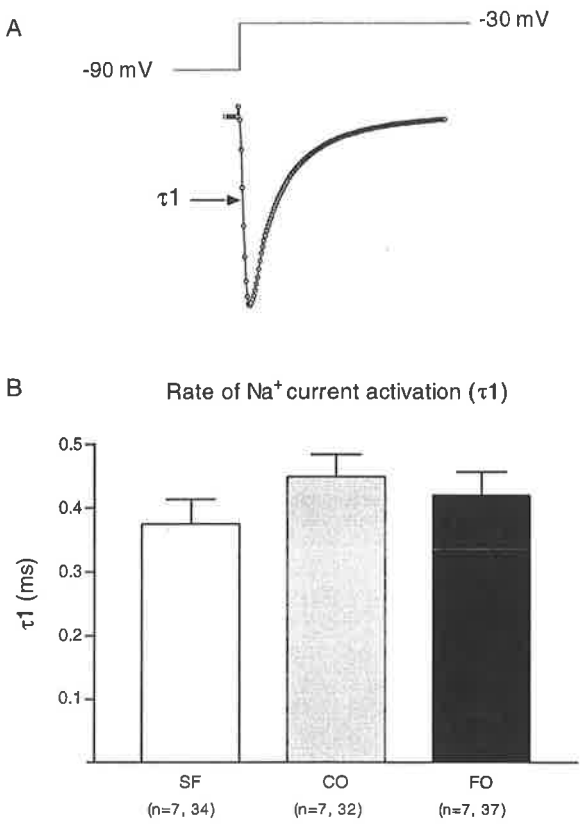


Figure 5.3 Na^+ current activation time constants.
A, Na^+ currents were evoked in a single cell by a voltage step to -30 mV from a holding potential of -90 mV. *B*, The time-constants of activation of the Na^+ currents were calculated from the Hodgkin-Huxley equation. Data were averaged as shown, for saturated fat (SF), canola oil (CO) or fish oil (FO) for the number of hearts and cardiomyocytes indicated in parentheses. Data represent mean ± SEM.

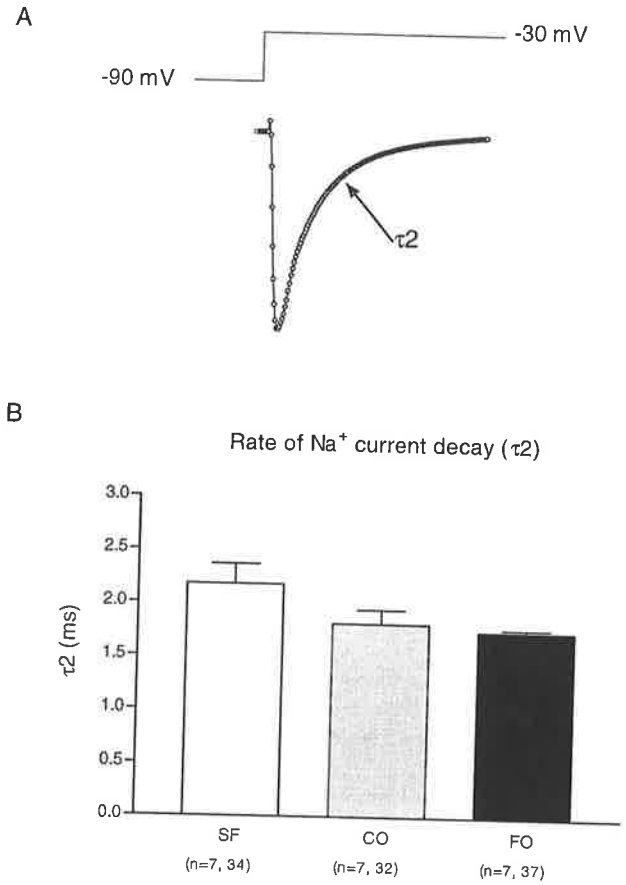


Figure 5.4 Na^+ current inactivation time constants.
A, Na^+ currents were evoked in a single cell by a voltage step to -30 mV from a holding potential of -90 mV. *B*, The time-constants of inactivation of the Na^+ currents were calculated from the Hodgkin-Huxley equation. Data were averaged as shown, for saturated fat (SF), canola oil (CO) or fish oil (FO) for the number of hearts and cardiomyocytes indicated in parentheses. Data represent mean ± SEM.

5.3.3.4 Time course of recovery from Inactivation

The rate of recovery from inactivation of Na^+ currents was investigated by the use of a two-pulse depolarisation protocol. Two identical voltage steps to -20 mV for 20 ms from a holding potential of -140 mV were given, separated by a variable time interval, t (as depicted in Figure 5.5A). At short intervals, the second pulse failed to evoke a Na^+ current since the channels had insufficient time to recover from inactivation induced by

the first pulse. As the interval was increased the amplitude of the current induced by the second pulse increased as the channels recovered from inactivation (Figure 5.5B). Ratios of the peak inward currents elicited by the second (I_2) and first (I_1) pulses (I_2/I_1) were plotted against the interpulse interval (t) and the time course of recovery was fitted to a single exponential function (as shown in Figure 5.5C);

$$I_2/I_1 = 1 - e^{(-t/\tau)}] \quad \text{.....4}$$

I_1 is the maximum current by the first pulse, I_2 is the maximum current elicited by the second pulse which was elicited by increasing the time interval from the first pulse (t), τ is the rate of recovery time constant (ms). The best fit for the time constants gave values of 1.72 ± 0.16 ms (n=6), 1.66 ± 0.11 ms (n=7), 1.54 ± 0.11 ms (n=7) for the SF, CO and FO groups, respectively (not significantly different).

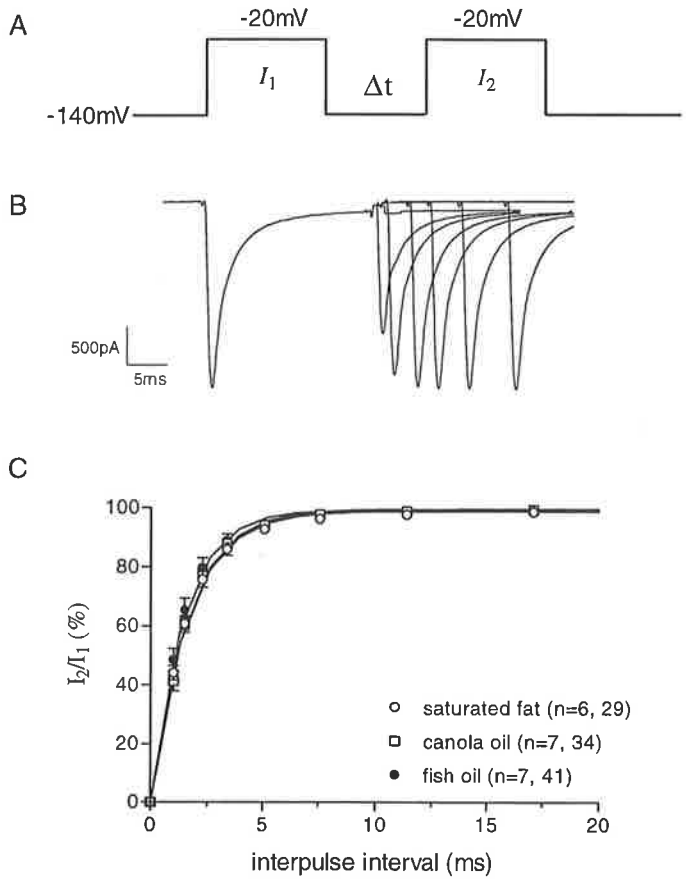


Figure 5.5 Rate of recovery from Na^+ current inactivation.
A two-pulse protocol was applied to determine the rate of recovery of Na^+ currents from inactivation. *A*, The first current was evoked by a pulse from a holding potential -140 mV. Subsequent voltage steps to -20 mV were given with a variable interval, t, between pulses as shown. *B*, Na^+ currents were generated followed by a second Na^+ current with gradually increasing time between pulses. *C*, The effect of with saturated fat (○), canola oil (□) or fish oil (●) for the number of hearts and cardiomyocytes indicated in parentheses. Data represent mean ± SEM.

5.3.4 Effect of dietary supplementation on rat cardiomyocyte transient outward K^+ currents

5.3.4.1 Voltage-dependence of activation of I_{to}

The average whole-cell capacitance of all cardiomyocytes from dietary supplemented rats was 128.2 ± 7.1 pF (*n*=17), 127.2 ± 5.6 pF (*n*=25) and 146.4 ± 7.0 pF (*n*=28) for SF, CO and FO supplemented rats, respectively (not significantly different). The effect of the

diets on the voltage-dependence of activation was determined by evoking currents by voltage steps to various potentials between -90 mV and +50 mV from a holding potential of -120 mV (as depicted in upper panel of Figure 5.6A). Figure 5.6A (lower panel) shows an example of typical I_{to} currents evoked in a single cardiomyocyte by a step in membrane potential from a holding potential of -120 mV to potentials of -70, -10, 0, +10, +20, +30 and +40 mV. I_{to} in all cells were activated at approximately -20 mV and reached a maximum current at approximately +50 mV (Figure 5.6B). The current density (maximum amplitude of the I_{to} currents/whole-cell capacitance) was plotted against the test potential, as shown in Figure 5.6B for cardiomyocytes isolated from rats supplemented for 3 weeks with either SF, CO or FO. The data points were fitted by the Boltzmann equation using a least squares fitting algorithm, where I_{max} is the maximum current, V' is the membrane potential for half inactivation of the channels, V is the test membrane potential, and k is a slope factor. The means of all the above parameters for the least squares fit of the equation are summarised in Table 5.5. Although the FO group had a 12% lower I_{max} compared with either SF or CO, the difference was not significant.

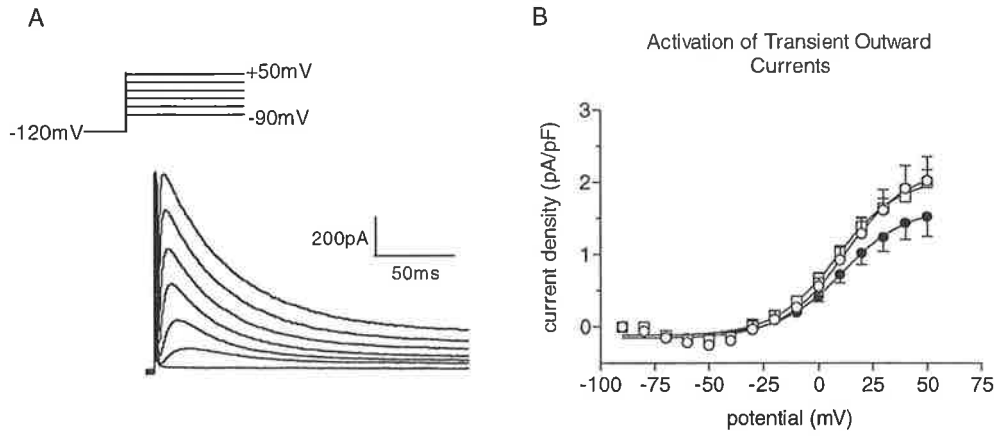


Figure 5.6 The voltage-dependence of activation of I_{to} in adult rat ventricular myocytes following dietary lipid supplementation.
A, Transient outward currents (I_{to}) were evoked by voltage steps from a holding potential of -120 mV to various voltages between -90 mV and +50 mV as depicted (upper panel). Plotted below are superimposed currents evoked at -70, -10, 0, 10, 20, 30 and 40 mV. *B*, The peak current density (peak current amplitude/cell capacitance (pA/pF) was plotted against the test potential. The solid lines show the least squares best fit of the Boltzmann equation. The parameters for the best fit in each case are shown in Table 5.5. Data represent mean ± SEM from animals supplemented with saturated fat (○; *n*=4, 17), canola oil (□; *n*=5, 24) or fish oil (●; *n*=6, 28).

Table 5.5 Activation parameters from least squares fit of the Boltzmann equation

Dietary group	I_{max} (pA/pF)	V_{50} (mV)	k (mV^{-1})
SF (*n*=4)	2.34 ± 0.35	-12.20 ± 0.76	13.45 ± 0.56
CO (*n*=5)	2.21 ± 0.15	-9.30 ± 1.95	14.47 ± 0.68
FO (*n*=6)	2.07 ± 0.29	-12.17 ± 2.76	14.79 ± 1.42

Abbreviations: SF, saturated fat diet; CO, canola oil diet; FO, fish oil diet. Data represent mean ± SEM (numbers in parentheses represent the number of hearts, *n*=17-28 cells examined for each dietary group).

5.3.4.2 Voltage-dependence of inactivation of I_{to}

The effect of the dietary supplementation on the voltage dependence of inactivation of the I_{to} currents was investigated by stepping the membrane potential to a test potential of +20 mV from holding potentials which varied between -120 and +30 mV (Figure 5.7A upper

panel). Figure 5.7A (lower panel) shows an example of typical I_{to} currents evoked in a single cardiomyocyte by a step to +20 mV from various holding potentials of -120, -80, -50, -40, -30, -20 and -10 mV. The current density (maximum amplitude of the I_{to} currents/whole-cell capacitance) was plotted against the holding potential, as shown in Figure 5.7B for cardiomyocytes isolated from rats supplemented with either SF, CO or FO. The data points were fitted using the Boltzmann equation (equation 2). The mean values for the parameters I_{max} , V' and k, for the least squares fit are given in Table 5.6. There was no significant difference between any of the dietary supplemented groups for any of the parameters examined.

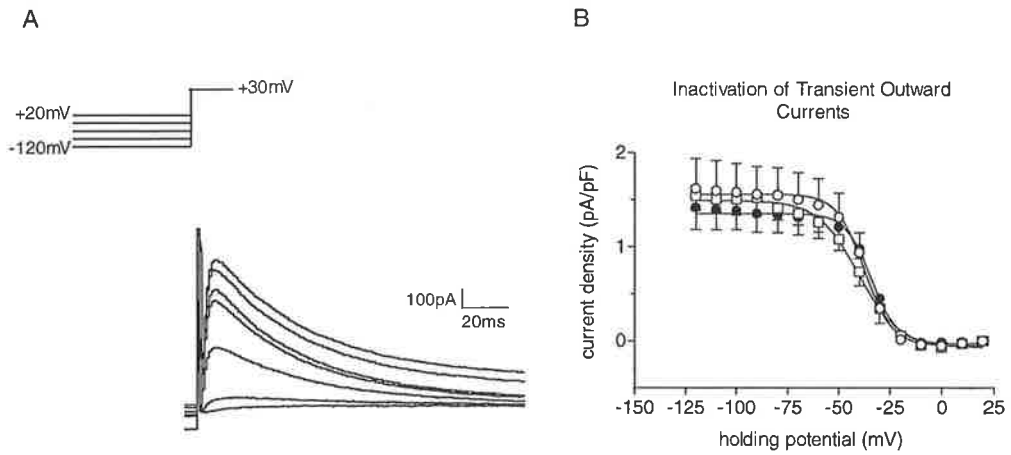


Figure 5.7 The voltage-dependence of inactivation of I_{to} in adult rat ventricular myocytes following dietary lipid supplementation.
A, Transient outward currents (I_{to}) were evoked by voltage steps to +30 mV from various holding potentials between -120 and +20 mV, as depicted (upper panel). Plotted below are currents evoked at -120, -80, -50, -40, ,-30, -20 and -10 mV, shown superimposed. *B*, The peak current density (peak current amplitude/cell capacitance (pA/pF) was plotted against the pulse potential. The solid line shows the least squares best fit of the Boltzmann equation. The parameters for the best fit in each case are shown in Table 5.6. Data represent mean ± SEM from animals supplemented with saturated fat (O; n=4, 7), canola oil (□; n=5, 24) or fish oil (●; n=6, 28).

Table 5.6 Inactivation parameters from least squares fit of the Boltzmann equation

Dietary group	I_{max} (pA/pF)	V_{50} (mV)	k (mV^{-1})
SF (n=4)	1.64 ± 0.30	-37.23 ± 1.82	-6.03 ± 0.39
CO (n=5)	1.57 ± 0.16	-39.89 ± 2.57	-7.51 ± 0.97
FO (n=6)	1.49 ± 0.17	-34.22 ± 1.61	-5.35 ± 0.57

Abbreviations: SF, saturated fat diet; CO, canola oil diet; FO, fish oil diet. Data represent mean ± SEM (numbers in parentheses represent the number of hearts, n=17-28 cells examined for each dietary group).

5.3.4.3 Kinetics of inactivation

The effect of the dietary lipid supplementation on the inactivation of the I_{to} currents was investigated by stepping the membrane potential to a test potential of +30 mV from a holding potential of -90 mV (Figure 5.8A). The time course of I_{to} inactivation was best fitted by a single exponential as shown in Figure 5.8A (lower panel). The time constant of inactivation (τ) was not significantly different between the dietary groups SF, CO or FO as shown in Figure 5.8B.

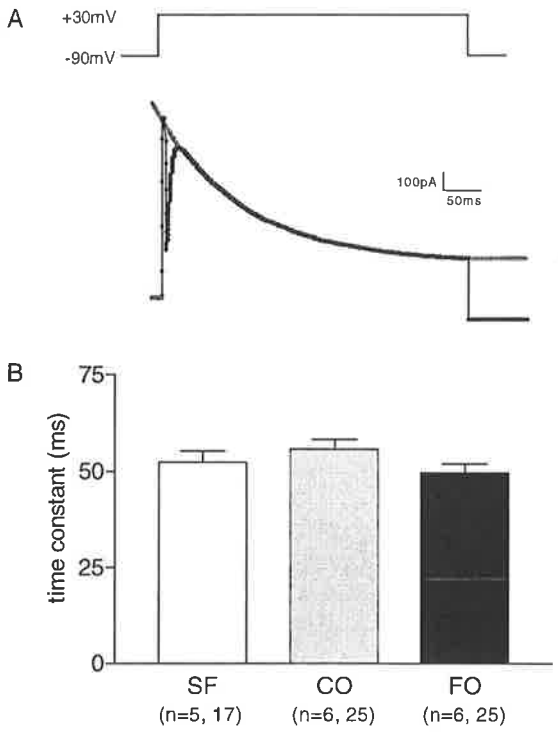


Figure 5.8 Inactivation time constants of I_{to}.
A, K^+ currents were evoked in a single cell by a voltage step to +30 mV from a holding potential of -90 mV. The time-constants of inactivation of the K^+ currents were calculated using a single exponential equation of the declining phase of the current as shown by the bold line (lower panel). *B*, Data were averaged for saturated fat (SF), canola oil (CO) or fish oil (FO) for the number of hearts and cardiomyocytes indicated in parentheses. Data represent mean ± SEM.

5.3.5 Cardiac phospholipid fatty acids from colony diet fed rats

In view of the unexpected relatively high n-3 PUFA proportion in the ventricular phospholipids in all dietary groups, particularly with regard to the proportion of 22:6n-3, the ventricular phospholipid fatty acid composition of rats maintained on rat colony diet *without* supplementation of any other oils was analysed (i.e, those rats maintained on the reference diet referred to in Table 5.1). The fatty acid profile (Table 5.7) was very similar to that observed for all dietary lipid supplemented rats with regard to the proportion of n-3 PUFAs present, and in particular, the relatively high proportion of 22:6n-3. Finally, fatty acid analysis of the rat colony diet (Table 5.1) revealed that the 18:3n-3 content of this

diet (4.6% of total fatty acids) was much higher than previously observed with such experimental diets (for example, compare to Chapter 4).

Table 5.7 Fatty acid composition (wt%) of ventricular cardiomyocyte phospholipids from rats fed the reference (colony) diet[a]

FAME[b]	Reference (Colony diet)
14:0	0.08 ± 0.08
16:0	14.21 ± 0.12
18:0	23.41 ± 0.45
18:1(n-9)	3.27 ± 0.16
18:1(n-7)	2.96 ± 0.12
18:2(n-6)	19.97 ± 0.63
18:3(n-3)	0.06 ± 0.06
20:0	0.08 ± 0.08
20:2	0.75 ± 0.44
20:3(n-6)	0.25 ± 0.01
20:4(n-6)	18.78 ± 0.68
20:5(n-3)	0.06 ± 0.06
22:5(n-3)	1.83 ± 0.06
22:6(n-3)	14.26 ± 0.30
Σ Sat.	37.77 ± 0.73
Σ Mono.	6.23 ± 0.28
Σ Poly.	55.96 ± 0.49
Σ n-6	39.00 ± 0.06
Σ n-3	16.22 ± 0.11
n-6/n-3	2.40 ± 0.02

[a]Data shown are mean ± SEM for 2 animals. [b]FAME, fatty acid methyl esters. There were no detectable levels of the fatty acids 20:1, 20:3 (n-3), 22:0,24:0 and 24:1.

5.3.6 Steady state fluorescence anisotropy of rat cardiomyocytes

The steady-state fluorescence anisotropy values (r_{ss}) were obtained using the probe TMAP-DPH in Ca^{2+} tolerant ventricular myocytes by the methods described in Chapter 2. No significant differences in the r_{ss} values, and hence the relative membrane fluidity, were

observed in cardiomyocytes isolated from rats following 3 weeks dietary lipid supplementation with either SF (0.263 ± 0.001, n=7), CO (0.258 ± 0.005, n=7) or FO (0.260 ± 0.004, n=7).

5.4 DISCUSSION

On analysis, the cardiomyocyte phospholipid composition revealed a higher than expected proportion of 22:6n-3 in the SF fed group and the CO group, with both of these groups having 22:6n-3 levels which approximated that seen with the FO fed group. Furthermore, cellular parameters associated with Na^+ and K^+ ion channels were unaffected following supplementation of the above dietary regimes.

While fatty acid analysis had been carried out on all dietary oil/fat supplements prior to the commencement of this experiment, such analysis on the normal rat colony diet supplied to the animal house was not carried out. On inspection of the above results, an immediate analysis only of the fatty acid composition of the normal rat colony diet was undertaken. This analysis revealed that the ALA (ie. 18:3n-3) content of the base diet was approximately 5% of the total fatty acids. This high level of "basal" ALA far exceeded previous levels measured in the commercial diet that was used at the CSIRO Health Sciences and Nutrition. This was likely to be due to the addition of soyabean oil and soyabean meal containing relatively high levels of ALA which had occurred during the manufacture of the diet by the supplier, and which our animal colony staff were not aware of prior to the start of this study.

Importantly, the results from the above study, while initially disappointing, have actually highlighted some important aspects related to the efficiency of n-3 PUFA incorporation into the ventricular phospholipids comprising cellular membranes. Firstly, it would appear that in the rat heart at least, if a small amount of ALA is present in the diet, it can still be metabolised through to the longer chain n-3 PUFAs, particularly EPA and DHA, and this conversion still occurs in the presence of high levels of saturated fat. For

example, in Chapter 4 the level of DHA (in cardiac phospholipids) in the OO and CO groups was approximately 9% and this was approximately 13% in the FO group. However, in this Chapter, using a colony diet that contained higher ALA levels, the proportion of DHA in the ventricular phospholipids was approximately 13-14% in the SF and CO groups. Therefore by extrapolation, ALA present in canola oil could, at least in the rat, provide a source of precursor n-3 PUFA for metabolism to the longer chain n-3 PUFAs even in the presence of high levels of dietary saturated fat. This is an important observation that opens up many areas for investigation relating to the protective effect of ALA and its elongation and desaturation products in cardiac arrhythmogenesis. Therefore, this dietary study needed to be modified and repeated to obtain greater differences between the phospholipid levels of DHA in the control and FO groups.

Study 2 "Effects of dietary fish oil on whole-cell Na^+ and K^+ currents using a fabricated diet"

5.5 INTRODCUTION

5.5.1 Study 2

Due to the unexpected excessive incorporation of n-3 PUFAs into the membrane phospholipids in the Study 1, Study 2 aimed to repeat the Na^+ and K^+ current measurements under conditions where incorporation of n-3 PUFAs would be expected to be significantly higher in only the fish oil supplemented group. To achieve this aim several major modifications to the diet and feeding conditions were made. Thus a new diet was completely fabricated with known amounts of fatty acids, contributed by the addition of 7% (wt/wt) Sunola oil (fatty acid composition described below). This oil was chosen as a dietary fat source since it provided essential fatty acids while maintaining a very low level of ALA, a potential precursor for EPA and DHA. Three dietary groups were compared in this study. A reference (REF) group consisting of animals maintained on the low-ALA diet and sham gavaged with a gavage tube lubricated in glycerol. A second group of rats was supplemented with 2 ml of saturated fat (SF) daily, and a third experimental group was supplemented with 2ml of fish oil extract (FO) by gavage.

In addition to the experiments carried out in the Study 1, the effects of dietary lipid supplementation on the development of isoproterenol-induced asynchronous contractile activity as well as various basal contractile properties of the cardiomyocytes were also investigated. Additional experiments were also carried out to determine 1. the characteristics of the time-dependent reactivation of Na^+ currents in greater detail and 2. the time-dependent reactivation of K^+ currents. In essence, all other general experimental

procedures used in the Study 1 were not changed in this Study 2 (for example, electrophysiological recording, isolation of cardiomyocytes and fatty acid analyses).

5.6 METHODS

5.6.1 Preparation of fabricated low ALA (reference) diet

The diets were prepared in the diet making facility at the CSIRO Health Sciences and Nutrition. Diets were prepared 2-3 weeks prior to use and stored at –20°C until use. The REF diet contained the following (based on the ICN AIN G-93 diet): 10% sucrose, 39.8% starch, 13.2% dextrinised starch, 5% cellulose, 20% casein, 7% sunola oil, 0.3% methionine, 0.25% choline chloride with 3.5% mineral mix (Table 5.8) and 1% vitamin mix (Table 5.9) as described below.

Table 5.8 The mineral mix contained the following as % of the mix

Potassium Iodate	0.0004
Copper Sulphate	0.065
Ferrous Sulphate	0.498
Magnesium Oxide	2.4
Manganese Chloride	0.032
Zinc Chloride	0.1245
Lime (fine calcium carbonate)	37.5
Salt (fine sodium chloride)	7.4
Chromium potassium sulphate	0.055
Potassium phosphate	19.6
Lithium Chloride	0.00174
Potassium sulphate	4.66
Potassium citrate	7.08
Glucose (dextrose)	20.59

Table 5.9 The vitamin mix contained the following as % of the mix

Vitamin A (500,000IU/g)	0.08
Vitamin D (500,000IU/g)	0.02
Vitamin E	0.75
Vitamin K	0.01
Niacin	0.3
Riboflavin	0.06
Ca^{2+} Pantothenate	0.16
Vitamin B12 (1%)	0.1
Thiamine	0.06
Pyridoxine	0.07
Folic acid	0.02
Biotin (2%)	0.1
Sucrose	98.3

Sucrose and dextrinised starch were from Inpak Foods, Alberton, SA, Australia. Casein was from Marsh Dairy Products, Port Melbourne, VIC, Australia. Magnesium oxide, lime, potassium sulphate and potassium citrate were from ACE Chemical company, Camden Park, SA, Australia. Lard was from Metro Quality Foods, Greenacres, NSW, Australia. Sunola oil was kindly donated by Meadow Lea Foods Ltd, Ryde, NSW, Australia. Fish oil (RoPUFA) was kindly donated by Hofmann La-Roche, Basel, Switzerland and all other products purchased from Sigma Chemical Company, Castle Hill, NSW, Australia.

5.6.2 Animals and dietary oils

Ethics approval for this study was obtained from the CSIRO Health Sciences and Nutrition Animal Ethics Committee and Adelaide University Animal Ethics Committee. Male Sprague Dawley rats were obtained from the Adelaide University Central Animal House

(Adelaide, Australia). All animals were housed in the CSIRO small animal colony in groups of no more than 5 per cage with food (fabricated laboratory rat chow containing 0.7 % w/w α-linolenic acid, 7 % w/w fat total (equivalent to 65 kJ energy per day, supplied by the addition of Sunola Oil, Meadow Lea Foods Ltd. (Ryde, NSW, Australia)) and water provided *ad libitum*. Room temperature was maintained at 23°C with constant (55 %) humidity, and lights were maintained on a 12 hour light (8am - 8pm)/dark cycle. Animals were maintained on the modified diet from 4 weeks of age and for the duration of the experiment. At 9 weeks of age the rats were gavaged daily with either a sham gavage using glycerol as lubricant, with this groups being termed the reference (REF) group; 2 ml of lard fat containing a high proportion of saturated fat (SF) or 2 ml fish oil (FO). SF was from Metro Quality Foods (Greenacres, NSW, Australia) and fish oil (FO) was from Hoffmann La Roche (Basel, Switzerland, source of n-3 PUFA). Animals were gavaged with one of the two lipid supplements once daily for 3 weeks (an additional 74 kJ of fat per day, giving a total of 29 % energy obtained from fat). The fatty acid composition of the fabricated rat diet and the dietary oils administered by gavage is given in Table 5.10.

5.6.3 Measurement of cardiomyocyte contraction and response to isoproterenol

Cardiomyocytes on coverslips were placed in a custom-designed superfusion chamber and superfused with Tyrode buffer containing 1mM Ca^{2+} at 37°C and allowed to equilibrate for 2 min. Cell length was determined as described previously (Leifert, 1997) and in Chapter 2. Contractility was induced in the presence of electrical-field stimulation using a Grass S4 stimulator. The cells were routinely stimulated with a pulse duration of 5 ms at a frequency of 1 Hz using two platinum wire electrodes located on either side of the superfusion chamber. Cellular arrhythmia, measured as asynchronous contractile activity, was induced by superfusion with progressively increasing isoproterenol concentrations

(0.01 μM to 3 μM) in the presence of electrical-field stimulation (1 Hz). Asynchronously contracting cells (in the presence of electrical-field stimulation) were defined as those cells that exhibited a contraction rate exceeding the rate of applied electrical stimulation (i.e., cells not contracting in synchrony with the applied electrical stimulus). Asynchronous contracting cells usually contracted at 2-4 contractions per second.

5.6.4 Statistics

Where necessary, statistical analysis was performed using the computer software program Instat version 3.0 (GraphPad Software, CA, USA). One-way or two-way ANOVA with Bonferroni multiple comparison test was used to compare differences between effects of the dietary treatment. Results are expressed as the mean ± SEM. For each comparison, the level of significance was set at $P<0.05$ for the indicated number of animals per dietary group. Where data appear as n = x,y; n = number of rat hearts and y = number of cells studied.

5.7 RESULTS

5.7.1 Fatty acid composition of dietary lipid supplements

Fatty acid analysis of the fabricated low ALA reference diet as well as the two lipid supplements is shown in Table 5.10. The low ALA reference diet that was formulated and used in this study contained significantly less ALA (0.68%) compared to the "colony diet " used in the Study 1 (4.6%). SF (beef lard) contained approximately 58% saturated fatty acids, mainly as 16:0 and 18:0. Apart from the presence of approximately 0.65% 18:3n-3 and 0.07% 20:5 n-3, no other n-3 PUFAs were present in this supplement. The major fatty acids in the FO supplement were 20:5n-3 (24%) and 22:6n-3 (12%).

5.7.2 Phospholipid fatty acid analysis

Ventricular phospholipid fatty acid composition for rats supplemented by gavage with either saturated fat (SF) or fish oil (FO), or from the REF (sham gavage) group are shown in Table 5.11. Ventricular phospholipid fatty acids of animals maintained on the REF diet alone exhibited a relatively low level of 22:6 n-3, 22:5 n-3 and 20:5 n-3 compared with the other two dietary groups (Table 5.11). Three weeks of daily gavage with the FO supplement significantly increased the proportion of total n-3 PUFAs due to increases in the proportions of 20:5 n-3, 22:5 n-3 and 22:6 n-3. Concomitant with n-3 PUFA supplementation was a reduction in the proportions of 18:1, 18:2 n-6 and 20:4 n-6, which led to a significant lowering in the ratio of n-6/n-3 PUFAs in this group. There was also an increase in the proportion of the total n-3 PUFAs in the SF group compared with the REF group, but not to the extent evident for the FO group. For SF dietary group, only the proportions of 22:5 n-3 and 22:6 n-3 were increased with little change occurring in the

proportion of 20:5 n-3 in comparison with the REF group. For the proportions of all the other major fatty acids, the SF group was similar to the REF group and significantly different to the FO group. The proportion of total saturated fatty acids in the ventricular phospholipids was not significantly different across all dietary groups even following the SF supplementation.

Table 5.10 Fatty acid composition (wt %) of the low ALA reference diet and the two dietary lipid supplements (SF and FO)

Major FAME[a]	Low ALA Reference Diet (REF)	Saturated Fat (SF)	Fish Oil (FO)
14:0	0.29	6.67	8.07
16:0	4.79	30.61	15.50
16:1	0.11	1.89	10.13
18:0	4.07	18.53	2.64
18:1[b]	75.25	35.97	15.58
18:2(n-6)	13.27	1.78	2.89
18:3(n-3)	0.68	0.65	1.13
20:4(n-6)	n/d	0.06	1.26
20:5(n-3)	n/d	0.07	24.33
22:5(n-3)	n/d	n/d	2.28
22:6(n-3)	n/d	n/d	12.14
Σ Sat.	10.43	58.04	27.31
Σ Mono.	75.50	39.39	28.16
Σ Poly.	13.96	2.56	44.57
Σ n-6	13.27	1.84	4.45
Σ n-3	0.68	0.72	40.00
n-6/n-3	19.38	2.55	0.11

Data shown are the means from diet/lipid supplement samples. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. n/d, not detected. Abbreviations: REF, reference group; SF, saturated fat group; FO, fish oil group.

Table 5.11 Fatty acid composition (wt %) of the ventricular phospholipids after dietary lipid supplementation

Major FAME[a]	REF (n = 8)	SF (n = 6)	FO (n = 6)
14:0	0.13 ± 0.02	0.27 ± 0.09	0.21 ± 0.02
16:0	16.84 ± 0.34	16.06 ± 0.51	15.89 ± 0.39
16:1	0.32 ± 0.04	0.76 ± 0.47	0.25 ± 0.07
17:0	0.23 ± 0.01[d,f]	0.36 ± 0.04	0.41 ± 0.04
18:0	24.75 ± 0.49	24.45 ± 0.71	25.28 ± 0.63
18:1[b]	15.38 ± 0.92[d]	14.36 ± 0.56[c]	10.13 ± 1.11
18:2(n-6)	6.29 ± 0.19[e,f]	5.37 ± 0.16[e]	3.72 ± 0.25
18:3(n-3)	0.34 ± 0.27	0.11 ± 0.05	n/d
20:2	0.63 ± 0.05	0.51 ± 0.04	tr.
20:3(n-6)	tr.	0.21 ± 0.03	0.21 ± 0.02
20:4(n-6)	29.6 ± 0.36[d]	28.66 ± 1.17[f]	24.47 ± 1.34
20:3(n-3)	0.21 ± 0.00	tr.	0.20 ± 0.00
22:0	0.30 ± 0.01	tr.	0.25 ± 0.06
20:5(n-3)	0.18 ± 0.07[e]	0.32 ± 0.17[e]	3.25 ± 0.79
24:0	0.76 ± 0.16	0.54 ± 0.08	tr.
22:5(n-3)	0.41 ± 0.14[e,g]	1.32 ± 0.22[e]	2.45 ± 0.07
22:6(n-3)	3.67 ± 0.65[e,f]	6.70 ± 0.87[e]	13.11 ± 0.53
Σ Sat.	43.12 ± 0.53	41.76 ± 0.85	42.29 ± 0.73
Σ Mono.	15.87 ± 0.93[d]	15.38 ± 0.98[c]	10.50 ± 1.13
Σ Poly.	41.01 ± 0.48[d]	42.85 ± 0.99[c]	47.21 ± 1.53
Σ n-6	36.08 ± 0.45[e]	34.24 ± 1.17[d]	28.36 ± 1.14
Σ n-3	4.30 ± 0.82[e,f]	8.10 ±1.030[e]	18.84 ± 0.64
n-6/n-3	8.39 ± 1.43[d]	4.23 ± 1.05	1.50 ± 0.06

Data shown are mean ± SEM for 6 to 8 animals per dietary group (indicated in brackets). Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. Superscripts indicate significant differences at [c]$P < 0.05$, [d]$P < 0.01$, [e]$P < 0.001$ vs FO and [f]$P < 0.05$, [g]$P < 0.01$, vs SF (by ANOVA with Bonferonni multiple comparison test). n/d, not detected, tr., less than 0.2 %. Abbreviations: REF, reference group; SF, saturated fat group; FO, fish oil group.

5.7.3 Cardiomyocyte contractile parameters

Body weights were not significantly different from rats supplemented for 3 weeks with SF or FO, or from the REF group (425 ± 12 g (n=7), 436 ± 11 g (n=6) and 429 ± 14 g (n=7), respectively). Growth rates from 4 weeks of age to 12 weeks of age are shown in Figure 5.9. Cell viability was determined by counting the number of rod-shaped cells

from each preparation. The viability of cardiomyocytes immediately after isolation was 70.2 ± 2.0 %, 63.1 ± 2.9 % and 63.0 ± 5.1 % for the REF, SF and FO groups respectively (not significantly different). However, after plating > 90 % of adhered cells were found to be viable (independent of dietary group) since many of the rounded cells do not adhere to the coverslips. Previous studies involving acute application of the n-3 PUFAs EPA and DHA, to electrical-field stimulated cardiomyocytes demonstrated that an increase in voltage was required to maintain cellular contractions upon addition of n-3 PUFAs and that this may be partially due to the inhibition of Na^+ currents, as shown in Chapter 3. Therefore, the voltage required to stimulate contractions in 90% of cardiomyocytes was determined (from the electrical stimulator). There was no significant difference between the groups as shown in Table 5.12. In response to electrical-field stimulation, cardiomyocytes contracted in synchrony with the applied pulse at 1 Hz. The contractile data are shown in Table 5.12. The resting cell length (diastolic) was similar in all three groups of cardiomyocytes studied. In response to steady-state electrical-field stimulation, cardiomyocytes shortened by approximately 3.3-3.9 % and this extent of shortening was similar for all groups. Since studies of post-rest contractile behaviour provide useful information on parameters associated with intracellular Ca^{2+} handling (Pieske *et al.*, 1999), the change in contraction amplitude following a 2 min rest (no stimulation) before reapplying electrical stimulation to initiate the post-rest potentiation (PRP) contraction, was also measured. As shown in Table 5.12, the PRP contraction amplitude was always greater than the contraction amplitude of contractions at steady-state (approximately 12.5 % in all groups, and was not significantly different). The greater contraction amplitude in PRP may reflect the ability of the sarcoplasmic reticulum to accumulate additional Ca^{2+}.

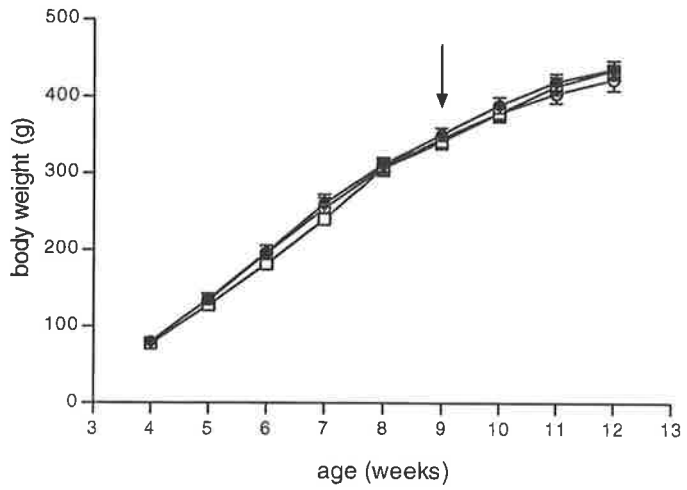


Figure 5.9 Body weights of lipid supplemented rats.
The arrow indicates the start of the dietary lipid supplementation, reference (□), saturated fat (○) or fish oil (●). Data represent mean ± SEM (n=6-7).

Table 5.12 Summary of cardiomyocyte contractile parameters

Contractile parameter	REF	SF	FO
V[a]	21.3 ± 1.7 (6, 31)	20.0 ± 0.5 (7, 37)	19.2 ± 0.5 (6, 34)
Diastolic cell length (μm)[b]	140.3 ± 3.3 (7, 35)	139.3 ± 3.9 (7, 36)	148.7 ± 3.8 (6, 29)
Systolic cell length (μm)[c]	135.3 ± 3.1 (7, 35)	130.3 ± 5.9 (7, 36)	143.9 ± 4.0 (6, 29)
Δ cell length (%)[d]	3.9 ± 1.0 (7, 35)	3.7 ± 0.5 (7, 36)	3.3 ± 0.7 (6, 29)
PRP (%)[e]	12.4 ± 0.7 (7, 35)	12.6 ± 0.5 (7, 36)	12.7 ± 0.7 (6, 29)

[a]The voltage setting required to stimulate 90% of cells in a field to contract. [b]Resting (diastolic) cell length. [c]Cell length at peak (systolic) cell shortening during steady-state. [d]Percent change in cell length [(systolic-diastolic)/diastolic]*100 during steady-state. [e]Post rest potentiation (PRP): (Post rest contraction length/steady state contraction length)*100. Data represent mean ± SEM (n = x,y; x = number of rat hearts and y = number of cells studied). Abbreviations: REF, reference group; SF, saturated fat group; FO, fish oil group.

5.7.4 *Isoproterenol induced asynchronous contractile activity*

The protocol developed to determine the effect of dietary lipids on cardiomyocyte contractility and the development of asynchronous contractile activity utilised quiescent, calcium-tolerant cells, in which contractile activity was synchronous with the applied electrical field stimulation. Asynchronous contractile activity could be elicited by the addition of increasing concentrations of the β-adrenergic receptor agonist, isoproterenol (3 min for each concentration). Asynchronous contractile activity first became evident within about 5 minutes of addition of 0.03 μM isoproterenol, and then became the predominant form of contractile activity after about 15 minutes (>3 μM isoproterenol). Using the above protocol, Figure 5.10 shows that asynchronous contractile activity was evident at significantly lower isoproterenol concentrations in cardiomyocytes isolated from the SF group in comparison with the REF group. The EC_{50} values being 347 ± 91 nM, (n=6) and 531 ± 91 nM, (n=6), for SF and REF, respectively. As shown in Figure 5.10, dietary FO supplementation significantly ($P<0.05$) prevented the development of isoproterenol-induced asynchronous contractile activity compared with the REF and SF groups. (EC_{50} = 892 ± 130 nM, n=6). The rank order potency for the effect of these dietary supplements on the development of asynchronous cardiomyocyte contractility was SF, REF, FO.

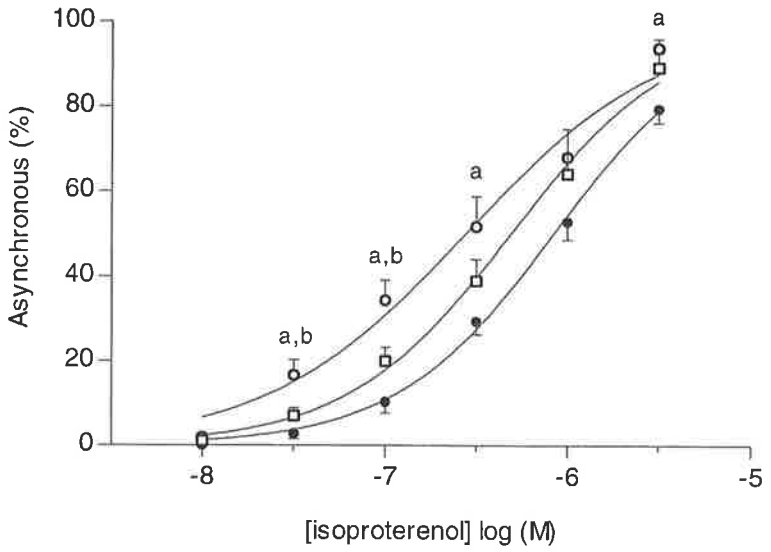


Figure 5.10 Development of asynchronously contracting cardiomyocytes.
Diets were reference (□), saturated fat (○) or fish oil (●). Data are means ± SEM for cardiomyocytes isolated from n=6 animals per dietary group. Significant differences at $P<0.05$ are designated as ^{a}FO vs SF and ^{b}REF vs SF. EC_{50} values for REF, SF and FO were 531 ± 91 nM, 347 ± 91 nM and 892 ± 130 nM, respectively ($P<0.05$).

5.7.5 Effect of dietary lipid supplementation on rat cardiomyocyte Na^+ currents

5.7.5.1 Voltage-dependence of activation of Na^+ currents

The average whole-cell capacitance of all cardiomyocytes from dietary supplemented rats was 130.8 ± 4.7 pF (n=28), 120.6 ± 6.1 pF (n=28) and 134.3 ± 6.3 pF (n=28) for REF, SF and FO supplemented rats, respectively (not significantly different). Na^+ currents in all cells were activated at approximately -60 mV and reached a maximum current at approximately -30 mV. The effect of the diets on the voltage-dependence of activation was determined by evoking currents by voltage steps to various potentials between -90 mV and +30 mV from a holding potential of -140 mV (as depicted in upper panel of Figure 5.11A). Figure 5.11A (bottom panel) shows an example of typical Na^+ currents evoked in a single cardiomyocyte by a step in membrane potential from a holding

potential of -140 mV to potentials of 0, -10, -20, -30, -40 and -50 mV. The maximum Na^+ current densities elicited by a voltage step from -140 mV to -30 mV for REF, SF and FO supplemented rats were -14.8 ± 1.6 (n=5), -15.5 ± 0.8 (n=5) and -15.4 ± 0.9 (n=5) pA/pF, respectively (Figure 5.11B). The data points were fitted using equation 1. The means of all the above parameters for the least squares fit of equation 1 are given in Table 5.13. There was no significant difference between any of the dietary supplemented groups for any of the Na^+ current activation parameters examined.

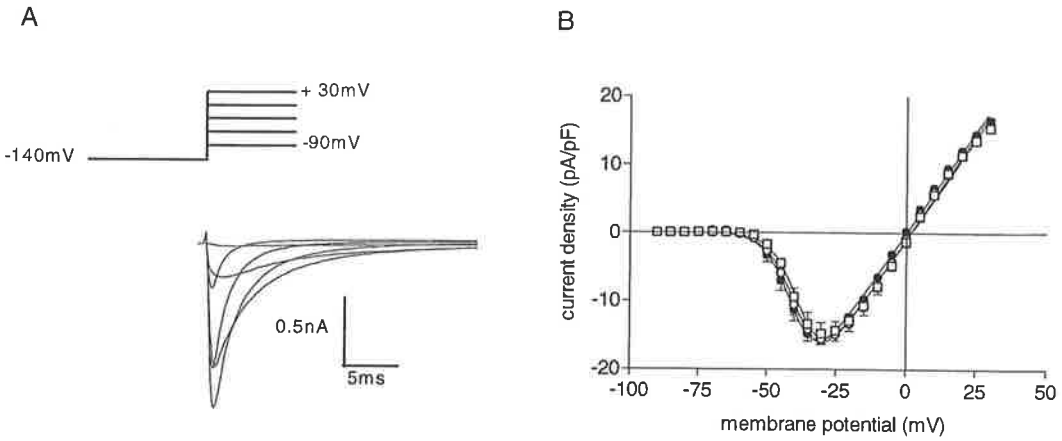


Figure 5.11. Effect of dietary supplementation of either reference, saturated fat or fish oil on the voltage-dependence of activation of Na^+ channels in adult rat ventricular myocytes.
A, Na^+ currents were evoked by voltage steps from a holding potential of -140 mV to various voltages between -90 mV and +30 mV as depicted (upper panel). Plotted below are superimposed currents evoked at 0, -10, -20, -30, -40 and -50 mV. *B*, The peak current density (peak current amplitude/cell capacitance (pA/pF) was plotted against the pulse (holding) potential). Data represent mean ± SEM from animals supplemented with reference (□; n=5, 28), saturated fat (○; n=5, 28), or fish oil (●; n=5, 28). The solid lines show the least squares best fit of equation 1.

Table 5.13 Activation parameters from least squares fit of equation 1

Dietary group	G_{max} (pS/pF)	V_{50} (mV)	E_{rev} (mV)	k (mV^{-1})
REF (n=5)	0.62 ± 0.05	-36.5 ± 0.9	2.2 ± 0.7	4.8 ± 0.3
SF (n=5)	0.61 ± 0.03	-38.6 ± 1.6	1.7 ± 1.6	4.1 ± 0.2
FO (n=5)	0.60 ± 0.04	-40.0 ± 1.0	0.5 ± 1.2	4.2 ± 0.2

Abbreviations: REF, reference diet; SF, saturated fat diet; FO, fish oil diet. Data represent mean ± SEM (numbers in parentheses represent the number of hearts. n=28 cells were examined for each dietary group).

5.7.5.2 *Voltage-dependence of inactivation of Na^+ currents*

The effect of the dietary lipid supplementation on the voltage dependence of inactivation of the Na^+ current was investigated by stepping the membrane potential to a test potential of -30 mV from holding potentials which varied between -140 and -35 mV (upper panel of Figure 5.12A). Figure 5.12A (bottom panel) shows an example of typical Na^+ currents superimposed from a single cardiomyocyte by a step in membrane potential from holding potentials of -130, -110, -90, -70, and -50 mV to a test potential of -30 mV. The current density (maximum amplitude of the Na^+ currents/whole-cell capacitance) was plotted against the holding potential, as shown in Figure 5.12B for cardiomyocytes isolated from rats supplemented for 3 weeks with either REF, SF and FO. The data points were fitted using the Boltzmann equation (equation 2). The mean values for the parameters I_{max}, V_{50} and k, for the least squares fit of equation 2 are given in Table 5.14. Maximum Na^+ current densities (I_{max} at -140 mV) in all cells from REF and SF rats were -13.4 ± 1.3 pA/pF and -13.5 ± 0.9 pA/pF, respectively. For FO supplemented rats the maximum current density of -12.7 ± 0.4 pA/pF was less compared with both REF and SF groups, however this was not statistically significant. Figure 5.12B shows that the voltage-dependence of inactivation was significantly left shifted in the FO group compared with the REF and SF groups $P<0.05$). The values for the membrane potential at which half of the Na^+ channels were inactivated (V_{50}) was -74.0 ± 0.6 mV and -73.5 ± 1.2 mV for REF and SF groups (not significantly different). The V_{50} for the voltage-dependence of inactivation for the FO group was significantly more negative compared with the SF group (-76.7 ± 0.7 mV, $P<0.05$).

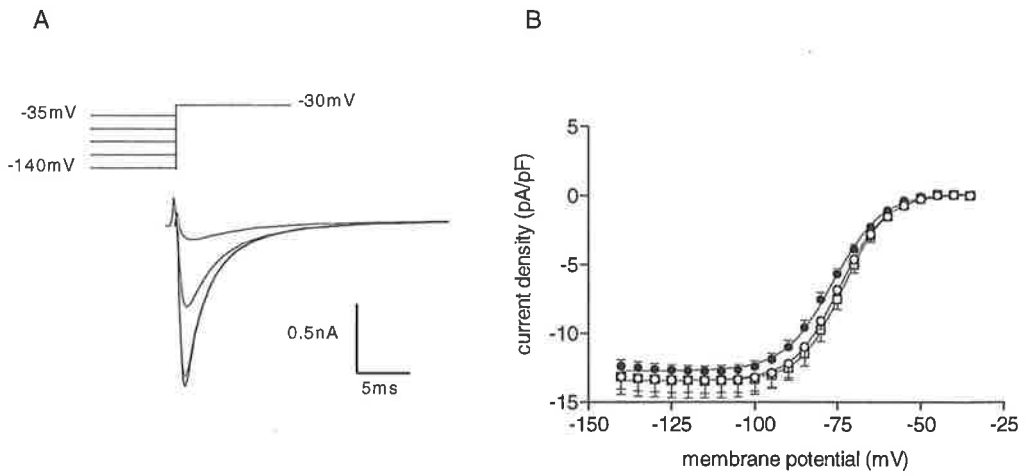


Figure 5.12 Effect of dietary supplementation of either reference, saturated fat and fish oil on the voltage-dependence of inactivation of Na^+ currents in adult rat ventricular myocytes.
A, Na^+ currents were evoked by voltage steps to -30 mV from various holding potentials between -140 and -35 mV, as depicted (upper panel). Plotted below are currents evoked at -130, -110, -90, -70, and -50 mV, shown superimposed. *B*, The peak current density (peak current amplitude/cell capacitance (pA/pF) was plotted against the pulse potential). Data represent mean ± SEM from animals supplemented with reference (□; *n*=5, 28), saturated fat (O; *n*=5, 28), or fish oil (●; *n*=5, 28). The solid line shows the least squares best fit of equation 2.

Table 5.14 Inactivation parameters from least squares fit of equation 2

Dietary group	I_{max} (pA/pF)	V_{50} (mV)	k (mV^{-1})
REF (*n*=5)	-13.4 ± 1.3	-74.0 ± 0.6	6.4 ± 0.2
SF (*n*=5)	-13.5 ± 0.9	-73.5 ± 1.2	6.2 ± 0.2
FO (*n*=5)	-12.7 ± 0.4	-76.7 ± 0.7[a]	6.6 ± 0.3

Abbreviations: REF, reference diet; SF, saturated fat diet; FO, fish oil diet. Data represent mean ± SEM (numbers in parentheses represent the number of hearts, *n*=28 cells examined for each dietary group). [a]$P<0.05$ vs SF

5.7.5.3 Kinetics of activation and inactivation of Na^+ currents

The effect of dietary lipid supplementation on the kinetics of activation and inactivation of the rat cardiomyocyte Na^+ current was investigated. The time course for the increase in the Na^+ current was taken as the rate of activation (τ_1) of the currents, while the time

course for the decline in the Na^+ current was taken as an indication of the rate of inactivation (τ_2). Figure 5.13A (upper panel) shows a representative recording of a Na^+ current in a single cell elicited by a voltage step to -30 mV from a holding potential of -90 mV. The data points shown were fitted with a modified Hodgkin-Huxley equation (Hodgkin & Huxley, 1952) (equation 3). Figure 5.13A (lower panel) shows the kinetic analysis of the rates of current activation for cells from REF, SF and FO groups were 0.330 ± 0.030 ms (*n*=5, 28), 0.290 ± 0.010 ms (*n*=5, 28) and 0.350 ± 0.020 ms (*n*=5, 27), respectively (not significantly different). Figure 5.13B (lower panel) shows the inactivation of whole-cell Na^+ currents (current decay) for the REF, SF and FO groups were 1.51 ± 0.11 ms, 1.21 ± 0.09 ms and 1.28 ± 0.10, respectively (not significantly different).

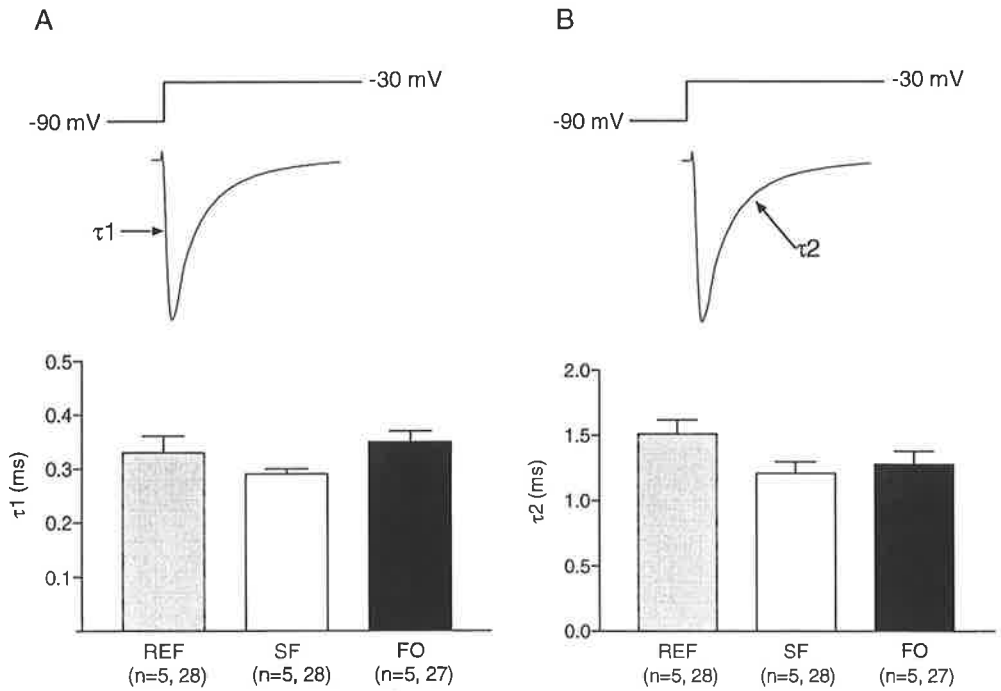


Figure 5.13 Na^+ current activation and inactivation time constants.
Na^+ currents were evoked in a single cell by a voltage step to -30 mV from a holding potential of -90 mV. *A*, The time-constants of activation (τ1) of the Na^+ currents and *B*, the time-constants of inactivation (τ2) of the Na^+ currents, were calculated from the Hodgkin-Huxley equation Data were averaged as shown, for reference (REF), saturated fat (SF), or fish oil (FO) for the number of hearts and cardiomyocytes (indicated in parentheses). Data represent mean ± SEM.

5.7.5.4 Time course of recovery from inactivation

The rate of recovery from inactivation of cardiomyocyte Na^+ currents was investigated by use of a two-pulse depolarisation protocol as described previously (Saint, 1998). Two identical voltage steps to -20 mV for 20 ms from a holding potential of -140 mV or -90 mV were applied, separated by a variable time interval, t (as depicted in upper panel of Figures 5.14A and E). Figures 5.14B and F show a representative recording of a Na^+ current in a single cell elicted by a voltage step to -30 mV from a holding potential of -90 mV. The two different holding potentials (i.e., -140 and -90 mV) were chosen because the time course for recovery of Na^+ currents would be expected to be different at these two holding potentials. At -140 mV, most channels would reside in the resting (closed), state whilst at -90 mV, there would be a mixed population of Na^+ channels existing in both resting and inactivated states, and these states may be differentially dependent on the incorporation of n-3 PUFAs into the cell membrane.

For the two protocols tested, at short intervals (less than 1 ms) the second pulse failed to evoke a Na^+ current since the channels had insufficient time to recover from inactivation induced by the first pulse. As this interval was increased the amplitude of the current elicited by the second pulse increased as the channels recovered from inactivation. Ratios of the peak inward currents elicited by the second (I_2) and first (I_1) pulses (I_2/I_1) were plotted against the interpulse interval (t) and the time course for recovery was fitted to a single exponential function (equation 4). The best fit for the time constants gave values of 1.40 ± 0.07 ms (n=5), 1.18 ± 0.14 ms (n=5) and 1.63 ± 0.17 ms (n=5) for the REF, SF and FO groups, respectively (Figures 5.14C and D, not significantly different). In addition, at a holding voltage of -90 mV (Figures 5.14G and H) there was no significant difference between the recovery time constants between the dietary groups. However, the recovery

time constants at a holding potential of -90 mV were substantially slower compared to the -140 mV holding potential protocol. The time constants were 20.50 ± 1.70 ms (n=5), 17.01 ± 2.88 ms (n=5) and 21.92 ± 2.88 ms (n=5) for REF, SF and FO, respectively (not significantly different).

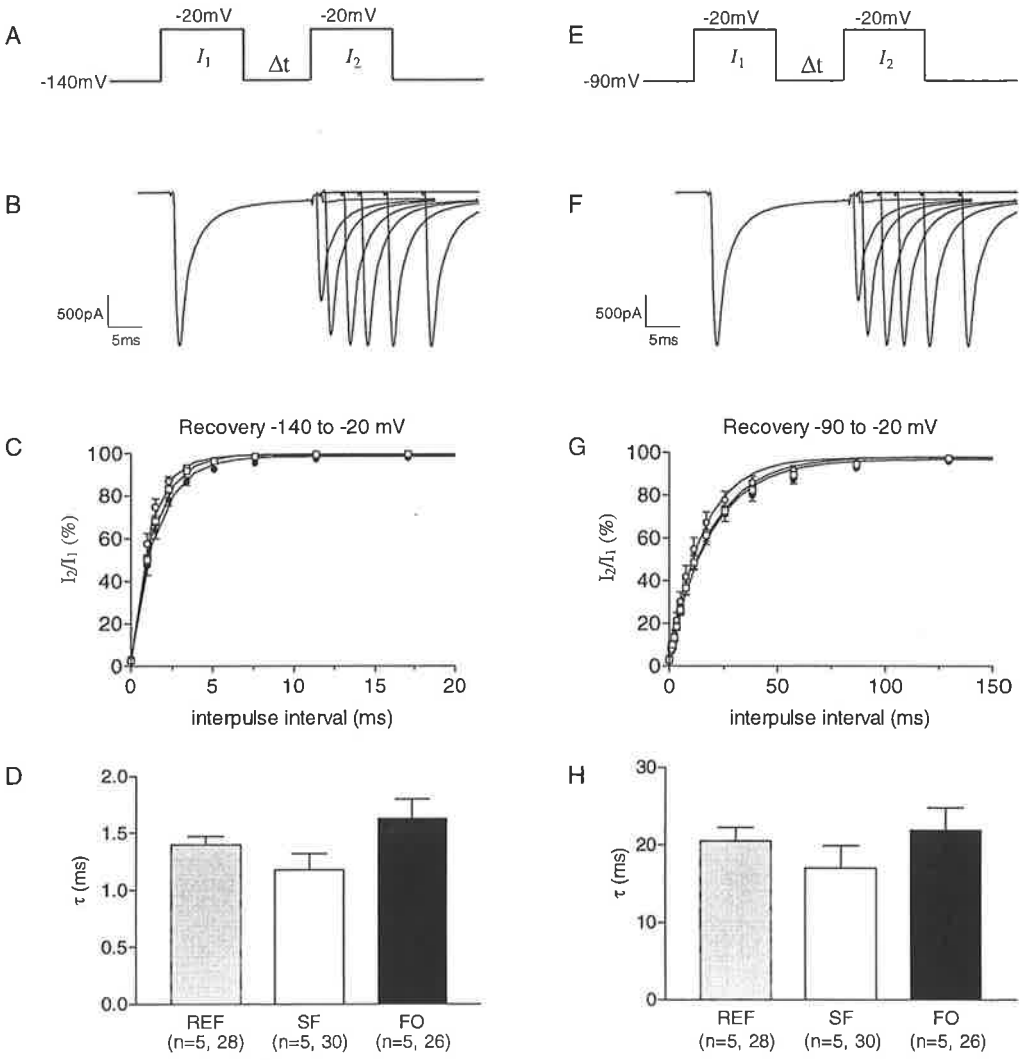


Figure 5.14 Rate of recovery of Na^+ currents from inactivation.
A two-pulse protocol was applied to determine the rate of recovery of Na^+ currents from inactivation. The first current was evoked by a pulse from a holding potential -140 mV (A) or –90 mV (E). Subsequent voltage steps to -20 mV were given with a variable interval, t, between pulses as shown. Example of Na^+ currents generated followed by a second Na^+ current with gradually increasing time between pulses are shown in B and F. Recovery curves for the effect of reference (□), saturated fat (○) or fish oil (●) are shown in C and G and the mean time constants derived from C and G are shown in D and H, for holding potentials of –140 mV and –90 mV respectively. Data represent mean ± SEM.

5.7.6 *Effect of dietary lipid supplementation on transient outward K^+ currents*

The average whole-cell capacitance of all cardiomyocytes from dietary supplemented rats was 119.3 ± 4.8 pF (n=29), 118.1 ± 5.0 pF (n=29) and 132.3 ± 4.3 pF (n=28) for REF, SF and FO supplemented rats, respectively (not significantly different).

5.7.6.1 Voltage-dependence of activation of I_{to}

The transient outward currents (I_{to}) in all cells were activated at approximately -20 mV and reached a maximum current at approximately +50 mV. The effect of the dietary treatments on the voltage-dependence of activation was determined by evoking currents by voltage steps to various potentials between -90 mV and +50 mV from a holding potential of -140 mV (as depicted in upper panel of Figure 5.15A). Figure 5.15A (lower panel) shows an example of typical I_{to} currents evoked in a single cardiomyocyte by a step in membrane potential from a holding potential of -140 mV to potentials of -70, -10, 0, +10, +20, +30 and +40 mV. The current density (maximum amplitude of the I_{to} currents/whole-cell capacitance) was plotted against the test potential, as shown in Figure 5.15B for cardiomyocytes isolated from rats supplemented for 3 weeks with either REF, SF and FO. The data points were fitted by the Boltzmann equation (equation 2) using a least squares fitting algorithm, where I_{max} is the maximum current, V' is the membrane potential for half inactivation of the channels, V is the test membrane potential, and k is a slope factor. The maximum I_{to} current densities elicited by a voltage step from -140 mV to +50 mV for REF, SF and FO supplemented rats were 2.07 ± 0.17 (n=5), 2.34 ± 0.16 (n=5) and 1.92 ± 0.19 (n=5) pA/pF. Although the FO group had a marginally lower maximum current density (at +50 mV) compared with either REF or SF, the difference

was not significant. The means of all the above parameters for the least squares fit of the equation are summarised in 5.15.

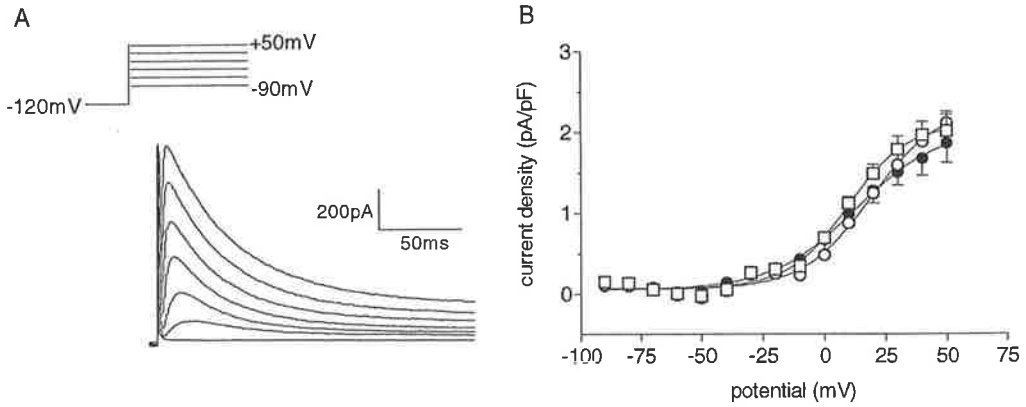


Figure 5.15 The voltage-dependence of activation of I_{to} in adult rat ventricular myocytes following dietary lipid supplementation.
A, Transient outward currents (I_{to}) were evoked by voltage steps from a holding potential of -120 mV to various voltages between -90 mV and +50 mV as depicted (upper panel). Plotted below are superimposed currents evoked at -70, -10, 0, 10, 20, 30 and 40 mV. *B*, The peak current density (peak current amplitude/cell capacitance (pA/pF) was plotted against the test potential. The solid lines show the least squares best fit of the Boltzmann equation. The parameters for the best fit in each case are shown in Table 5.15. Data represent mean ± SEM from reference (□; n=5, 28), saturated fat (○; n=5, 28) or fish oil (●; n=5, 28) groups.

Table 5.15 Activation parameters from least squares fit of the Boltzmann equation

Dietary group	I_{max} (pA/pF)	V_{50} (mV)	k (mV^{-1})
REF (n=5)	2.11 ± 0.21	8.33 ± 1.70	12.23 ± 0.72
SF (n=5)	2.34 ± 0.16	17.67 ± 1.85	13.55 ± 0.31
FO (n=5)	1.92 ± 0.19	7.39 ± 4.52	13.50 ± 0.80

Abbreviations: REF, reference diet; SF, saturated fat diet; FO, fish oil diet. Data represent mean ± SEM (numbers in parentheses represent the number of hearts, n=28 cells examined for each dietary group). [a]P<0.05 vs SF

5.7.6.2 *Voltage-dependence of inactivation of* I_{to}

The effect of the dietary lipid supplementation on the voltage dependence of inactivation of the I_{to} currents were investigated by stepping the membrane potential to a test potential

of +30 mV from holding potentials which varied between -120 and +20 mV (upper panel of Figure 5.16A). Figure 5.16A (lower panel) shows an example of typical I_{to} currents evoked in a single cardiomyocyte by a step to +30 mV from various holding potentials of -120, -80, -50, -40, -30, -20 and -10 mV. The current density (maximum amplitude of the I_{to} currents/whole-cell capacitance) was plotted against the holding potential, as shown in Figure 5.16A for cardiomyocytes from either REF, SF or FO groups. The mean values for the parameters I_{max} , V_{50} and k, for the least squares fit of the Boltzmann equation (equation 2) are given in Table 5.16. There was no significant difference between any of the groups for any of the parameters examined, however, there was a slightly lower current density in the FO group compared with the REF or SF groups.

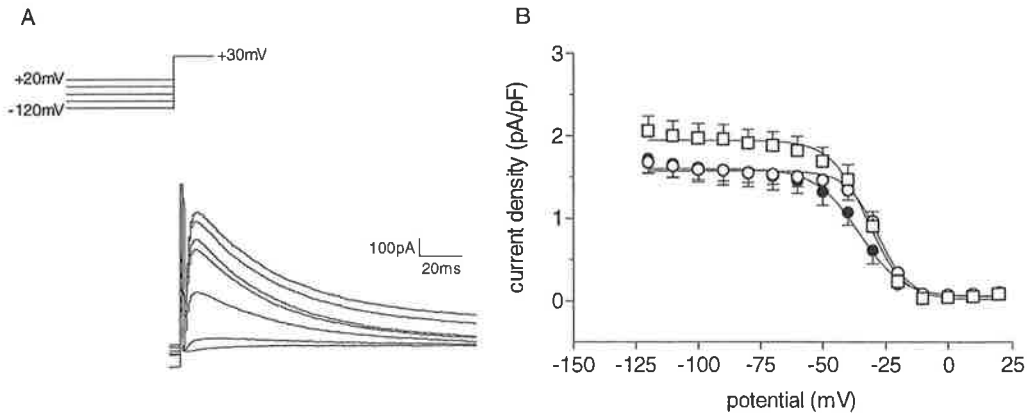


Figure 5.16 The voltage-dependence of inactivation of I_{to} in adult rat ventricular myocytes following dietary lipid supplementation.

A, Transient outward currents (I_{to}) were evoked by voltage steps to +30 mV from various holding potentials between -120 and +20 mV, as depicted (B, upper panel). Plotted below are currents evoked at -120, -80, -50, -40, ,-30, -20 and -10 mV, shown superimposed. *B*, The peak current density (peak current amplitude/cell capacitance (pA/pF) was plotted against the pulse potential. The solid line shows the least squares best fit of the Boltzmann equation. The parameters for the best fit in each case are shown in Table 5.16. Data represent mean ± SEM from animals supplemented with reference (□; *n*=5, 28), saturated fat (○; *n*=5, 28) or fish oil (●; *n*=5, 28).

Table 5.16 Inactivation parameters from least squares fit of the Boltzmann equation

Dietary group	I_{max} (pA/pF)	V_{50} (mV)	k (mV^{-1})
REF (*n*=5)	1.94 ± 0.17	-32.72 ± 1.29	-5.25 ± 0.22
SF (*n*=5)	1.57 ± 0.12	-29.03 ±0.41	-5.70 ± 0.50
FO (*n*=5)	1.56 ± 0.16	-33.98 ± 3.40	-6.30 ± 0.44

Abbreviations: REF, reference diet; SF, saturated fat diet; FO, fish oil diet. Data represent mean ± SEM (numbers in parentheses represent the number of hearts, *n*=28 cells examined for each dietary group).

5.7.6.3 Kinetics of inactivation

The effect of the dietary supplementation on the inactivation of the I_{to} currents was investigated by stepping the membrane potential to a test potential of +30 mV from a holding potential of -90 mV (Figure 5.17A upper panel). The time course of I_{to} inactivation was best fitted by a single exponential of the declining phase of the currents as shown in Figure 5.17B (lower panel). Kinetic analysis of the rates of I_{to} inactivation for REF, SF and FO groups were 49.1 ± 2.6 ms (*n*=5, 29), 41.3 ± 3.2 ms (*n*=5, 27) and 42.9 ± 4.4 ms (*n*=5, 27), respectively (not significantly different) as shown in Figure 5.17B.

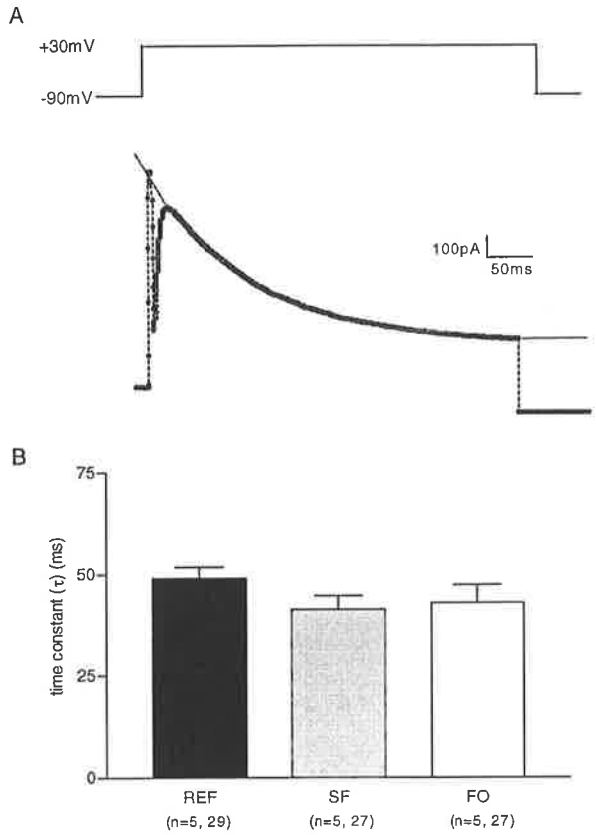


Figure 5.17 Inactivation time constants of I_{to}.
A, K^+ currents were evoked in a single cell by a voltage step to +30 mV from a holding potential of -90 mV. The time-constants of in activation of the K^+ currents were calculated using a single exponential equation of the declining phase of the current as shown by the solid line (lower panel). *B*, Exponential time constants were averaged for reference (REF), saturated fat (SF) or fish oil (FO) for the number of hearts and cardiomyocytes indicated in parentheses. Data represent mean ± SEM.

5.7.6.4 Time course of recovery from Inactivation of I_{to}

Rate of recovery from inactivation of K^+ currents was investigated by use of a two-pulse depolarisation protocol. Two identical voltage steps to +30 mV for 300 ms from a holding potential of -90 mV were given, separated by a variable time interval, t (as depicted in upper panel of Figure 5.18A). For this protocol, at short time intervals (less than 1 ms), the second pulse failed to evoke a K^+ current since the channels had insufficient time to recover from inactivation induced by the first pulse. As the interval was increased, the amplitude of the current elicited by the second pulse increased as the channels recovered

from inactivation (Figure 5.18B). Ratios of the peak currents elicited by the second (I_2) and first (I_1) pulses (I_2/I_1) were plotted against the interpulse interval (t) and the time course of recovery were fitted to a single exponential function (equation 4). τ is the rate of recovery time constant (ms) as shown in Figure 5.18C. The best fit for the time constants gave values of 21.7 ms (n=1, 5), 16.8 ± 3.2 ms (n=3, 15) and 16.9 ± 5.5 ms (n=3, 15) for the reference, SF and FO groups, respectively (Figure 5.18D, not significantly different).

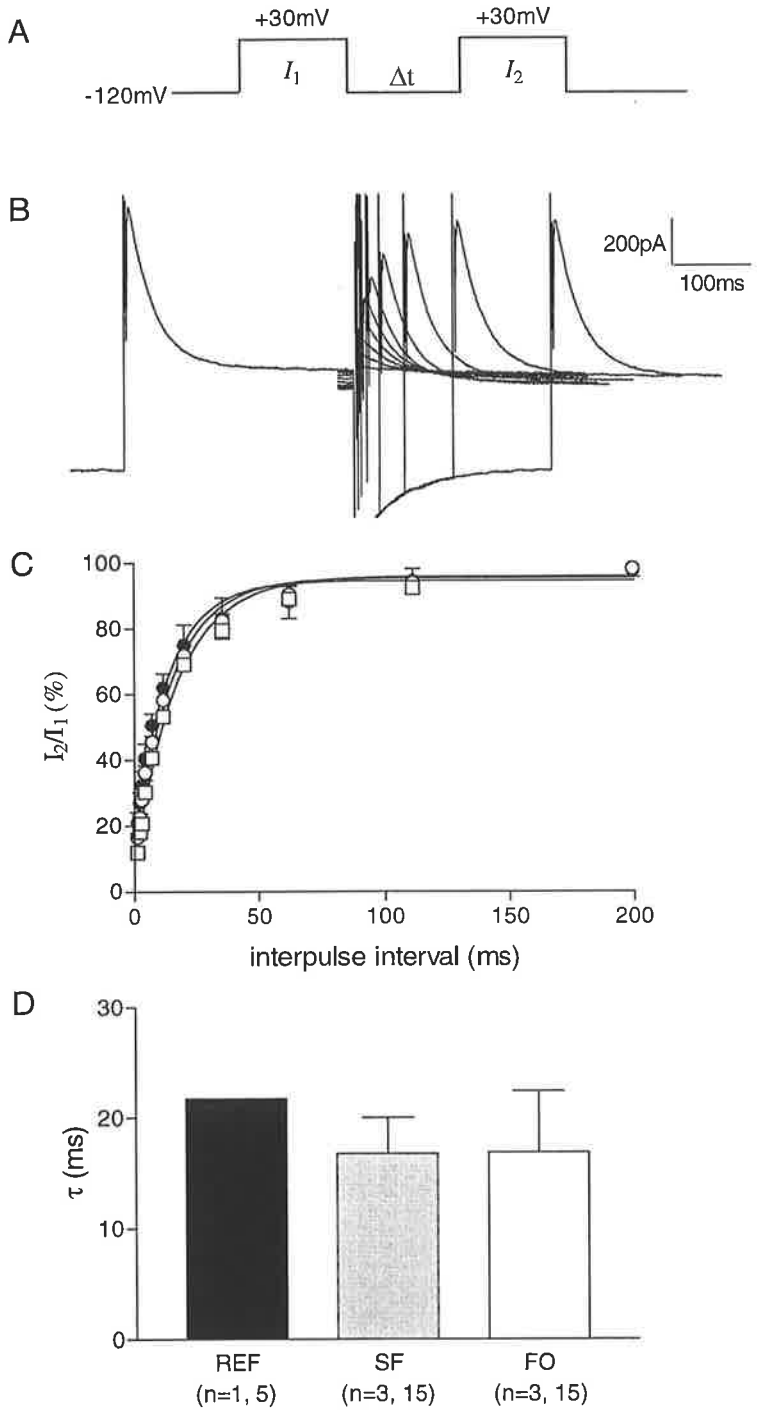


Figure 5.18 Rate of recovery of I_{to} from inactivation.
A two-pulse protocol was applied to determine the rate of recovery of K^+ currents from inactivation. *A*, The first current was evoked by a pulse from a holding potential -120 mV. Subsequent voltage steps to +30 mV were given with a variable interval, t, between pulses as shown. *B*, Example of K^+ currents generated followed by a second K^+ current with gradually increasing time between pulses are shown. *C*, Recovery curves for the effect of reference (□), saturated fat (○) or fish oil (●) are shown. *D*, mean time constants derived from C are shown. Data represent mean ± SEM. Abbreviations: REF, reference diet; SF, saturated fat diet; FO, fish oil diet.

5.8 DISCUSSION

The results of Study 2 shows that cardiomyocytes isolated from the hearts of rats supplemented with either n-3 PUFAs or a predominantly saturated fatty acid mix exhibited distinct changes in phospholipid fatty acid composition with respect to the proportion of n-3 and n-6 PUFAs. While differential effects were observed on the incidence of isoproterenol-induced asynchronous contractile activity, only a small (but significant) effect was seen on cardiomyocyte ion current activity. This effect could be interpreted as a shift to more hyperpolarised potentials during inactivation as a result of fish oil supplementation (Figure 5.12 and Table 5.16).

The reference diet provided a relatively low, but not deficient level of 18:3 n-3 in the diet in order to minimise the extent of conversion of 18:3 n-3 to the longer chain n-3 PUFAs, particularly 22:6 n-3. As a result, the proportion of 22:6 n-3 in the ventricular phospholipid fatty acids of the REF dietary animals was about 3.7% of the total fatty acids, with the total proportion of n-3 PUFAs being 4.3%. These proportions are significantly lower than those reported in previous publications (McLennan, 1993; Pepe & McLennan, 1996) and indicate that the level of dietary 18:3 n-3 dramatically influences the proportions of both n-3 and n-6 PUFAs in ventricular phospholipids due presumably to the competition between 18:3 n-3 and 18:2 n-6 for further metabolism to the longer chain PUFAs by the Δ6 desaturase pathway (Hrelia *et al.*, 1995). Supplementation of the reference diet by gavage with an n-3 PUFA enriched fish oil diet (mainly comprising 20:5 n-3), resulted in a substantial increase in the proportion of total n-3 PUFAs (including 22:6 n-3). These changes in ventricular phospholipid fatty acid composition may influence cardiomyocyte contractile activity in terms of susceptibility to the development

of asynchronous contractions, with higher proportions of 22:6 n-3 being associated with the antiarrhythmic effects, and the higher proportion of 20:4 n-6 being associated with the proarrhythmic effects via the production (and changing the ratio) of pro- and antiarrhythmic prostanoids and thromboxanes (Abeywardena & Charnock, 1995; Oudot *et al.*, 1995; Li *et al.*, 1997). Dietary induced differences in cardiomyocyte arrhythmia susceptibility were not attributable to differences in the proportion of the total ventricular phospholipid fatty acid saturated fatty acids which remained similar from all three groups.

Using the cellular model of isolated rat ventricular cardiomyocytes it was possible to demonstrate that dietary lipid supplementation with n-3 PUFA enriched fish oil for a period of 3 weeks resulted in significant protection towards the development of asynchronous contractile activity induced by the β-adrenergic receptor agonist, isoproterenol. In this regard the effects of dietary n-3 PUFAs at the cellular level are similar to those reported when using a whole animal model of ischaemic and reperfusion induced cardiac arrhythmias (McLennan, 1993), indicating the relevance of this cellular model to the laboratory rat model in terms of dietary lipid studies.

Since Na^+ and K^+ currents are important for the initiation and progression of the excitation-contraction coupling process in cardiomyocytes, it was possible that dietary supplementation with fish oil which is known to increase the incorporation of n-3 PUFAs into cardiac membrane phospholipids, may also alter these major ion currents as has been shown in other cells (Park & Ahmed, 1992; Wu *et al.*, 1997). Indeed, the earlier study reported in this thesis demonstrated potent effects of acute (as opposed to dietary) application of the n-3 PUFAs on cardiac Na^+ currents, which included inhibition of whole-cell Na^+ currents and a shift in the voltage-dependence of inactivation to more

negative potentials. Similarly, in the present study, dietary fish oil supplementation resulted in a significant shift of the voltage-dependence of inactivation of Na^+ currents to more negative potentials. These data indicate that more Na^+ channels may be residing in an inactivated state in cardiomyocytes isolated from fish oil treated rats at membrane potentials close to the physiological resting membrane potential (approximately -80 mV), or under conditions that partially depolarise the cell (eg. myocardial ischaemia and/or excessive β-adrenergic receptor stimulation). However, significant decreases in the peak Na^+ currents in the fish oil group were not observed. It is likely that the action of incorporated n-3 PUFAs is most pronounced when the cell membrane becomes partially depolarised, thus holding the Na^+ channels in an inactivated state for a longer period and preventing the development of triggered activity and tachyarrhythmias. The effects of FO supplementation may therefore result in the cell membranes being less "excitable" to aberrant depolarising stimuli that would normally result arrhythmias. This would be amplified were there to be an increase in the release of (free) fatty acids from membrane phospholipids under cellular stress such as anoxia or elevated $[Ca^{2+}]_i$. This change in the voltage-dependence of Na^+ current activity following FO supplementation closely parallels the mechanism of action of certain class I antiarrhythmic agents such as lidocaine.

Although previous investigators have shown that acute addition of n-3 PUFAs can inhibit cardiac K^+ currents (I_{to}) (Bogdanov *et al.*, 1998), this study demonstrated that under the conditions used, dietary supplementation of n-3 PUFAs did not significantly effect rat cardiomyocyte outward K^+ current density nor the voltage-dependence or kinetic properties of such currents. It is likely that the antiarrhythmic action of the n-3 PUFAs

when they are incorporated into membrane phospholipids is not directly due to the modulation of I_{to}.

The evidence from this study suggests that the sensitivity of cardiomyocytes to β-adrenergic receptor stimulation and therefore the extent of arrhythmogenesis (as measured by asynchronous contractile activity), is modulated in part by a dietary lipid effect on n-3 PUFA levels in phospholipids, which may influence β-adrenergic receptor or post-receptor sensitivity. The effects of dietary lipid supplementation on cell contractile activity appear mainly independent of their effects on K^+ current activity. The steady-state voltage-dependence of inactivation of Na^+ currents was more hyperpolarised by dietary FO supplementation (approximately –3 mV), and this effect may be of physiological significance.

CHAPTER 6

6 "Effect of dietary fish oil on Ca^{2+} handling in cardiomyocytes"

6.1 INTRODUCTION

Current evidence suggests that in mammalian cardiac muscle the process of contraction is mainly initiated by the action potential activating the sarcolemmal L-type Ca^{2+} channels to cause an influx of Ca^{2+} into the cells (Opie, 1998b). The voltage-dependent Ca^{2+} influx across the sarcolemma promotes further release of stored Ca^{2+} from the sarcoplasmic reticulum (SR) by a process known as calcium-induced calcium release (CICR) (Fabiato, 1983), although voltage-sensitive release mechanisms have also been recently implicated (Mason & Ferrier, 1999; Ju & Allen, 2000; Piacentino, III *et al.*, 2000). Together, these sources of Ca^{2+} initiate contractions via activation of troponin/actin-myosin linked processes. Relaxation takes place as a result of two main systems acting together to decrease the cytoplasmic $[Ca^{2+}]_i$. The sarco-endoplasmic reticulum Ca^{2+} pump (SERCA) pumps Ca^{2+} back into the SR making it available for release at the next excitation-contraction coupling cycle. Additionally, the sarcolemmal Na^+/Ca^{2+} exchanger extrudes the Ca^{2+} that entered the cell via Ca^{2+} channels (Bassani *et al.*, 1992; Bassani *et al.*, 1994). Although other mechanisms are involved in the maintenance of a low cytoplasmic $[Ca^{2+}]_i$ during diastole, including sarcolemmal and mitochondria Ca^{2+}-ATPase pumps, SERCA and the Na^+/Ca^{2+} exchanger perform the major roles on a beat to beat basis (Bers & Perez-reyes, 1999).

6.1.1 *Cardiac disease and Ca^{2+} handling - role of the SR and Na^+/Ca^{2+} exchanger*

In various cardiovascular disease states, intracellular Ca^{2+} regulation of the myocardium has been shown to be altered, resulting in abnormal myocardial contractility. In failing human hearts it has been shown that there is a reduction in the ability of the SR Ca^{2+} content to increase sufficiently and the alterations are likely to be secondary to decreased expression of SERCA and overexpression of the Na^+/Ca^{2+} exchanger (Studer *et al.*, 1994; Schlotthauer *et al.*, 1998; Pieske *et al.*, 1999; Hasenfuss *et al.*, 1999). In various heart failure models, the Ca^{2+} releasing function of SR is altered (Yamamoto *et al.*, 1999) as well as the activity of Ca^{2+} influx and efflux pathways (Zhang *et al.*, 1999), resulting in decreased amplitude of contractions in cardiomyocytes and depressed Ca^{2+} transients (Holt *et al.*, 1998). Alterations in cellular Ca^{2+} transport mechanisms and excitation-contraction coupling have also been reported in animal models of hypertrophy and diabetes (Yu *et al.*, 1994; Yu *et al.*, 1995; Lagadic-Gossmann *et al.*, 1996; Delbridge *et al.*, 1997; Tamada *et al.*, 1998; Maier *et al.*, 1998; Mccall *et al.*, 1998; Kotsanas *et al.*, 2000), and in cellular models of arrhythmia (Terada *et al.*, 1994; De Ferrari *et al.*, 1995). The mechanism mediating the increased likelihood of arrhythmias is considered to be partly related to disturbances in the $[Ca^{2+}]_i$. Elevated $[Ca^{2+}]_i$ levels have been suggested to play a significant role in the genesis of cardiac arrhythmias following a variety of conditions, such as after myocardial ischaemia and reperfusion and exposure to catecholamines and digitalis (Leaf & Kang, 1997). Furthermore, an increase in $[Ca^{2+}]_i$ was shown to result in the initiation of various arrhythmias and that the process of arrhythmia *per se* induced a further rapid increase in $[Ca^{2+}]_i$, further exacerbating the situation (Thandroyen *et al.*, 1991).

6.1.2 *L-type Ca^{2+} channels and Ca^{2+} handling*

Voltage-gated L-type Ca^{2+} channels (dihydropyridine receptors) control depolarization-induced Ca^{2+} entry in cardiac myocytes. Ca^{2+} is released from the SR when its release channels (ryanodine receptors) are activated by Ca^{2+} influx through the L-type Ca^{2+} channels. Coupling between L-type Ca^{2+} channels and ryanodine receptor plays an important role in excitation-contraction coupling in cardiac myocytes. Compounds such as Bay K8644 which are well known for their agonist effects on the cardiac L-type Ca^{2+} channel, have also been recently reported to alter ryanodine receptor gating (Katoh *et al.*, 2000) and increase the frequency of Ca^{2+} sparks (Satoh *et al.*, 1998). Interestingly, acute addition of DHA was shown to inhibit the activation of L-type Ca^{2+} channel currents and increased Ca^{2+} transients induced by Bay K8644 in adult rat cardiomyocytes (Pepe *et al.*, 1994; Xiao *et al.*, 1997). Although it has not been verified to date, modulation of the L-type Ca^{2+} channels by dietary as opposed to acute addition of n-3 PUFAs may be one mechanism partly responsible for their antiarrhythmic effects.

6.1.3 *β-adrenergic receptor activation*

β-adrenergic receptor (over)-stimulation can promote ectopic beats, ventricular automaticity and lead to cardiac tachyarrhythmias which can all contribute to arrhythmogenesis in the whole heart (Priori & Corr, 1990; Opie, 1998a; Du *et al.*, 1999). Myocardial ischaemia also evokes an excessive release of norepinephrine in the ischaemic region of the myocardium (Akiyama & Yamazaki, 2001). The effects of β-adrenergic receptor stimulation by isoproterenol are known to affect myocardial contractility via adenylate cyclase activation and elevated cAMP production resulting in earlier and larger peaks of contraction associated with a rapid phase of relaxation. Indeed,

increased exposure to β-adrenergic stimulation may be related to myocardial Ca^{2+} overload and aftercontractions or triggered activity (Marban *et al.*, 1986; Priori & Corr, 1990; Williams, 1993; De Ferrari *et al.*, 1995; Delbridge *et al.*, 1996b; Tamada *et al.*, 1998). Several groups have used isoproterenol to determine whether differences in cardiomyocyte responsiveness (in the sensitivity of Ca^{2+} handling and contractility) occur, in various types of cardiovascular disease (Terada *et al.*, 1994; Maier *et al.*, 1998; Holt *et al.*, 1998). Furthermore, results cited in Chapter 4 demonstrate that cardiomyocytes from FO supplemented rats are less likely to undergo isoproterenol-induced spontaneous and asynchronous contractile activity, indicating that cardiomyocyte Ca^{2+} handling may be different when comparing SF and FO supplemented rats. Such an observation requires further investigation at the cellular level particularly with regard to defining the likely mechanisms whereby dietary lipids influence cellular transport mechanisms for Ca^{2+}.

6.1.4 Ca^{2+} handling and n-3 PUFAs

Recent evidence suggests that acute addition of n-3 PUFAs to rat cardiomyocytes can reduce both the availability of Ca^{2+} for SR uptake and inhibit the SR release mechanism (Taffet *et al.*, 1993; Negretti & O'Neill, 1997; Negretti *et al.*, 2000). Changes of this nature could potentially prevent spontaneous release of SR Ca^{2+} and therefore, the development of arrhythmias. Indeed, it has been suggested that this may be a mechanism underlying the antiarrhythmic effects of acute n-3 PUFAs at the level of the SR (Negretti & O'Neill, 1997; Rodrigo *et al.*, 1999; Negretti *et al.*, 2000). Previous studies of this type have provided useful insight into the possible mechanism(s) of the antiarrhythmic action of these fatty acids when acutely applied (i.e., when they are not esterified into the membrane phospholipids). However, the effects of dietary n-3 PUFA modification of the

ventricular membrane phospholipid composition on cardiomyocyte Ca^{2+} handling have not been reported.

6.1.5 This study

The purpose of this study was to determine whether incorporation of n-3 PUFAs (supplemented as a dietary fish oil concentrate) into cardiac membrane phospholipids (as evidenced by changes in the ventricular phospholipid fatty acid profile) alter SR Ca^{2+} content and Ca^{2+} transients in adult rat cardiomyocytes. This study therefore aimed firstly, to establish whether an antiarrhythmic effect of a fish oil dietary supplement (compared with saturated fat), could be observed in isolated rat cardiomyocytes and, secondly, to determine the Ca^{2+} handling properties of the cardiomyocytes since Ca^{2+} overload plays a central role in arrhythmogenesis (Opie, 1998a). This study was also designed to establish whether dietary lipid supplementation influenced SR function as has been shown to occur with respect to the acute addition of n-3 PUFAs (Rodrigo *et al.*, 1999; Negretti *et al.*, 2000). This could provide evidence that a dietary lipid induced change in Ca^{2+} handling is associated with the antiarrhythmic effects of n-3 PUFAs.

6.2 METHODS

6.2.1 Animals and diets

Room temperature was maintained at 23°C with constant (55%) humidity, and lights were maintained on a 12 hour light (8am - 8pm)/dark cycle. Young male Sprague Dawley rats consumed the fabricated laboratory rat chow (based on the AIN 96 G mix with 7% fat supplied by the addition of Sunola Oil) and water provided *ad libitum* from 4 weeks to 9 weeks of age. Animals were then randomly assigned to one of two groups containing the above diet supplemented with either saturated fat (SF) or fish oil (FO) (both 10% added lipid) for a period of 3 weeks prior to the preparation of isolated cardiomyocytes. Details of the vitamin and mineral mix are shown in Chapter 5, Study 2. The final fatty acid composition of the diets are shown in Table 6.1. Diets were prepared 2-3 weeks prior to use and stored at –20°C until use.

6.2.2 Measurement of cardiomyocyte contraction and response to isoproterenol

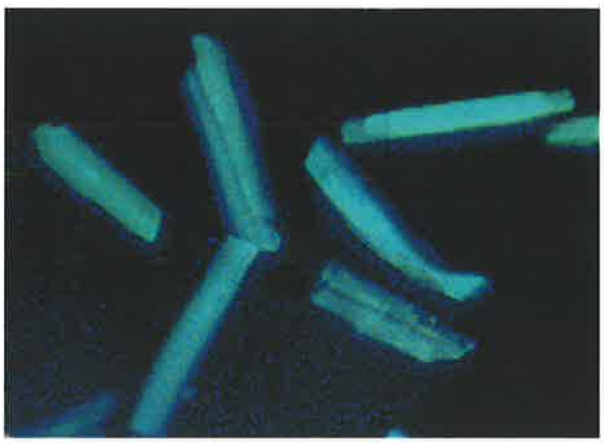
Cardiomyocytes on coverslips were placed in a custom-designed superfusion chamber and superfused with Tyrode buffer containing 1 mM Ca^{2+} and allowed to equilibrate for 2 min. A video camera mounted on an inverted Olympus microscope housed in a perspex chamber maintained at 37°C, transferred images to a computer connected between the camera and a monitor, as described in Chapter 2. An on-line, real-time computer program was used (LabVIEW, National Instruments, Victoria, Australia) to measure cell length. Contractility was induced by electrical-field stimulation using a Grass S4 stimulator. The cells were routinely stimulated with a pulse duration of 5 ms at a frequency of 1 Hz using two platinum wire electrodes located on either side of the superfusion chamber. Asynchronous contractile activity was induced by superfusion with progressively

increasing isoproterenol concentrations (0.01 μM to 3 μM) in the presence of electrical-field stimulation. Asynchronously contracting cells (in the presence of electrical-field stimulation) were visually scored and defined as those cells that exhibited a contraction rate exceeding the rate of applied electrical stimulation (i.e., cells not contracting in synchrony with the applied electrical stimulus). Four preparations per heart, consisting of at least 80 cardiomyocytes were counted for each rat heart.

6.2.3 Measurement of $[Ca^{2+}]_i$ using Fura-2

6.2.3.1 Instrumentation

The second generation fluorescent calcium ion (Ca^{2+}) indicators such as Fura-2 have been used for measuring intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a variety of cell types since 1985 (Grynkiewicz *et al.*, 1985). One of the main advantages of these dyes is their ability to measure fast changes in $[Ca^{2+}]_i$. Fura-2 was chosen to measure $[Ca^{2+}]_i$ in these studies since it has several advantages over other dyes (for review see (Sipido & Callewaert, 1995)). Fura-2 excitation is at two wavelengths and emission is measured at one. The emission wavelength is approximately 512 nm and is well within the visible wavelength range, therefore allowing for direct observation of cell loading (see panel 1 - photograph) via the fluorescent microscope.



Panel 1. Photograph of isolated adult rat cardiomyocytes. Viewed at low magnification showing incorporation of Fura-2 and emitted green fluorescence following consecutive excitation at 340 and 380 nm (200 Hz). For purposes of this photograph, cardiomyocytes were incubated with 5 μM Fura-2 for 30 min at 24°C to produce overloading of the dye.

To load the cardiomyocytes with the Fura-2, cells were incubated with the Fura-2 acetomethoxy ester (AM) form of the dye which rapidly partitions into the cell membrane and across into the cytoplasm. Once inside of the cell, intracellular esterases cleave the ester group liberating the salt form of the dye, thus trapping the dye inside the cell. Compartmentalisation of the dye may occur to some extent within the mitochondria but this can be partly overcome by incubating the cells at low temperature and loading the dye more slowly with lower concentrations of the ester (Sipido & Callewaert, 1995). Typically, when free Ca^{2+} binds to the Fura-2 the fluorescence increases at 340 nm (F_{340}) and decreases at 380 nm (F_{380}). The fluorescence ratio ($F_{340/380}$) is measured as an indication of intracellular [Ca^{2+}] following cellular calibration as described in "experimental techniques". Since this is a ratiometric method for determining [Ca^{2+}], the extent of dye loading is eliminated as an experimental variable. The time resolution of the final $[Ca^{2+}]_i$ measurement is partly limited by the sampling rate of the fluorescence ratio. In cardiac myocytes a fast sampling rate was required since the upstroke of the Ca^{2+} transient is very fast (time to peak is usually less than 50 ms in rapidly contracting cells). Therefore, with Fura-2, switching of the excitation light beam between 340 nm and 380 nm becomes the rate-limiting step. For these studies, the excitation light beam was rapidly switched between the two wavelengths, by using a Lambda DG-4 high-speed filter changer (Sutter Instrument Company, Novato, CA, USA) which uses rapidly oscillating mirrors mounted on galvanometers. This resulted in a signal time resolution of 2 ms. Additionally, this instrument lacks some of the inherent vibration problems associated with the traditional high speed "filter wheel" beam choppers (wavelength changers).

6.2.3.2 Experimental techniques

Cardiomyocytes were prepared as described in Chapter 2 and measurement of total phospholipid fatty acids was carried out as described in Chapter 4.

Freshly isolated cardiomyocytes on laminin coated coverslips (#0 glass) were transferred to a petri dish containing Tyrode solution supplemented with 1 mM Ca^{2+}, 2 % (w/v) BSA and 30 mM 2,3-butane-dione monoxime and 5 μM Fura-2 (acetomethoxy (AM) form) at 24°C for 15 min. The coverslips were then transferred to a custom-designed superfusion chamber (#0 glass base) located on the stage of an inverted epifluorescence microscope (Nikon, Tokyo, Japan) and superfused with Tyrode buffer containing 2 mM Ca^{2+}. For fluorescence measurements, cells were illuminated sequentially at 340 ± 10 nm and 380 ± 10 nm with a UV light source (175 W Xenon lamp) and Lambda DG-4 high-speed filter changer. The emitted fluorescence was detected with a photomultiplier provided with a lightpath filter set at 510 ± 40 nm. An adjustable rectangular diaphragm in the light-path preceding the photomultiplier restricted measurement of fluorescence to a single cell (prepared and designed by Professor David Allen, Department of Physiology, University of Sydney, Australia). Cells were were displayed on a TV monitor and aligned along their horizontal axis. Routinely, the diaphragm was opened and the entire cell was placed within the borders of the rectangular aperture, so as not to include signals derived from background fluorescence. The signals from the photomultiplier were fed through an A-D converter (BIOPAC Systems, Santa Barbara, CA, USA), digitized at 200 Hz and passed through a low pass band filter and output to a computer for real-time analysis (as described below). After subtraction of any background (signal obtained from a cell-free region in the field of view), the ratio (R) of fluorescence signals at 340 nm/380 nm were

calculated and converted to $[Ca^{2+}]_i$ using the following formula (Grynkiewicz *et al.*, 1985),

$$[Ca^{2+}]_i = K_d\beta[(R-R_{min})/(R_{max}-R)]$$

where R_{max} represents the maximum fluorescence ratio (calcium saturation) which was determined at the end of an experimental protocol by treating cells with the membrane permeating agent, digitonin (20 μM). The minimum fluorescence ratio R_{min}, was determined by treating cardiomyocytes with 5 mM EGTA and 20 mM caffeine, pH 7.3. K_d is the dissociation constant for Fura-2 in simple ionic solution (225 nM) (Grynkiewicz *et al.*, 1985), and β represents the ratio of emission intensities at 380 nm excitation at saturating and calcium-free conditions.

Cardiomyocytes were routinely superfused at 2.5 ml/min at 24°C with standard Tyrode buffer containing 2 mM Ca^{2+}. After 5 min equilibration, cells were then stimulated at 0.5 Hz for 2 min, using two platinum wire electrodes placed within the superfusion chamber and connected to a DS9A Digitimer stimulator (Herts, England). This procedure allowed for the measurement of baseline calcium transients which included the end-diastolic $[Ca^{2+}]_i$ (the $[Ca^{2+}]_i$ between successive contractions), and the systolic $[Ca^{2+}]_i$ (the maximum $[Ca^{2+}]_i$ during contractions). In all experiments, at least 5 consecutive Ca^{2+} transients were ensemble averaged to obtain the Ca^{2+} transient parameters using AcqKnowledge™ software (SDR Clinical Technology, Sydney, NSW, Australia). All cardiomyocyte experiments were performed within 6 h of isolation.

6.2.3.3 SR Ca^{2+} content and Ca^{2+} transients in cardiomyocytes

Sarcoplasmic reticulum (SR) Ca^{2+} content was determined using two procedures. Firstly, after steady-state Ca^{2+} transients were established at 0.5 Hz, a 30 s rest period was applied to allow the SR to load and then electrical stimulation was resumed at 0.5 Hz, as described previously (Negretti *et al.*, 1995). The magnitude of the first systolic Ca^{2+} transient (termed the rest potentiation) has been shown to be correlated with changes in the amount of Ca^{2+} stored in the SR and was greater than that obtained during the steady-state period. The peak height of the first Ca^{2+} transient after the rest period was analysed and taken as an indirect measure of relative SR Ca^{2+} content. SR Ca^{2+} content can also be determined by rapid application of caffeine which unloads the stores of Ca^{2+} in the SR (Capogrossi *et al.*, 1986b; Wolska & Lewartowski, 1993; Delbridge *et al.*, 1996a; Holt & Christensen, 1997; Negretti *et al.*, 2000). Steady-state Ca^{2+} transients were recorded for 1 min (in the presence of electrical field stimulation) and then caffeine (20 mM) was rapidly applied to the cardiomyocyte of interest via a rapid-application system using a reservoir placed 30 cm above the level of the chamber (in the absence of electrical stimulation). The outlet tube for caffeine application was placed approximately 500 μm upstream from the cell of interest and delivered at a flow rate of 5 ml/min. To test the effect of the SR Ca^{2+}-ATPase inhibitor, 2,5-di-tert-butylhydroquinone (DBHQ) (Kabbara & Stephenson, 1997) on SR Ca^{2+} pump function and Ca^{2+} transients, cardiomyocytes were stimulated at 0.2 Hz and Ca^{2+} transients measured at steady-state before and after addition of 10 μM DBHQ for 4 min. This procedure allows determination of the proportion of Ca^{2+} that is normally contributed by the SR to the excitation-contraction coupling process. The effect of DBHQ was completely reversible following a 4 min washout protocol. Activation of the L-type Ca^{2+} channel was achieved following addition of 0.1 μM Bay K8644 for 4 min, and β-adrenergic agonist stimulation was carried out

using 0.5 μM isoproterenol at stimulation rates between 0.2 and 2 Hz. To determine the rate of rise of $[Ca^{2+}]_i$, transients were ensemble averaged and the time-to-peak as well as the slope of the rise in $[Ca^{2+}]_i$ were calculated. To determine the time constant (τ) of the declining phase (decay) of all Ca^{2+} transients, exponential rates of decay were fitted to a single exponential function:

$$([Ca^{2+}] = ([Ca^{2+}]_{max} / e^{(t/\tau)}) + [Ca^{2+}]_{min})$$

where t = time (sec) and τ is the time constant for Ca^{2+} transient decay. Separate experiments were carried out on cardiomyocytes to determine effects of SR Ca^{2+} content, DBHQ and isoproterenol sensitivity.

6.2.4 Statistics

Statistical analysis was performed using the computer software program Instat version 3.01 (GraphPad Software, San Diego, CA, USA). Student's unpaired t-test, two-way ANOVA with Bonferroni multiple comparison test or Fishers' Exact test were used to compare differences between effects of the various dietary lipid treatments. Results are expressed as the mean ± SEM. For each comparison, the level of significance was set at $P<0.05$ for the indicated number of animals per dietary group. Where data appear as n = x, y; n = number of rat hearts and y = number of cells studied.

6.3 RESULTS

6.3.1 Preliminary experiments on Ca^{2+} transients

Following acquisition and set up of the apparatus used for fluorescent microscopy, several experiments were carried out to determine whether the measurement of $[Ca^{2+}]_i$ was sensitive to changes in fluorescent microscope magnification (comparing 20x and 40x fluorescent objectives). It was found that the 40x objective yielded twice the intensity of fluorescence at the individual excitation wavelengths compared with the 20x objective, as expected. However, since the analysis of Ca^{2+} is a ratiometric method, the $F_{340/380}$ ratios were the same using both objectives. Furthermore, preliminary experiments (n=5-10) demonstrated that the ratio values obtained were reproducible (CV=5%) and the objective magnification did not significantly alter these values. Nevertheless, the 40x objective was used for all subsequent studies. An initial concern was that adding a #0 glass coverslip with adhering cells, on to another #0 glass coverslip (which made up the base of the superfusion chamber) may provide difficulties in obtaining fluorescence data, particularly due to the low excitation wavelengths and the thickness of the glass through which the light emitted by the cells must travel to be measured by the photomultiplier tubes. However, preliminary experiments plating cells directly onto the base of the superfusion chamber alone provided no further benefit over that of layering two #0 glass coverslips in the manner described above. Therefore, for convenience, #0 coverslips containing adhering cardiomyocytes were placed onto the bottom of a #0 glass coverslip which provided the base of the superfusion chamber for $[Ca^{2+}]_i$ measurements.

The fastest sampling rate attained without loss of sensitivity was found to be 200 Hz. Although transients adequate for measurement could still be determined at 250 to 300 Hz,

this faster rate data acquisition was not deemed necessary. At 200 Hz, an adequate number of data points (Fura-2 fluorescence) were recorded during each contractile cycle to enable the acquisition of definable Ca^{2+} transients which did not exhibit excessive "noise". Figure 6.1A shows the electrical stimuli and Figure 6.1 B and C show a representative example of the individual fluorescence transients at 340 nm and 380 nm. Figure 6.1D demonstrates a low pass filtering of the data and subsequent fluorescent ratio analysis, $F_{340/380}$, representing uncalibrated intracellular [Ca^{2+}] (in arbitrary units).

Isolated cardiomyocytes have been shown to display similar Ca^{2+} dependent systolic and diastolic contractile properties as that observed in intact cardiac muscle (Capogrossi *et al.*, 1986a). To determine whether the cellular Ca^{2+} transients were responding in a similar manner to that expected from intact cardiac muscle tissue, the following preliminary experiments were carried out. Thapsigargin was used to block SR Ca^{2+} uptake as shown previously (Negretti *et al.*, 1993a). Treatment with thapsigargin for 2 min resulted in a significant decrease in systolic $[Ca^{2+}]_i$ as would be expected due to the reduced level of free cytoplasmic Ca^{2+} available during each contractile cycle. However, DBHQ (Kabbara & Stephenson, 1997) was used as the choice of SR Ca^{2+} uptake inhibitors in the following dietary study, since in contrast to DBHQ, the effects of thapsigargin were not reversible. To measure SR Ca^{2+} load, rapid cooling contractures were elicited by rapid application of Tyrode buffer maintained at 2°C. This procedure can be used to estimate the relative amount of the releasable myocardial SR Ca^{2+} pool present in rat and human myocardium (Yu *et al.*, 1994; Stauffer *et al.*, 1997; Maier *et al.*, 2000). The typical reduction in cell length following this protocol was approximately 25%. Rapid application of 20 mM caffeine produced similar results. Under these conditions, the Ca^{2+} released is removed from the cytoplasm by non-SR pathways and the caffeine-induced Ca^{2+} transient declined

more slowly than electrically stimulated Ca^{2+} transients, probably due to the inhibition of SR Ca^{2+} uptake by caffeine (Varro *et al.*, 1993; Donoso *et al.*, 1994). Of the two methods utilised in this study to measure SR Ca^{2+} load, the caffeine-induced contracture method was chosen for the dietary study, since the rapid cooling contracture method resulted in significant condensation and "fogging" on the glass within the superfusion chamber reducing the emitted fluorescence detectable. The β-adrenergic receptor agonist, isoproterenol, and the L-type Ca^{2+} channel agonist, Bay K8644 were used at 0.5 μM and 0.1 μM, respectively in the dietary study. These particular concentrations were chosen since they elicited a positive inotropic effect and an increase in Ca^{2+} transients without causing immediate hypercontracture and/or arrhythmic contractions.

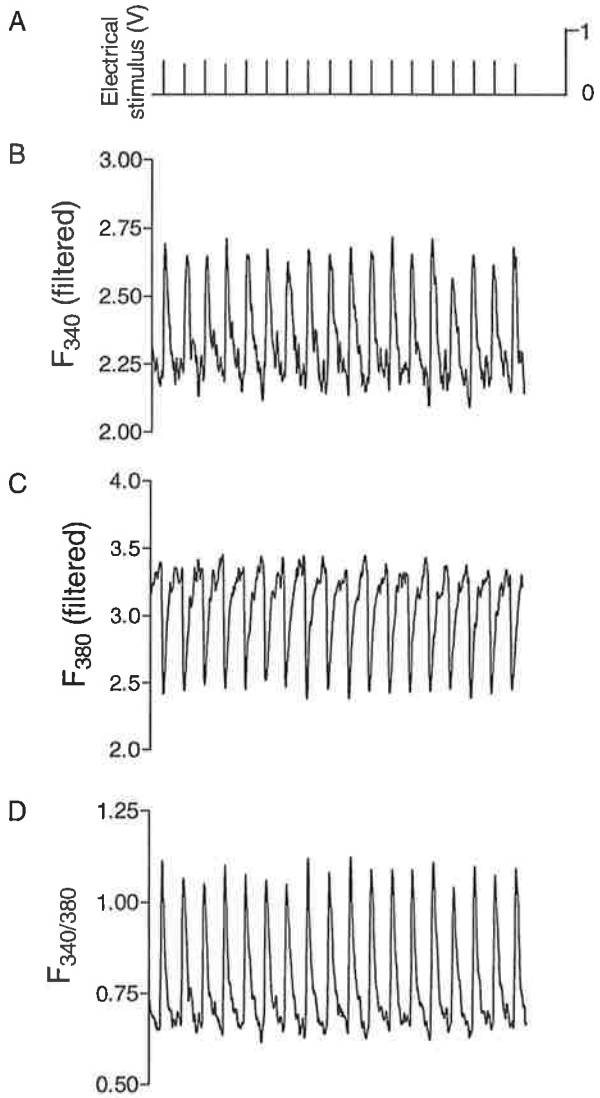


Figure 6.1 Ca^{2+} transients using Fura-2.
A, Electrical stimulation pulses were at 1 Hz. *B*, low-pass filtered (340 nm) fluorescence data . *C*, low-pass filtered (380 nm) fluorescence data. *D*, Ratio of fluorescence taken from *B* and *C*, representing changes (uncalibrated) in cytoplasmic $[Ca^{2+}]_i$. Sampling frequency was 200 Hz, following 10 min incubation in 5 μM Fura-2, using a 40x fluorescent objective. Data are expressed in arbitrary units.

6.3.2 *Effects of dietary fish oil on body weight*

Prior to dietary lipid supplementation, body weights were not significantly different. The body weights at 9 weeks of age were 360.7 ± 9.2 g (n=8) and 374.7 ± 9.5 g (n=8), respectively, and following 3 weeks dietary lipid supplementation with either SF or FO, increased to 474.2 ± 10.3 g (n=8) and 474.6 ± 2.4 g (n=8), respectively (not significantly different) as shown in Figure 6.2.

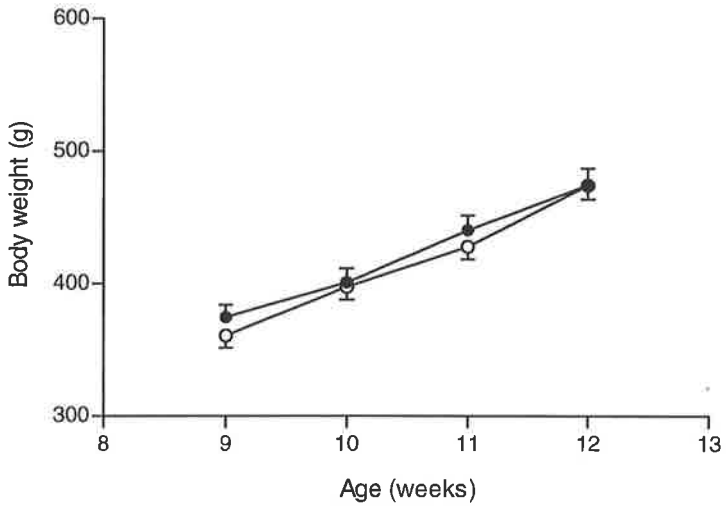


Figure 6.2 Body weight of rats during the dietary supplementation period with saturated fat or fish oil.
Diets were supplemented with either 10 % saturated fat, SF (O) or 10 % fish oil, FO (●). Data are means ± SEM for n = 8 animals per dietary group

6.3.3 *Effects of dietary fish oil on cardiomyocyte viability*

Following isolation of cardiomyocytes, viability was determined as the percentage of rod-shaped cells not exhibiting membrane blebs, and was 73.1 ± 3.9 % in the SF group and 76.7 ± 2.2 % in the FO group (not significantly different).

6.3.4 *Fatty acid composition of rat ventricular phospholipids*

The fatty acid composition of the lipid supplemented diets are shown in Table 6.1. The FO diet contained approximately 29% total n-3 polyunsaturated fatty acids (PUFAs) whilst the SF diet supplement contained approximately 1% n-3 PUFAs. The FO diet also contained comparatively less saturated fat and monounsaturated fat.

Table 6.1 Fatty acid composition (wt %) of the lipid supplemented diets

Major FAME[a]	SF	FO
14:0	4.3	4.8
16:0	19.9	10.5
16:1	1.1	6.7
18:0	10.6	2.6
18:1[b]	53.1	36.7
18:2 (n-6)	7.1	6.6
18:3 (n-3)	1.2	0.9
20:4 (n-6)	n/d[c]	1.0
20:5 (n-3)	n/d[c]	17.8
22:5 (n-3)	n/d[c]	1.7
22:6 (n-3)	n/d[c]	8.9
Σ Sat.	36.4	18.6
Σ Mono.	55.1	44.0
Σ Poly.	8.5	37.4
Σ n-6	7.1	7.8
Σ n-3	1.4	29.4
n-6/n-3	5.2	0.3

Data shown are mean for two samples per dietary group. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. [c] n/d, not detected. SF; saturated fat group, FO; fish oil group.

Following 3 weeks of dietary lipid supplementation, significant changes were evident in the cardiac phospholipid fatty acid profile (Table 6.2). In comparison with the SF diet treatment, dietary FO increased the total n-3 PUFAs (6.9 % and 26.2 % in the SF and FO groups, respectively) as a result of significantly increased proportions of docosahexaenoic acid (22:6, n-3), docosapentaenoic acid (22:5, n-3) and eicosapentaenoic acid (20:5, n-3). For example, the proportion of DHA was 6.0 ± 0.8 % in the SF group compared with 20.5 ± 0.9 % in the FO group ($P<0.001$). Concomitantly, the proportion of the total n-6 PUFAs in the FO supplemented rats was reduced as a result of the decrease in the proportions of arachidonic acid (20:4, n-6) and linoleic acid (18:2, n-6). Collectively, this resulted in a significant reduction in the n-6/n-3 PUFA ratio in the FO fed rats. The overall proportion of saturated fatty acids was not significantly influenced by the nature of the dietary lipid supplement (37.6 ± 2.4 % and 36.0 ± 0.7 % in the SF and FO groups, respectively). The other significant change of note in the FO supplemented rats was the lowering of the proportion of total monounsaturated fatty acids due primarily to the lowered proportion of oleic acid (18:1) in the ventricular phospholipids. In both dietary groups, α-linolenic acid (18:3, n-3) was below the limits of detection.

Table 6.2 Fatty acid composition (wt %) of the ventricular phospholipids after dietary lipid supplementation

Major FAME[a]	SF	FO
16:0	9.4 ± 0.9	11.9 ± 0.5[d]
18:0	25.9 ± 1.3	23.1 ± 0.4
18:1[b]	14.5 ± 0.6	10.2 ± 0.5[f]
18:2 (n-6)	5.9 ± 1.1	3.7 ± 0.6
18:3 (n-3)	n/d[c]	n/d[c]
20:4 (n-6)	33.6 ± 2.4	22.2 ± 2.0[e]
20:5 (n-3)	n/d[c]	3.2 ± 0.1
22:5 (n-3)	0.8 ± 0.1	2.5 ± 0.1[f]
22:6 (n-3)	6.0 ± 0.8	20.5 ± 0.9[f]
Σ Sat	37.6 ± 2.4	36.0 ± 0.7
Σ Mono	14.9 ± 0.6	10.9 ± 0.5[f]
Σ Poly	47.2 ± 1.5	53.1 ± 0.9[f]
Σ n-6	40.1 ± 2.4	26.7 ± 1.7[f]
Σ n-3	6.9 ± 0.9	26.2 ± 0.9[f]
n-6/n-3	5.8 ± 0.6	1.0 ± 0.1[f]

Data shown are the mean ± SEM for 6 animals per dietary group. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. [c]n/d, not detected. Superscripts indicate significant differences at [d]P<0.05, [e]P<0.01, [f]P<0.001 vs SF (by Students unpaired t-test). SF; saturated fat group, FO; fish oil group.

6.3.5 Isoproterenol induced asynchronous contractile activity of cardiomyocytes

Figure 6.3A shows the change in cell length of a synchronously contracting cardiomyocyte before and after treatment with the β-adrenergic receptor agonist, isoproterenol, a positive inotropic effect which also induces asynchronous contractile activity (Figure 6.3B) in cardiomyocytes (Priori & Corr, 1990; Tweedie *et al.*, 1997; Jahangiri *et al.*, 2000). Figure 6.3C shows that the onset of asynchronous contractile activity was evident at lower isoproterenol concentrations in cardiomyocytes isolated from the SF group in comparison with the FO group. This effect was statistically

significant between the SF and FO groups at the concentrations of isoproterenol indicated in Figure 6.3. It was not possible to accurately determine the ED_{50} dose of isoproterenol needed to elicit asynchronous contractile activity due to the fact that maximum levels of asynchronous contractility were not achieved in the particular experimental protocol followed in this study. However, cardiomyocytes isolated from the SF rats would appear to exhibit significantly greater significantly to isoproterenol (at concentrations ≥ 30 nM) with regard to the development of asynchronous contractile behaviour than do cells isolated from rats fed the FO supplemented diet.

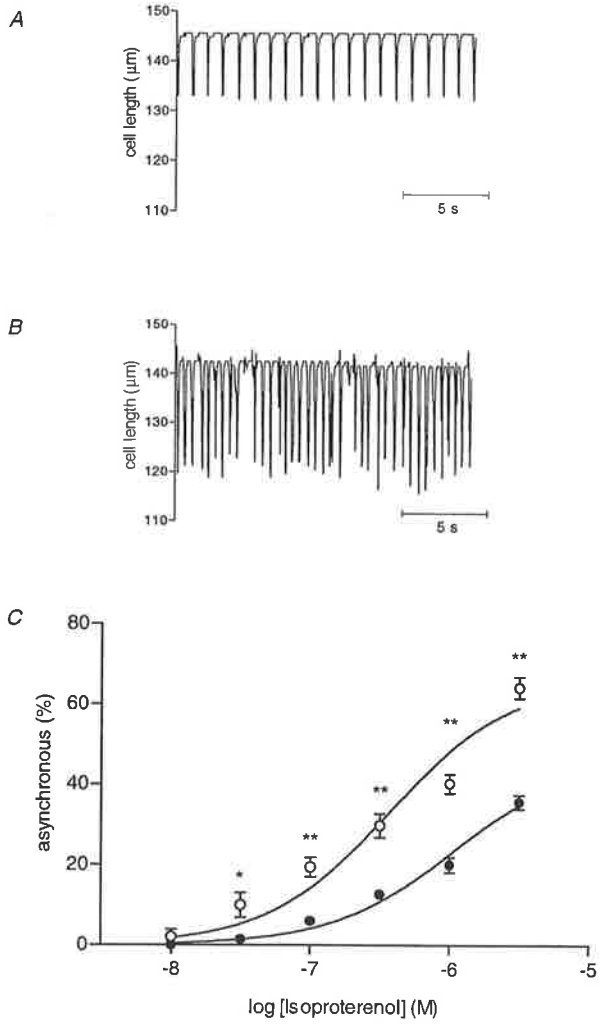


Figure 6.3 Isoproterenol-induced asynchronous contractility in rat cardiomyocytes.
A, original record demonstrating the change in cell length during steady-state (synchronous) contractility induced by electrical stimulation at 1 Hz. *B*, showing the development of a positive inotropic response and asynchronous contractility following treatment with 1 µM isoproterenol with electrical stimulation maintained at 1 Hz. *C*, percentage of asynchronously contracting cardiomyocytes in response to increasing concentrations of isoproterenol. Diets were supplemented with either 10 % saturated fat, SF (○) or 10 % fish oil, FO (●). Data are means ± SEM for $n = 6$ animals per dietary group. *P<0.05, **P<0.001 for FO vs SF.

6.3.6 Effect of dietary lipid supplementation on Ca^{2+} transients in cardiomyocytes

6.3.6.1 SR Ca^{2+} content

To determine whether there was a difference in the level of Fura-2 loading, the value of the fluorescence (F_{380}) was compared in cells during the diastolic phase (non contracting

phase) and was lower in cells from the SF group (3.68 ± 0.12 fluorescent units, n = 96) compared with the FO group (4.03 ± 0.14 fluorescent units, n = 96), however this difference was not significant. To determine SR Ca^{2+} content, two alternate experimental protocols were used on the same cell. A representative recording of Ca^{2+} transients is shown in Figure 6.4. Figure 6.4 shows Ca^{2+} transients under steady-state conditions while stimulated at a frequency of 0.5 Hz. This was then followed by a 30 s rest period with no electrical stimulation. The amplitude of the first Ca^{2+} transient (as shown) immediately after this rest period was used as an indirect measure of SR Ca^{2+} content by measuring the developed $[Ca^{2+}]_i$ (calculated by subtracting the minimum (end diastolic) $[Ca^{2+}]_i$ concentration from the maximum (systolic) $[Ca^{2+}]_i$ concentration). In addition, an alternative procedure for determining SR Ca^{2+} content using caffeine (20 mM) was carried out on the same cells as described in "methods" and shown in Figure 6.4.

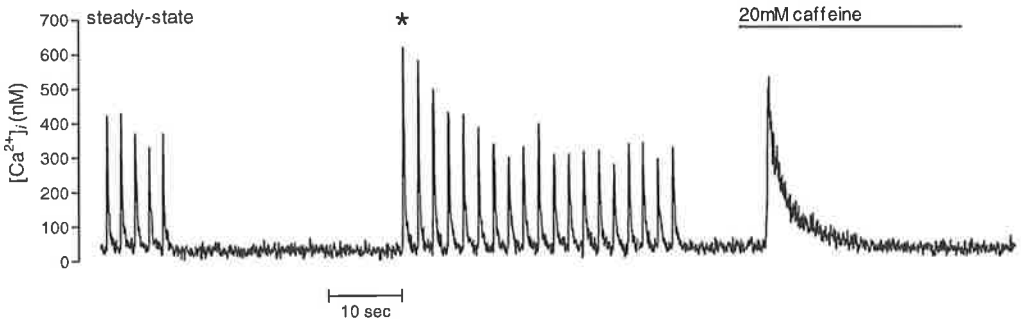


Figure 6.4 Indirect measurement of SR Ca^{2+} content in rat cardiomyocytes.
Representative Ca^{2+} transients in an isolated rat cardiomyocyte measured using Fura-2. Steady-state Ca^{2+} transients were elicited at a stimulation frequency of 0.5 Hz. Following a 30 s rest period (no electrical stimulation), the negative staircase effect was observed, characteristic of rat cardiomyocytes prior to the establishment of a new steady-state. The first developed $[Ca^{2+}]_i$ transient following the 30 s rest (shown by the asterisk, *) indicates the level of SR loading during the rest period. Following steady-state again, 20 mM caffeine was rapidly applied in the absence of electrical stimulation, indicated by the horizontal bar.

The means of the $[Ca^{2+}]_i$ during the diastolic and systolic phases as well as the developed $[Ca^{2+}]_i$ from all cells studied by the protocol shown in Figure 6.4, is shown in Figure 6.5A-F. The diastolic $[Ca^{2+}]_i$ was approximately 50 nM in SF and FO cardiomyocytes and was increased to approximately 400 nM during steady-state contractions at 0.5 Hz resulting in a developed $[Ca^{2+}]_i$ of approximately 350 nM on a beat-to-beat cycle (Figure 6.5A-C).

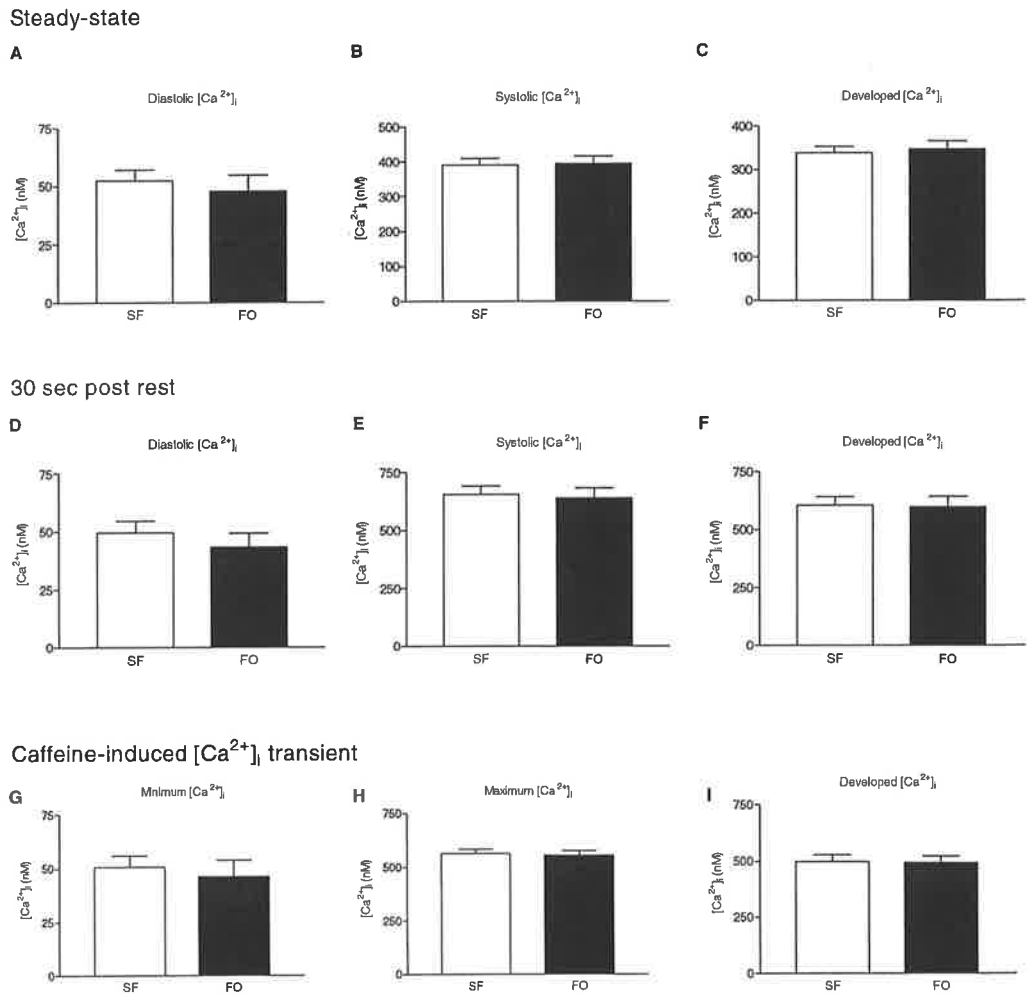


Figure 6.5 Changes in $[Ca^{2+}]_i$.
A, Diastolic $[Ca^{2+}]_i$, *B*, systolic$[Ca^{2+}]_i$, *C*, developed $[Ca^{2+}]_i$ during steady-state electrical stimulation at 0.5Hz. The first Ca^{2+} transient was analysed following the 30 s rest period and the diastolic $[Ca^{2+}]_i$, systolic$[Ca^{2+}]_i$ and developed $[Ca^{2+}]_i$ are shown in *D-F*, respectively. The minimum, maximum and developed caffeine-induced rise in $[Ca^{2+}]_i$, is shown in *G-I*, respectively. Data are means ± SEM for n = 32 cardiomyocytes from 8 animals per dietary group. Abbreviations, saturated fat (SF) and fish oil (FO).

Immediately following the 30 s post rest period, electrical stimulation was resumed resulting in the first systolic Ca^{2+} transient being greater than that at steady-state. The systolic $[Ca^{2+}]_i$ was increased to approximately 650 nM in both the SF and FO groups (not significantly different) as shown in Figure 6.5E. Once steady-state was attained (following the characteristic negative staircase effect) electrical stimulation was ceased and caffeine was rapidly applied. Under these conditions, the Ca^{2+} released from the SR is removed from the cytoplasm by non-SR pathways and the caffeine-induced Ca^{2+} transient declined more slowly than electrically stimulated Ca^{2+} transients, probably due to the inhibition of SR Ca^{2+} uptake by caffeine (Figure 6.4). The maximal concentration of $[Ca^{2+}]_i$ as measured by this protocol (approximately 550 nM) as shown in Figure 6.5H, was less than that achieved following the 30 s post rest potentiation protocol described above (650 nM) and was similar in value for both the SF and FO groups. The difference in values of the $[Ca^{2+}]_i$, estimated by the two different methods, is probably because the post rest transient includes Ca^{2+} contributed from both the SR as well as the sarcolemma. However, the results obtained by this latter protocol similarly demonstrate that there was no significant difference between cells from the SF and FO dietary supplemented groups in relation to cardiac SR Ca^{2+} content. Additionally, the diastolic $[Ca^{2+}]_i$ was lower in the FO group compared with the SF group under basal (control) conditions and following caffeine application, however this difference was not significant. The time constants (τ) of the exponential decay in $[Ca^{2+}]_i$ following caffeine was increased in the FO group (2.001 ± 0.141 s, n=8) compared with the SF group (1.777 ± 0.118 s, n=8), however this did not reach statistical significance.

6.3.6.2 *Effect of DBHQ on Ca^{2+} transients*

DBHQ was used to inhibit the uptake of Ca^{2+} by the SR as shown previously (Ju & Allen, 1999). Figure 6.6A shows a representative recording of electrically stimulated Ca^{2+} transients at a frequency of 0.2 Hz before and after application of 10 μM DBHQ for 4 min. It should be noted that experiments on cardiomyocytes involving DBHQ treatment could not be carried out at faster stimulation frequencies, since the declining (decay) phase of the Ca^{2+} transients were substantially increased (i.e., the rate of decay was decreased) resulting in a progressive increase in diastolic $[Ca^{2+}]_i$ and hypercontracture at stimulation frequencies greater than 0.2 Hz in the presence of this inhibitor. The ensemble average of the Ca^{2+} transients from Figure 6.6A is shown in Figure 6.6B on an expanded time scale. This demonstrates that DBHQ treatment resulted in a significant reduction in the value of the peak (systolic) $[Ca^{2+}]_i$ and an increase in the time constant of decay of the Ca^{2+} transient.

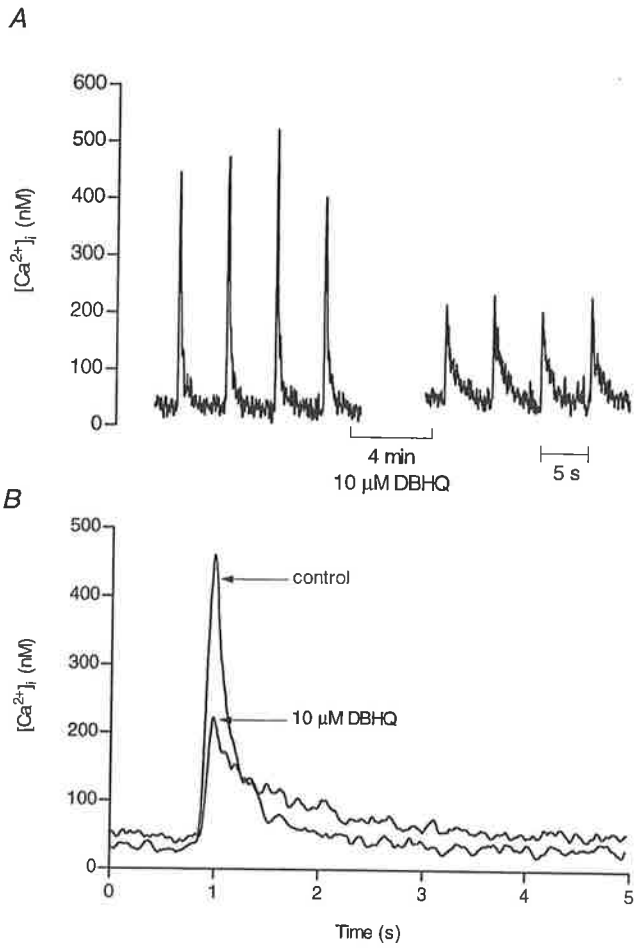


Figure 6.6 Effects of the SR Ca^{2+} pump inhibitor, DBHQ, on Ca^{2+} transients in rat cardiomyocytes. *A*, representative Ca^{2+} transients in an isolated rat cardiomyocyte measured using Fura-2 in the absence (control) or following 4 min treatment with 10 µM DBHQ at a stimulation frequency of 0.2 Hz. *B*, ensemble average of Ca^{2+} transients from panel *A*.

Figure 6.7A shows that the diastolic $[Ca^{2+}]_i$ before (control) DBHQ treatment was lower in the FO group compared with the SF group, however this was not significant. Figure 6.7B-C demonstrates that the systolic and developed $[Ca^{2+}]_i$ were similar in cardiomyocytes isolated from both dietary groups. Figure 6.7D-F shows that the diastolic, systolic and developed $[Ca^{2+}]_i$ respectively, following DBHQ treatment was lower in the FO group compared with the SF group, however this difference was not significant.

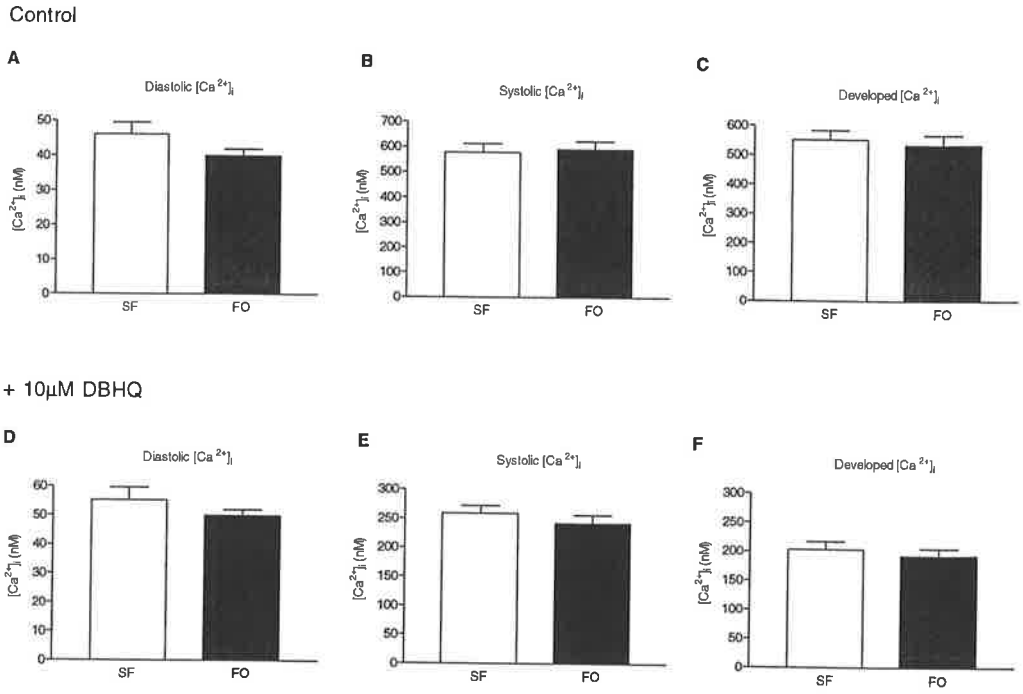


Figure 6.7 Changes in $[Ca^{2+}]_i$ following DBHQ treatment.
Comparison of SF and FO dietary groups during steady state at 0.2 Hz. *A*, Diastolic $[Ca^{2+}]_i$, *B*, systolic$[Ca^{2+}]_i$, *C*, developed $[Ca^{2+}]_i$ during steady-state electrical stimulation at 0.2Hz and, *D*, diastolic $[Ca^{2+}]_i$, *E*, systolic$[Ca^{2+}]_i$, *F*, developed $[Ca^{2+}]_i$ following 4 min with 10 μM DBHQ. Data are means ± SEM for saturated fat (SF, n=8, 32) and fish oil (FO, n=8, 32).

Since the rate of rise in the Ca^{2+} transients of cardiomyocytes is indicative of Ca^{2+} transport into the cytoplasm by SR and non-SR pathways (i.e., mainly sarcolemmal L-type Ca^{2+} channel), the rise in the Ca^{2+} transients was also analysed. To determine whether dietary FO treatment affected the initial rise in $[Ca^{2+}]_i$, the data were analysed in two ways. Firstly, the time-to-peak was determined by calculating the time between the initial upstroke of the Ca^{2+} transient and the peak of the Ca^{2+} transient. Secondly, the slope of the upward (systolic) phase was calculated. The results are shown in Figure 6.8. These particular measurements of the rise in $[Ca^{2+}]_i$ transients demonstrated that there was no significant difference in the time-to-peak or slope of the rise in $[Ca^{2+}]_i$ between

cardiomyocytes from the SF and FO groups in the absence or presence of DBHQ, suggesting that fish oil supplementation does not modulate the Ca^{2+} influx mechanisms either via SR or sarcolemmal pathways. However, it was noted that DBHQ significantly increased the time taken to reach the peak systolic $[Ca^{2+}]_i$ in both dietary groups, suggesting that the SR may play an important role in the upstroke of the Ca^{2+} transients. However, the time constant of $[Ca^{2+}]_i$ decay in the FO group was greater under control conditions (although not significant, Figure 6.9) and was significantly greater ($P<0.05$) compared with the SF group following DBHQ treatment as shown in Figure 6.9. This suggests a more rapid Ca^{2+} efflux via sarcolemmal Ca^{2+} exchangers in the SF group since Ca^{2+} re-uptake into the SR is blocked by DBHQ. The contribution of the SR to the $[Ca^{2+}]_i$ transient was calculated (i.e., the DBHQ-sensitive component of the Ca^{2+} transient) to provide approximately 62 % of the Ca^{2+} available for transients in both groups and therefore, for contraction. This result compares to 42 % in toad pacemaker cells (Ju & Allen, 1999) and 32 % in guinea-pig ventricular cells (Lewartowski & Wolska, 1993) calculated by similar methods.

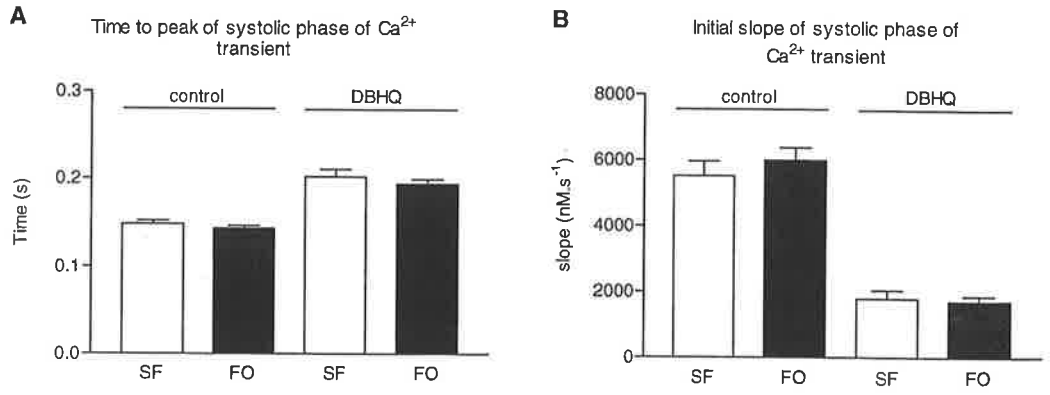


Figure 6.8 Time to peak and slope of upward phase of Ca^{2+} transients following treatment with DBHQ.

A, time between the initial upstroke and the peak of the Ca^{2+} transient before (control) and after 10 μM DBHQ. *B*, Slope of the upstroke of the Ca^{2+} transient before (control) and after 10 μM DBHQ. Data are means ± SEM for saturated fat (SF, n=8, 32) and fish oil (FO, n=8, 32).

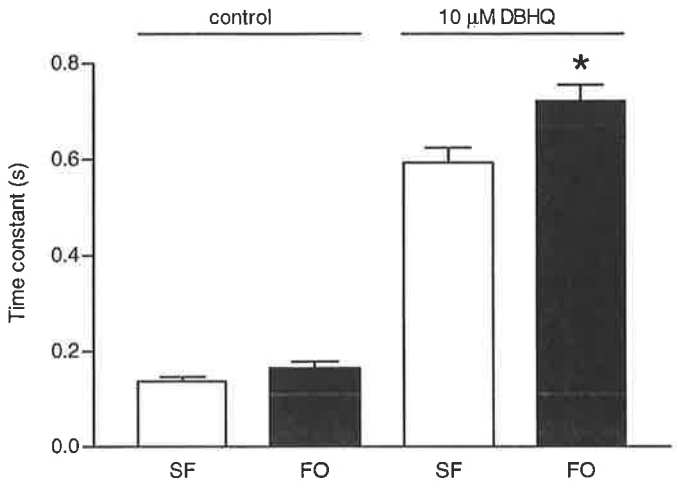


Figure 6.9 Time constant of decay of Ca^{2+} transients following treatment with DBHQ.
The time constants (τ) of the declining phase of Ca^{2+} transients were determined by single exponential fit ($[Ca^{2+}] = ([Ca^{2+}]_{max} / e^{(t/\tau)}) + [Ca^{2+}]_{min}$). Data are means ± SEM for saturated fat (SF, n=8, 32) and fish oil (FO, n=8, 32). *P<0.05.

6.3.6.3 *Effect of isoproterenol on Ca^{2+} transients*

To investigate whether Ca^{2+} handling following β-adrenergic receptor stimulation was different between cardiomyocytes isolated from the two dietary groups, isoproterenol (0.5 μM) was used to increase the cellular Ca^{2+} load. This concentration of isoproterenol increased the amplitude of Ca^{2+} transients at all stimulation frequencies tested. A representative recording of Ca^{2+} transients over the frequency range 0.2 - 2 Hz is shown in Figure 6.10A. The ensemble average of five Ca^{2+} transients from a single cardiomyocyte stimulated at 0.5 Hz in the absence and presence of isoproterenol is shown in Figure 6.10B. Isoproterenol increased the amplitude of Ca^{2+} transients and decreased the time constant for $[Ca^{2+}]_i$ decay. The time constant for $[Ca^{2+}]_i$ decay in cardiomyocytes from the FO group was significantly greater (P<0.05) than the time constant of the SF group at a frequency of 0.2 Hz, as shown in Figure 6.10C. Furthermore, β-stimulation with isoproterenol significantly decreased the time constant of the decay phase of Ca^{2+}

transients (in both groups) indicating a more rapid Ca^{2+} sequestration by the SR and/or Ca^{2+} efflux via sarcolemmal Ca^{2+} exchangers. In the presence of isoproterenol, the time constant for the decay phase of Ca^{2+} transients was significantly higher in the FO group compared with the SF group at 0.2 Hz ($P<0.01$).

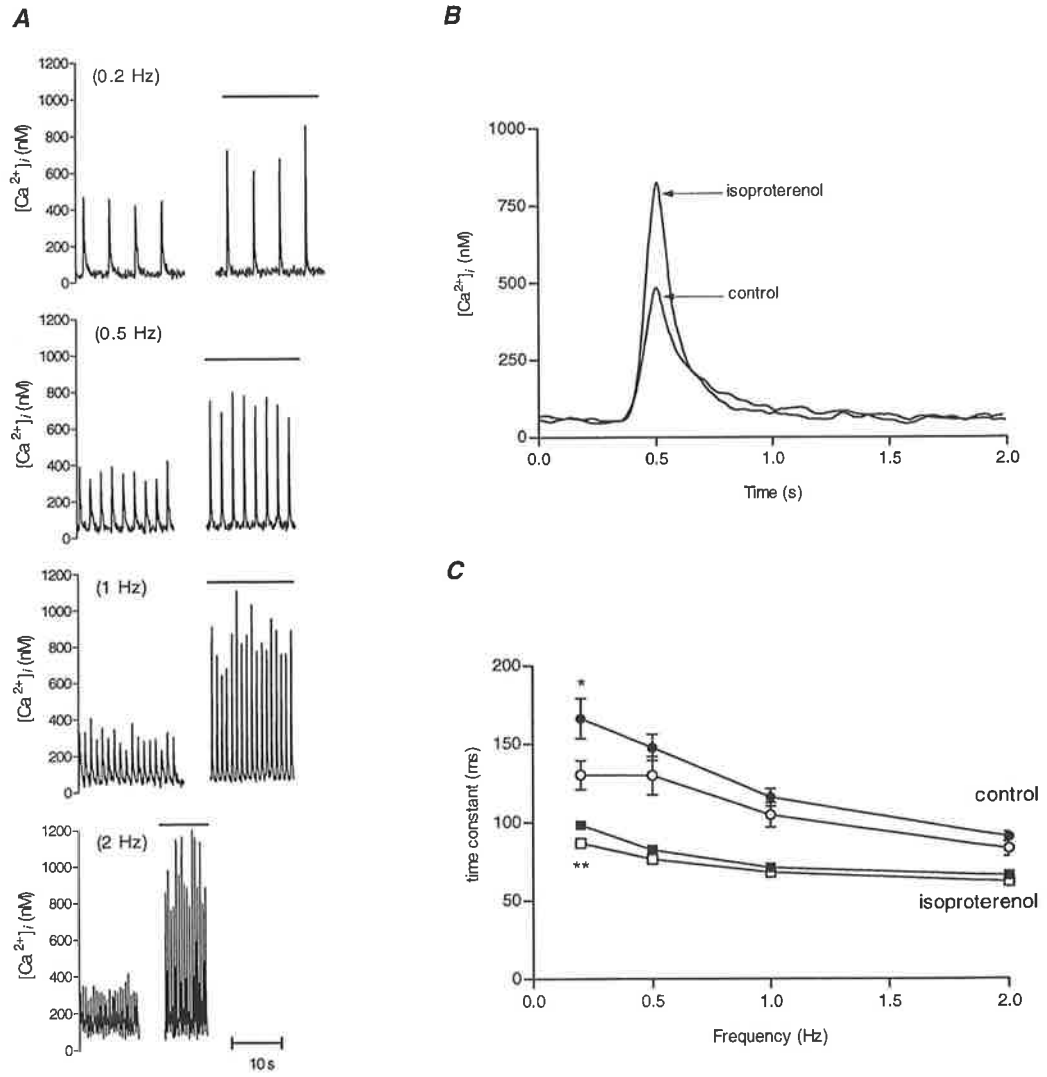


Figure 6.10 Effects of isoproterenol on Ca^{2+} transients and decay at 0.2 to 2 Hz in rat cardiomyocytes. *A*, representative Ca^{2+} transients in an isolated rat cardiomyocyte measured using Fura-2, in the absence or presence (indicated by the horizontal bar) of 0.5 μM isoproterenol at stimulation frequencies of 0.2, 0.5, 1 and 2 Hz. *B*, representative ensemble average of five Ca^{2+} transients following electrical stimulation at 0.5 Hz under control conditions or in the presence of 0.5 μM isoproterenol as indicated. *C*, time constants determined by single exponential fit ($[Ca^{2+}] = ([Ca^{2+}]_{max} / e^{(t/\tau)}) + [Ca^{2+}]_{min}$) of the decay phase of the electrically-stimulated Ca^{2+} transients following electrical stimulation at frequencies of 0.2 to 2 Hz during control conditions, comparing saturated fat, SF (○) and fish oil, FO (●) supplementation, and following 0.5 μM isoproterenol treatment, SF (□), FO (■). Data are means ± SEM for SF (n=8, 32) and FO (n=8, 31). *P<0.05 SF vs FO under control conditions, **P<0.01 SF vs FO in the presence of isoproterenol.

The level of diastolic $[Ca^{2+}]_i$ was frequency dependent and increased significantly with increasing frequency of stimulation from 0.2 to 2 Hz both in the absence and presence of isoproterenol (Figure 6.11A) for both dietary groups. Isoproterenol treatment resulted in an increase in the end-diastolic $[Ca^{2+}]_i$ (Figure 6.11A), the peak systolic $[Ca^{2+}]_i$ (Figure 6.11B) and the developed $[Ca^{2+}]_i$ (Figure 6.11C) at all stimulation frequencies tested. The systolic and developed $[Ca^{2+}]_i$ levels (Figure 6.11B,C), both in the absence or presence of isoproterenol were higher in the SF group compared with the FO group over all stimulation frequencies, although this did not reach statistical significance. Furthermore, the number of cardiomyocytes contracting asynchronously at 1 Hz during Ca^{2+} transient measurements in the presence of 0.5 μM isoproterenol was 1 of 29 (3 %) in the FO group compared with 4 of 32 (12.5 %) in the SF group. In addition, at a stimulation rate of 2 Hz, 5 of 31 cardiomyocytes (16 %) contracted asynchronously from the SF group compared to 0 of 29 cardiomyocytes from the FO group (P=0.053). This level of asynchronous contractile activity is consistent with the results shown in Figure 6.3 and may be partly explained by a combination of higher values for end diastolic and peak systolic $[Ca^{2+}]_i$, and a significant decrease in the time constant of the Ca^{2+} transient decay phase in the SF group.

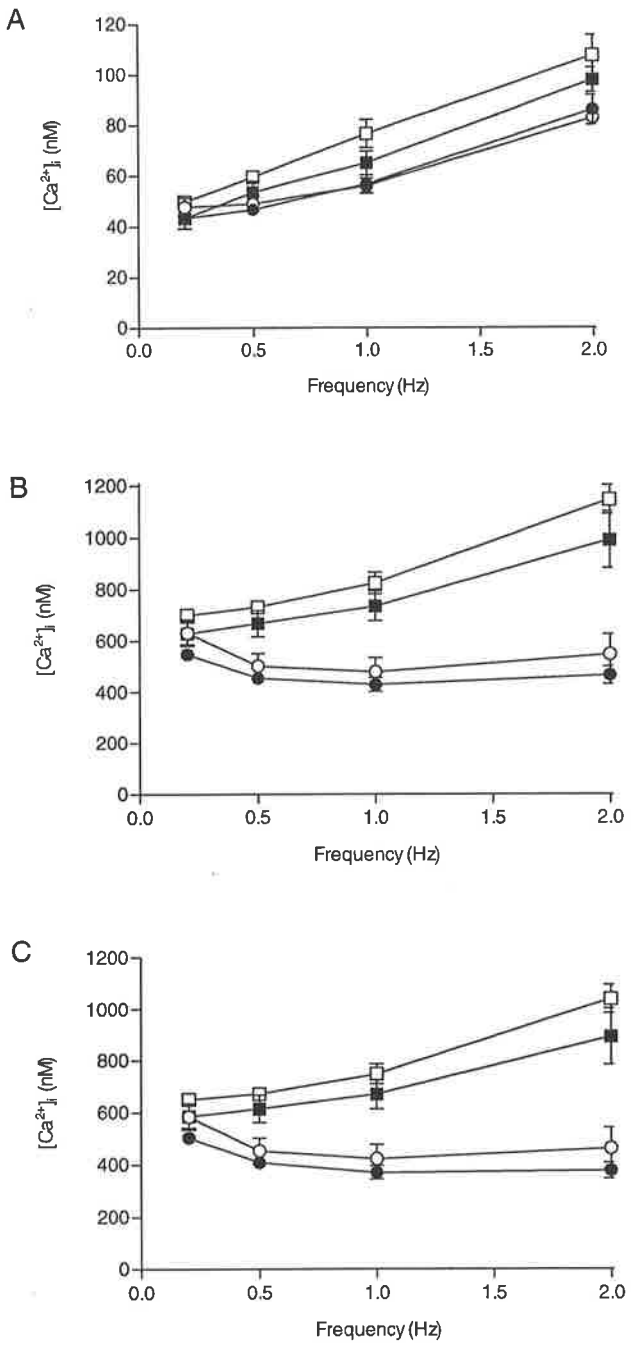


Figure 6.11 Effects of electrical stimulation frequency and isoproterenol on $[Ca^{2+}]_i$.
Ca^{2+} transients in isolated rat cardiomyocytes were measured using Fura-2 at stimulation frequencies of 0.2, 0.5, 1 and 2 Hz during control conditions comparing saturated fat, SF (○) and fish oil, FO (●) supplementation, and following 0.5 μM isoproterenol treatment, SF (□), FO (■). *A*, end-diastolic $[Ca^{2+}]_i$, *B*, systolic (peak) $[Ca^{2+}]_i$, *C*, developed $[Ca^{2+}]_i$ (systolic minus end diastolic). Data are means ± SEM for SF (n=8, 32) and FO (n=8, 31).

6.3.6.4 Effect of Bay K8644 on Ca^{2+} transients

The L-type Ca^{2+} channel opener, Bay K8644 (0.1 μM) was used to increase Ca^{2+} load on the cardiomyocytes since previous studies have demonstrated that the acute addition of n-3 PUFAs modulates the activity of L-type Ca^{2+} channels in the presence of the L-type Ca^{2+} channel activator, Bay K8644 (Pepe *et al.*, 1994). Figure 6.12A shows the effect of 0.1 μM Bay K8644 on Ca^{2+} transients at a stimulation frequency of 0.5 Hz. Figure 6.12B demonstrates the increase in both diastolic and systolic $[Ca^{2+}]_i$ in the presence of 0.1 μM Bay K8644. Bay K8644 increased the maximum amplitude (systolic) of Ca^{2+} transients as well as the resting (diastolic) $[Ca^{2+}]_i$ in cardiomyocytes isolated from both dietary groups. Figure 6.13 shows that there was no significant difference between the diastolic, systolic and developed $[Ca^{2+}]_i$ when comparing SF and FO groups over a range of stimulating frequencies in the presence of Bay K8644.

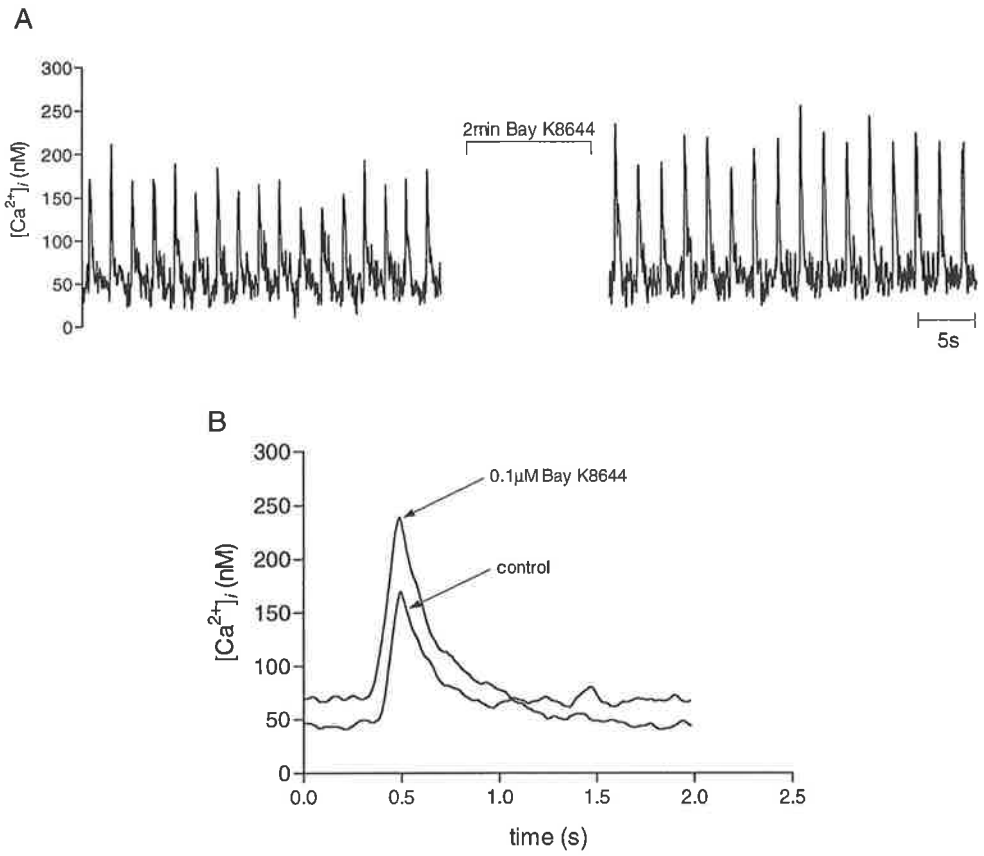


Figure 6.12 Effect of 0.1 μM Bay K8644 on Ca^{2+} transients.
A, Representative Ca^{2+} transients in an isolated rat cardiomyocyte. Steady-state Ca^{2+} transients were elicited before and after treatment with Bay K8644 for 2 min at a stimulation frequency of 0.5 Hz. *B*, representative ensemble average of the Ca^{2+} transients from (*A*), under control conditions or in the presence of 0.1 μM Bay K8644 as indicated.

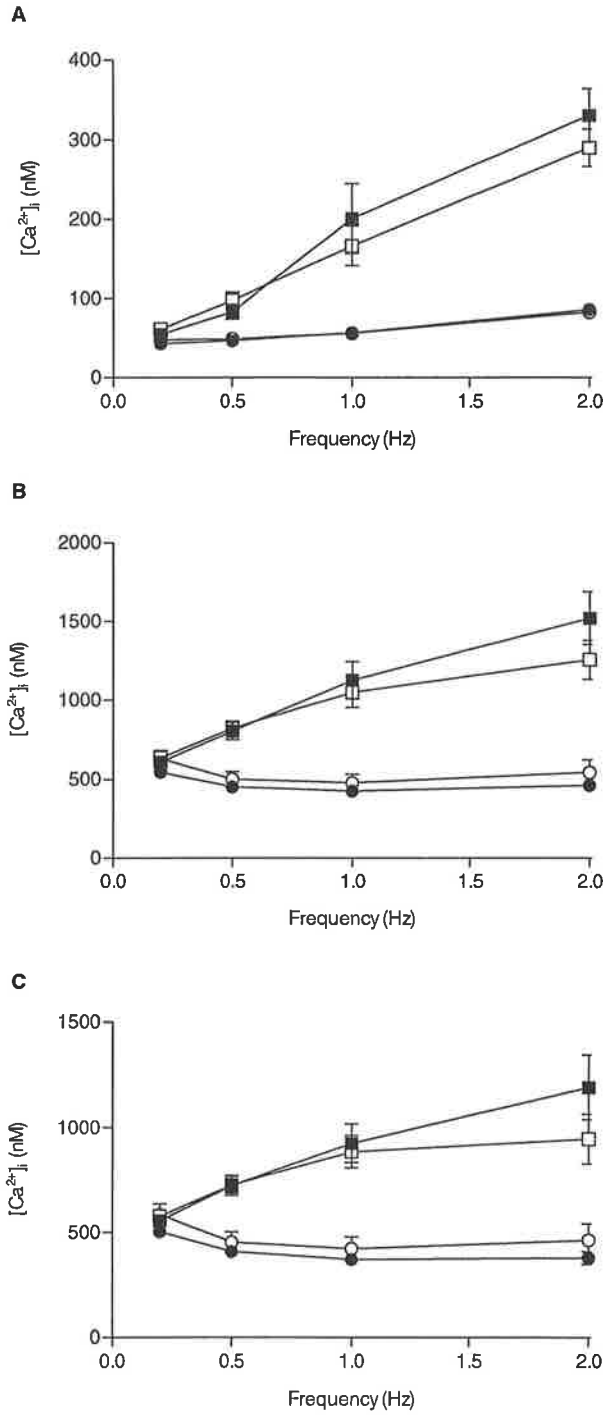


Figure 6.13 Effects of electrical stimulation frequency and Bay K8644 on $[Ca^{2+}]_i$.
Ca^{2+} transients in isolated rat cardiomyocytes were measured using Fura-2 at stimulation frequencies of 0.2, 0.5, 1 and 2 Hz during control conditions (taken from figure 6.11) comparing saturated fat, SF (○) and fish oil, FO (●) supplementation, and following 0.1μM Bay K8644 treatment, SF (□), FO (■). *A*, end-diastolic $[Ca^{2+}]_i$, *B*, systolic (peak) $[Ca^{2+}]_i$, *C*, developed $[Ca^{2+}]_i$ (systolic minus end diastolic). Data are means ± SEM for SF (n=8, 32) and FO (n=8, 31).

6.3.7 *Steady-state fluorescence anisotropy of rat cardiomyocytes.*

This study investigated whether the incorporation of n-3 PUFAs into membrane phospholipids by dietary fish oil supplementation (compared with saturated fat) influenced membrane fluidity as measured using the fluorescence anisotropy value of the reporter probe TMAP-DPH. This study involved supplementing FO in the diet (rather than by gavage) and supplementation of dietary lipid by this method appeared to be more efficient at incorporating n-3 PUFAs into the membrane phospholipids, as demonstrated in Table 6.2. Therefore, it was felt appropriate to investigate whether membrane fluidity was altered following incorporation of relatively higher proportions of n-3 PUFAs into the phospholipids via this dietary supplementation protocol.

The fluorescence anisotropy value (r_{ss}) of the FO cardiomyocytes (0.241 ± 0.003, n=8) was not significantly different from that obtained for SF cardiomyocytes (0.241 ± 0.005, n=6), indicating the membrane fluidity (as detected by TMAP-DPH) was unchanged by the nature of the dietary fat supplementation.

6.4 DISCUSSION

Laboratory-based studies on potential antiarrhythmic mechanisms have focussed on the action of free fatty acids and their acute effects on various trans-sarcolemmal ion currents. The predominant effects of the n-3 PUFAs appear to be one of dampening the electrical excitability of cardiac myocytes and preventing unwanted aberrant electrical disturbances (as discussed in Chapter 3) and Ca^{2+} sparks (Ca^{2+} sparks are thought to represent "elementary" quantal Ca^{2+} release events, which arise from one or more ryanodine receptor channels in the SR) leading to arrhythmic contractility (Hallaq *et al.*, 1992; Pepe *et al.*, 1994; Xiao *et al.*, 1995; Kang & Leaf, 1996b; Xiao *et al.*, 1997). In contrast, this study investigated the antiarrhythmic effects following dietary supplementation of the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by dietary fish oil (FO) supplementation for 3 weeks. Significant increases in the proportions of EPA and DHA in the ventricular phospholipids (which would represent changes in the cardiac membrane phospholipids), in comparison with that following dietary supplementation with saturated fat (SF) were observed. This was associated with a significant decrease in the number of cardiomyocytes contracting in an asynchronous manner in response to the β-adrenergic receptor agonist, isoproterenol. Dietary-induced changes in cardiomyocyte arrhythmia susceptibility to isoproterenol were not attributable to differences in the proportion of the total ventricular saturated fatty acids since these remained similar in both groups but did appear to be correlated with higher proportions of total n-3 PUFAs. The protective effect of FO supplementation was associated with an increase in the time constant for the decay of Ca^{2+} transients and a general lowering of diastolic $[Ca^{2+}]_i$ in the FO group, but was not associated with a change in the sarcolemmal membrane lipid fluidity as determined by the fluidity probe TMAP-DPH.

Prior to dietary lipid supplementation, the diet used in this study provided a relatively low, but not deficient level of 18:3 n-3 (0.68% of total fatty acids) in order to minimise the extent of conversion of 18:3 n-3 to the longer chain n-3 PUFAs, particularly 22:6 n-3. The proportion of total n-3 PUFAs (including 22:6 n-3) in the phospholipid fraction was substantially increased following three weeks of dietary lipid supplementation with an n-3 PUFA enriched fish oil diet (mainly enriched with 20:5 n-3). These changes in membrane membrane lipid composition may influence cardiomyocyte contractile activity in terms of susceptibility to the development of asynchronous contractions, with higher proportions of 22:6 n-3 possibly underlying the antiarrhythmic effects, and the higher proportion of 20:4 n-6 underlying the proarrhythmic effects via the production of pro- and antiarrhythmic prostanoids and thromboxanes (Abeywardena & Charnock, 1995; Oudot *et al.*, 1995) as discussed in Chapter 1.

6.4.1 Cellular Ca^{2+}

It has been reported that in guinea-pig cardiomyocytes an increase in SR Ca^{2+} content results in arrhythmias (aftercontractions) (Tweedie *et al.*, 2000) and the primary trigger for spontaneous Ca^{2+} release from the SR may be raised cytoplasmic $[Ca^{2+}]_i$ (Egdell *et al.*, 2000). The SR has been reported to account for approximately 87% of Ca^{2+} removal from the cytoplasm during electrically-driven contractions in rat cardiomyocytes (Negretti *et al.*, 1993b). Therefore, it was plausible to hypothesise that animals on a diet that is potentially "cardioprotective" (antiarrhythmic) would have a decreased level of Ca^{2+} in their SR stores and/or a lower diastolic $[Ca^{2+}]_i$. However, using Fura-2, this study showed that the SR Ca^{2+} content was similar in cardiomyocytes isolated from both the FO and SF dietary groups indicating that there must be an alternative explanation to reconcile the antiarrhythmic properties attributable to the FO diet. Although the diastolic $[Ca^{2+}]_i$ was

always found to be lower under the conditions tested, but this did not reach statistical significance.

When dietary n-3 PUFAs were incorporated into ventricular cardiac membrane phospholipids, the steady-state cell contraction amplitude (and post rest potentiation) were similar when comparisons were made between cardiomyocytes isolated from rats from the SF and FO dietary groups (as shown in Chapter 5). The above result contrasts to that obtained by others (Macleod *et al.*, 1998; Negretti *et al.*, 2000) when n-3 PUFAs are added acutely to isolated cardiomyocytes where a rapid, negative inotropic effect is accompanied by a decrease in amplitude of Ca^{2+} transients. The acute effect of n-3 PUFAs is believed to be related to inhibition of the sarcolemmal L-type Ca^{2+} current (Pepe *et al.*, 1994; Xiao *et al.*, 1997) as well as to direct effects of n-3 PUFAs on the Ca^{2+} content of the SR via SR uptake and SR release processes (Negretti & O'Neill, 1997; Rodrigo *et al.*, 1999; Negretti *et al.*, 2000). In contrast, arachidonic acid (AA) has been shown to induce a doubling in the amplitude of Ca^{2+} transients and lead to a positive inotropic effect (two-fold increase in cell shortening) in adult rat ventricular myocytes (Damron & Summers, 1997). Liberation of AA in the heart may also lead to the synthesis of pro-arrhythmic metabolites including the 2-series prostanoids and thromboxane A_2. These metabolites of AA have been shown to induce pro-arrhythmic effects in neonatal rat cardiomyocytes (Li *et al.*, 1997) which may be partly due to their ability to disrupt the normal intracellular Ca^{2+} handling process.

Although this study demonstrated that the resting cell $[Ca^{2+}]_i$ was slightly higher in the SF group compared with the FO dietary group, it is possible that this slightly higher $[Ca^{2+}]_i$ may result from an increased propensity for Ca^{2+} sparks in cells isolated from the SF

group. This could predispose cells to increased arrhythmic contractile activity in the presence of isoproterenol. The slower rate of Ca^{2+} extrusion from the cytoplasm in cells from the FO group at low stimulation rates, as evident from the greater time constant (τ), appeared independent of the SR Ca^{2+} ATPase pump activity since, in the presence of DBHQ, similar values for the DBHQ-sensitive Ca^{2+} transient amplitudes were observed between the two dietary groups. This result suggests that the differences observed in the extrusion of Ca^{2+} from the cytoplasm may involve sarcolemmal Ca^{2+} transporters, in particular the Na^{+}-Ca^{2+} exchanger which accounts for approximately 67% of the cytoplasmic Ca^{2+} removal not mediated by the SR (Bassani *et al.*, 1992; Negretti *et al.*, 1993b; Bassani *et al.*, 1994). The resultant effect of increasing the time constant of Ca^{2+} transient decay would likely change the shape of the action potential prolonging the refractory period and/or decreasing the plateau potential (as shown previously following acute addition of DHA to neonatal cardiomyocytes, (Fournier *et al.*, 1995)). Indeed, some clinically relevant antiarrhythmic drugs such as amiodarone exert their antiarrhythmic effects by prolonging the action potential duration (Bosch *et al.*, 1999).

6.4.2 Ca^{2+} and depolarisation

Direct measurements with intracellular electrodes in single myocytes undergoing spontaneous Ca^{2+} release have shown that each spontaneous contraction is accompanied by a small depolarisation, rather than being triggered by an action potential alone. Under conditions where there are multiple sites of elevated Ca^{2+} within a cell at a given time, summation of these small depolarisations may be sufficient to exceed the threshold to induce an action potential (Priori & Corr, 1990; Williams, 1993). Therefore, future studies investigating the effects of dietary fish oil on the number and duration of Ca^{2+} sparks under basal conditions may prove to greatly aid the understanding of whether

dietary n-3 PUFAs regulate the SR's ability to control Ca^{2+} flux. Although Ca^{2+} release from the SR provides the main source of intracellular Ca^{2+} for cardiac contractility, Ca^{2+} influx through the sarcolemma may also contribute to activation of cell shortening. Recently, studies in isolated ventricular myocytes have provided evidence for a second mechanism of excitation-contraction coupling (Mason & Ferrier, 1999; Piacentino, III *et al.*, 2000). This mechanism is believed to be a voltage-sensitive release mechanism (VSRM) which couples sarcolemmal depolarisation directly to SR Ca^{2+} release. For example, the local anaesthetic tetracaine, was reported to preferentially inhibit cardiomyocyte contractions by the VSRM in contrast to the calcium-induced-calcium release (CICR) mechanism(s). It is feasible that in the studies presented here, dietary esterification of n-3 PUFAs into membrane phospholipids, may alter $[Ca^{2+}]_i$ by processes similar to those involved in the VSRM, since acute n-3 PUFAs (Chapter 3) or dietary fish oil (Chapter 5) altered the voltage dependence of Na^+ channels and would therefore alter characteristics of membrane depolarisation and membrane potential. Furthermore, isoproterenol was previously shown to shift the voltage-dependence of the L-type Ca^{2+} channel activation to more negative potentials resulting in an increased inward current. It is likely that this effect of isoproterenol could have partly contributed to the susceptibility of the SF cardiomyocytes to Ca^{2+} overload and to the generation of subsequent asynchronous contractile activity. Therefore, the antiarrhythmic action of the n-3 PUFAs may be partly related to their ability to prevent this shift in the voltage-dependence of the L-type Ca^{2+} channel activation induced by isoproterenol, however this was not tested in the present study. A recent report demonstrated that acute addition of EPA to cardiomyocytes resulted in a negative inotropic effect induced by a lowering of the availability of Ca^{2+} for uptake and by inhibiting the SR Ca^{2+}-release mechanism (Negretti *et al.*, 2000). In combination, these effects would likely lower the frequency of

spontaneous Ca^{2+} sparks and waves of Ca^{2+} release, thus reducing asynchronous (arrhythmic) contractile activity.

6.4.3 β-adrenergic stimulation

It is known that myocardial ischaemia evokes an excessive release of norepinephrine in the ischaemic region of the myocardium (Akiyama & Yamazaki, 2001). The effects of β-adrenergic receptor stimulation by isoproterenol are known to influence myocardial contractility via adenylate cyclase activation and elevated cAMP production resulting in increased sarcolemmal Ca^{2+} currents, reduced myofilament Ca^{2+} sensitivity and enhanced SR Ca^{2+} uptake. Collectively, these effects result in an earlier and larger peak of contraction associated with more rapid phases of contraction and relaxation and this was also observed in this study with regard to the Ca^{2+} transients following isoproterenol treatment. Increasing concentrations of isoproterenol induced a steady increase in the percentage of cells undergoing asynchronous contractile activity and such an effect may be related to Ca^{2+} overload (Marban *et al.*, 1986; Williams, 1993; Delbridge *et al.*, 1996b; Tamada *et al.*, 1998). At the cellular level this form of aberrant contractile behaviour in part reflects the behaviour of the whole myocardium to arrhythmogenic stressors associated with ischaemic arrhythmias, excessive β-adrenergic receptor activation and/or calcium overload.

One possible antiarrhythmic mechanism by which the incorporated n-3 PUFAs could manifest their effects, could relate to an altered membrane lipid environment. The n-3 PUFA induced changes in membrane-composition are likely to alter membrane physical properties, which, in turn could affect many membrane-bound enzyme activities (McMurchie, 1988; Nalbone *et al.*, 1990; Fournier *et al.*, 1995; de Jonge *et al.*, 1996).

Collectively, this could reduce sensitivity and response of the β-adrenergic receptor signalling pathway (Grynberg *et al.*, 1996; Reithmann *et al.*, 1996).

This study demonstrated that membrane fluidity as measured using the probe TMAP-DPH was not significantly changed by dietary incorporation of n-3 PUFAs into ventricular phospholipids, which reflects the fatty acid composition of the many membranes of the cardiomyocytes (a situation opposite to that seen following acute addition of n-3 PUFAs). However, others have reported a decrease in membrane fluidity with a concomitant rise in membrane cholesterol (and are known to "rigidify" the membranes) levels in neonatal cardiomyocytes cultured in an EPA-enriched culture medium (de Jonge *et al.*, 1996). It is possible that changes in membrane fluidity were not detected due to the nature of the probe used as discussed earlier. TMAP-DPH inserts into the polar head group of the bilayer (Lentz, 1989). Although changes in fluidity were not detected in this membrane region in the dietary study, other probes that are known to penetrate deeper in the lipid bilayer, including diphenylhexatriene, may have provided useful information on fluidity within these more non-polar regions. The results of the present study contrast with the earlier studies in which the addition of free, n-3 PUFAs to cardiomyocytes increased membrane fluidity (see Chapter 2 and 3). This suggests that the free form of n-3 fatty acids, as opposed to esterified n-3 PUFAs associated with membrane phospholipids, are able to perturbate the lipid membrane bilayer within the polar head group region, resulting in membrane fluidity increases and ion channel blockade.

6.4.4 Conclusions

In conclusion, a possible mechanism of action pertaining to the antiarrhythmic effects of the n-3 PUFAs may relate to the fact that n-3 PUFAs when incorporated into the membrane phospholipids following short-term dietary administration modulate Ca^{2+} transient activity and Ca^{2+} decay rates. The n-3 PUFAs incorporated into membrane phospholipids following dietary n-3 PUFA supplementation may also act as a pool for the generation of free n-3 PUFAs which are released by the action of phospholipases which in turn are activated during ischaemia or in other circumstances (Nair *et al.*, 1997). These free n-3 PUFAs would then be able to undergo metabolic alterations to produce antiarrhythmic eicosanoids and prostaglandins (Abeywardena & Charnock, 1995) and/or to act directly on various ion channels and membrane-associated enzyme systems at the cell surface as well as at the SR. Of particular interest is the possibility that the antiarrhythmic n-3 PUFAs influence the activity and functioning of the β-adrenergic receptor, G-protein transducers and the adenylate cyclase complex in the modulation of the excitation-coupling processes of the myocardium. It is therefore likely that membrane incorporated n-3 PUFAs exert multiple effects on the cardiac myocardium and act synergystically to elicit antiarrhythmic and cardioprotective effects at a number of functionally important sites.

CHAPTER 7

7 "Effects of dietary lipid supplements on cardiac phospholipid species and non-esterified fatty acids"

7.1 INTRODUCTION

It is likely that the n-3 PUFAs exhibit their antiarrhythmic effects by first being incorporated into cardiac cell membrane lipids (Abeywardena & Charnock, 1995; Li *et al.*, 1997). Previous studies using cultured neonatal cardiomyocytes reported that acute addition of n-3 PUFAs prevented or terminated arrhythmic activity by slowing the rate of spontaneous contractions (Kang & Leaf, 1996d). Such a positive effect of n-3 PUFAs was also shown in surgically-prepared ischaemia-susceptible dogs (Billman *et al.*, 1994; Billman *et al.*, 1997). Studies by this group concluded that there was no antiarrhythmic effect due to an increased fraction of EPA and DHA in membrane phospholipids, rather, it was the unesterified PUFAs that were able to protect cultured neonatal cardiomyocytes from the development of arrhythmias (Weylandt *et al.*, 1996). However, that study was carried out in the presence of a single high concentration of isoproterenol, and data were not presented in relation to possible changes in the isoproterenol dose-response curves as a function of PUFA treatment. Therefore, if lower concentrations of isoproterenol had been used in that study, it may have been possible to show that the incorporated (esterified) n-3 PUFAs exhibit antiarrhythmic effects in neonatal cardiomyocytes, as shown in Chapter 4 in this thesis using electrically field stimulated adult rat cardiomyocytes.

A recent study using pigs fed fish oil for 6 weeks demonstrated that under normoxic conditions the fish oil group had significantly higher concentrations of the n-3 non-esterified fatty acids (NEFAs), EPA, DPA and DHA in the myocardial tissue compared with saturated fat supplemented pigs (Nair *et al.*, 1999). Furthermore, the concentration of AA in the NEFA (as well as the myocardial phospholipid fraction) was significantly lower in the fish oil group compared with the saturated fat group. In an earlier study by Charnock (Charnock *et al.*, 1992), rats supplemented for 11 months with fish oil (from weaning) exhibited an increase in the DHA content of the total cardiac phospholipids as well as a small (but significant) increase in the DHA of the NEFA fraction.

7.1.1 This study

This study aimed to determine whether 3 weeks of dietary fish oil supplementation to rats was sufficient to increase n-3 PUFAs in the NEFA fraction of the myocardium and to determine the extent to which the different classes of phospholipids exhibited changes in their proportions of n-3 PUFAs. Therefore, it would be of considerable interest to understand whether dietary supplementation of fish oil to rats (as done in the studies presented in Chapters 4-6) elevated the free n-3 fatty acid concentrations to levels similar to those that were used in the acute studies. Three different dietary lipid supplements were chosen, representing a diet high in mainly saturated fat (SF), monounsaturated fat (control; CON) or polyunsaturated fat (mainly as fish oil n-3 PUFAs; FO). The effects of the different dietary lipid supplements on both the amount and fatty acid composition of the myocardial NEFAs were compared with concurrent changes in the fatty acid composition of their total cardiac phospholipids as well as the major phospholipid classes.

7.2 METHODS

7.2.1 Diets and animals

The use of all animals used in this study was approved by the CSIRO Health Sciences and Nutrition Animals Ethics and Experimentation Committee. Male Sprague Dawley rats were obtained from the Adelaide University Central Animal House (Adelaide, South Australia). The animals were housed in the CSIRO small animal colony in wire cages at a room temperature of 23°C with constant humidity (55%). The animals were on a 12-light/dark cycle and were allowed free access to the diet, and water was provided *ad libitum*. These rats were maintained on the prepared diet from 4 weeks of age and for the duration of the experiment.

The diets were prepared using the diet making facility at the CSIRO Health Sciences and Nutrition. The standard fabricated diet was administered from weaning to 9 weeks of age and contained 7% (w/w) sunola oil (high oleic acid oil) as the lipid source. Further details of the diet components and diet preparation are described in Chapter 5 - Study 2. However, in this dietary study, rats between 9-10 weeks of age were placed in separate cages and supplemeted with a fabricated diet containing an additional 10% of either sunola oil (control, CON), saturated fat (SF) or fish oil (FO) for 3 weeks.

7.2.2 Heart preparation for lipid analysis

All hearts were treated with collagenase and protease (as per the method for preparation of cardiomyocytes) and subsequently washed with Tyrode solution to remove excess

protein. Atrial and extracardiac tissue was discarded, leaving the ventricular tissue for analysis. Isolated ventricular myocardium was blotted and weighed.

7.2.3 Extraction of total lipids

Total lipids were extracted from approximately 1 g ventricular tissue from each animal and from the standard colony diet and the dietary oils using a slight modification of the method of Bligh and Dyer (Bligh & Dyer, 1959). For analysis of ventricular lipid composition, the partially digested tissue was homogenised in 1 ml of water using a Tenbröeck hand-held homogeniser. Eight millilitres of 2-propanol was added and the mixture boiled for 30 s. After cooling, 16 ml of chloroform was added, the mixture shaken, and the organic phase collected. The organic phase was evaporated to dryness under N_2. The phospholipids were separated from the other lipid classes by thin layer chromatography (TLC) on silica gel 150A-LK5D plates (Whatman, Clifton, NJ, USA), and developed in a solvent system of petroleum ether:acetone (3:1 [v/v]). The phospholipids remaining at the origin were scraped from the plate.

7.2.4 Phospholipid class analysis

Phospholipid classes were separated using TLC by the following method. Total lipid extracts (approximately 100 mg/ml; mg total lipid/ml $CHCl_3$) were spotted (20 µl) alongside a standard mix, which contained 10 mg/ml of the following; sphingomyelin (SPM), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and cardiolipin (CLP, also termed diphosphatidylglycerol). Individual phospholipid classes were separated on Whatman Linear LK5D Silica Gel plates (Whatman Inc, Clifton, NJ, USA) in a polar solvent comprising chloroform /

methanol / isopropanol / water / triethylamine (30:9:25:7:25, by volume) as described previously (Touchstone *et al.*, 1979). The phospholipid classes were identified by co-migration with known standards on the same plate and visualised by spraying with fluorescein. Sample bands were scraped from the silica gel plate and transferred to glass (kimble) tubes.

7.2.5 Non-esterified fatty acids (NEFAs)

Extraction of NEFAs was performed using Alltech Ultra-Clean solid phase extraction disposable cartridges (Alltech Associates Pty. Ltd., Baulkham Hills, NSW, Australia) as described previously (Kim & Salem, 1990). The cartridge column was equilibrated by rinsing with 4 ml hexane. All solutions were added to the top of the cartridge column and forced through under pressure using a stream of nitrogen, which resulted in the eluate flowing at a rate of 3-5 ml/min. 100 µl of total lipid extract (containing 10 mg lipid) was loaded onto the column and forced through as described above. Thereafter, the column was eluted with 4 ml of chloroform:isopropanol (2:1 v/v) to remove neutral lipids. Then, 4 ml of diethyl ether:acetic acid (100:2 v/v) was added and the eluate collected for analysis of NEFAs. The ether fraction was then washed with 2 ml of water to remove excess acetic acid and ether peroxides so that possible decomposition of NEFAs was prevented (Kim & Salem, 1990). The ether fraction was then dried under nitrogen for FAME analysis.

7.2.6 *Preparation of fatty acid methyl esters for GC analyses*

To each isolated class of lipid, a known amount (1-10 μg) of heptadecanoic acid (17:0) was added as an internal standard prior to methylation, to permit quantitation of the lipid classes (Charnock *et al.*, 1992; Kramer *et al.*, 1998; Nair *et al.*, 1999;). Phospholipid fatty acid methyl esters (FAMEs) were prepared as described previously (McMurchie *et al.*, 1983) by heating the samples (total phospholipids, phospholipid classes or non-esterified fatty acids) at 50°C overnight in 2ml 1% (v/v) H_2SO_4 in dried methanol. 200 μl water was added and FAMEs were extracted using hexane. Contaminants were removed using a Florisil column (activated magnesium silicate). All solvents used for lipid extraction, TLC and preparation of FAMEs contained the antioxidant butylated hydroxytoluene (0.05% w/v). FAMEs from lipid extracts of the lipid supplemented diets, and the myocardial phospholipid extracts, were analysed by GLC. GLC was performed using a Hewlett Packard HP 5710 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) fitted with a 50 m BPX70 capillary column (Scientific Glass Engineering, Melbourne, Victoria, 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/min. A cold on-column injector was used with the flame ionisation detector temperature set at 250°C. FAMEs were identified using authentic lipid standards (Nu-Chek-Prep Inc., Elysian, MN, USA) by GLC and using Delta Chromatography software (Delta chromatography data systems version 5.0, DataworX Pty. Ltd., Scarborough, QLD, Australia). The proportions of the total fatty acids were normalised to a value of 100%.

7.3 RESULTS

7.3.1 Fatty acid composition of diets

The fatty acid composition of the lipid-supplemented diets is shown in Table 7.1. The FO diet contained approximately 29% total n-3 polyunsaturated fatty acids (PUFAs) whilst the CON and SF dietary supplements contained approximately 0.5-1.4% n-3 PUFAs, respectively. The FO diet also contained comparatively less saturated fat and monounsaturated fat. The CON diet contained mainly oleic acid (18:1 n-9) and linoleic acid (18:2 n-6), whilst the SF diet contained mainly palmitic acid (16:0), stearic acid (18:0) and oleic acid.

Table 7.1 Fatty acid composition (wt%) of lipid supplemented diets

Major FAME[a]	CON	SF	FO
14:0	0.1	4.3	4.8
16:0	4.0	19.9	10.5
16:1	0.1	1.1	6.7
18:0	3.5	10.6	2.6
18:1[b]	79.5	53.1	36.7
18:2 (n-6)	11.0	7.1	6.6
18:3 (n-3)	0.5	1.2	0.9
20:4 (n-6)	n/d[c]	n/d[c]	1.0
20:5 (n-3)	n/d[c]	n/d[c]	17.8
22:5 (n-3)	n/d[c]	n/d[c]	1.7
22:6 (n-3)	n/d[c]	n/d[c]	8.9
Σ Sat.	8.7	36.4	18.6
Σ Mono.	79.8	55.1	44.0
Σ Poly.	11.5	8.5	37.4
Σ n-6	11.0	7.1	7.8
Σ n-3	0.5	1.4	29.4
n-6/n-3	22.5	5.2	0.3

Two samples per dietary group were analysed and the mean of data is shown. Computational parameters (Σ and n-6/n-3) were derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. [c]n/d, not detected. Abbreviations, control (CON), saturated fat (SF), fish oil (FO).

7.3.2 *Total cardiac lipids*

The total lipid/heart weight (wet wt) ratio was 40.1 ± 3.2 mg/g, 39.8 ± 2.1 mg/g and 35.6 ± 2.5 mg/g for CON, SF and FO, respectively (not significantly different). These values compare well to values previously reported in pig hearts of 32-37 mg lipid/g wet heart (Nair *et al.*, 1999).

7.3.3 *Total cardiac phospholipids*

Following 3 weeks of dietary lipid supplementation, significant changes were evident in the cardiac total phospholipid fatty acid profile (Table 7.2). In comparison with the control (CON) and saturated fat (SF) treatment, dietary fish oil (FO) resulted in an increase in the total n-3 PUFAs as a result of a significant increase in the proportions of docosahexaenoic acid (22:6, n-3), docosapentaenoic acid (22:5, n-3) and eicosapentaenoic acid (20:5, n-3). Concomitantly, the proportion of the total n-6 PUFAs in the ventricular tissue from the FO supplemented rats was reduced as a result of the decrease in the proportions of arachidonic acid (20:4, n-6) and linoleic acid (18:2, n-6). Collectively, this resulted in a significant reduction in the n-6/n-3 PUFA ratio in the FO fed rats. The overall proportion of saturated fatty acids was not significantly influenced by the nature of the dietary lipid supplement (34 - 35% in all groups). The other significant change of note in the FO supplemented rats was the lowering of the proportion of total monounsaturated fatty acids due mainly to the lowered proportion of oleic acid (18:1) in the ventricular total phospholipid fatty acids. However, in all dietary groups, α-linolenic acid (18:3, n-3) was similar in the cardiac phospholipids (at the limit of detection, approximately 0.04%).

Table 7.2 Fatty acid composition (wt %) of the total ventricular phospholipids after dietary lipid supplementation

Major FAME[a]	CON (n = 6)	SF (n = 8)	FO (n = 8)
14:0	0.08 ± 0.01	0.14 ± 0.02	0.12 ± 0.03
16:0	7.44 ± 1.27	8.37 ± 1.22	9.82 ± 0.54
16:1	0.11 ± 0.03	0.12 ± 0.03	0.31 ± 0.06[i,f]
18:0	25.42 ± 0.17	25.29 ± 0.39	23.33 ± 0.24[h,k]
18:1[b]	14.46 ± 1.09	12.73 ± 0.35	10.31 ± 0.26[h,i]
18:2 (n-6)	11.19 ± 0.59	10.33 ± 0.79	5.85 ± 0.37[h,k]
18:3 (n-3)	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
20:0	0.18 ± 0.04	0.14 ± 0.04	0.18 ± 0.04
20:1	0.15 ± 0.01	0.10 ± 0.01	0.24 ± 0.02[g,k]
20:2	0.35 ± 0.02	0.78 ± 0.03[e]	0.18 ± 0.03[g,k]
20:3 (n-6)	0.29 ± 0.01	0.49 ± 0.03[e]	0.37 ± 0.02[j]
20:4 (n-6)	30.50 ± 1.74	29.30 ± 0.88	17.54 ± 0.24[h,k]
22:0	0.30 ± 0.16	0.34 ± 0.02	0.18 ± 0.07
22:1	n/d	n/d	0.38 ± 0.02
20:5 (n-3)	0.05 ± 0.01	0.076 ± 0.004	2.79 ± 0.14[h,k]
24:0	1.09 ± 0.09	0.87 ± 0.06[c]	0.12 ± 0.01[h,k]
24:1	0.07 ± 0.01	0.10 ± 0.01	0.10 ± 0.04
22:5 (n-3)	0.28 ± 0.05	0.95 ± 0.15[d]	2.89 ± 0.12[h,k]
22:6 (n-3)	8.12 ± 0.77	10.12 ± 0.96	25.11 ± 0.73[h,k]
Σ Sat.	34.47 ± 0.99	35.31 ± 1.28	34.07 ± 0.52
Σ Mono.	14.72 ± 1.09	13.01 ± 0.35	11.14 ± 0.26[g]
Σ Poly.	50.81 ± 2.00	51.67 ± 1.58	54.78 ± 0.75
Σ n-6	41.98 ± 1.35	40.13 ± 0.76	23.77 ± 0.32[h,k]
Σ n-3	8.48 ± 0.73	11.16 ± 1.00	30.84 ± 0.93[h,k]
n-6/n-3	4.95 ± 0.31	3.76 ± 0.27[d]	0.78 ± 0.03[h,k]

Data shown are mean ± SEM for 6 - 8 animals per dietary group. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. n/d, not detected. Superscripts indicate significant differences at [c]P<0.05, [d]P<0.01, [e]P<0.001 for CON vs SF; [f]P<0.05, [g]P<0.01, [h]P<0.001 for CON vs FO; [i]P<0.05, [j]P<0.01, [k]P<0.001 for SF vs FO (by ANOVA). CON; control group, SF; saturated fat group, FO; fish oil group.

7.3.4 *Cardiac phospholipid classes*

Comparison of the major phospholipid classes of rat myocardium shows the major phospholipid present was phosphatidylcholine (PC) representing approximately 44% of all phospholipids analysed (Table 7.3). The second most abundant phospholipid species was phosphatidylethanolamine (PE, 32%), followed by cardiolipin (CLP, 17%). Phosphatidylinositol (PI) and phosphatidylserine (PS) which were pooled (as the resolution of these two phospholipids was incomplete in the TLC system chosen), collectively contributed approximately 5%, whilst lysophosphatidylcholine (LPC) and sphingomyelin (SPM) were approximately 1.4% and 0.55%, respectively. There was no significant difference due to the different lipid supplements on the relative amounts of any of the phospholipid classes analysed (see Table 7.3). The relative amounts of the phospholipid classes PC, PE and CLP are similar to that reported previously (Lamers *et al.*, 1992; Kramer *et al.*, 1998), with PI + PS and SPM being slightly lower than that found in pig heart and cultured neonatal rat ventricular cardiomyocytes possibly representing species and/or developmental differences .

Table 7.3 Phospholipid class proportions (wt %) from rat myocardium

Phospholipid class	**CON** (n=6)	**SF** (n=8)	**FO** (n=8)
SPM	0.57 ± 0.06	0.62 ± 0.10	0.45 ± 0.04
LPC	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
PC	45.0 ± 1.5	42.1 ± 1.4	43.2 ± 1.1
PI + PS	5.1 ± 0.3	5.2 ± 0.3	5.0 ± 0.2
PE	30.7 ± 1.2	32.2 ± 1.0	32.0 ± 1.1
CLP	17.0 ± 0.4	18.5 ± 0.9	17.8 ± 0.5

Data shown are mean ± SEM for 6 - 8 animals per dietary group. Class proportions were derived from comparing the full fatty acid set against an internal standard. There was no significant difference between the dietary groups. Abbreviations: CON; control group, SF; saturated fat group, FO; fish oil group, SPM; sphingomyelin, LPC; lysophosphatidylcholine, PC; phosphatidylcholine, PI; phosphatidylinositol, PS; phosphatidylserine, PE; phosphatidylethanolamine, CLP; cardiolipin.

7.3.5 Fatty acid proportions in cardiac phospholipid classes

Dietary supplementation of FO for 3 weeks resulted in significant incorporation of DHA into the PE fraction such that it represented 39% of total fatty acids (compared with approximately 16% in both the CON and SF groups, $P<0.001$). Furthermore, the significantly lower proportions of AA in the FO group compared to CON and SF ($P<0.001$) were also evident as shown in Table 7.4. The highest proportions of DHA was found in the PE fraction of all dietary groups as compared with all other phospholipid species examined (although the FO group had the highest proportion of DHA (39.1%) compared with the REF (15.6%) and SF (16.6%) groups). For example, Table 7.5 shows that the percentage of DHA in the PC fraction was lower than the PE fraction for all dietary groups (approximately 14% DHA from the FO group and only 2.7% and 3.8% in the REF and SF groups, respectively). Therefore, it appears that the majority of DHA is preferentially incorporated into PE phospholipids, whilst AA was preferentially incorporated into PC phospholipids (32%, 31% and 25% for CON, SF and FO, respectively). The large proportion of DHA present in the PE phospholipids from the FO group resulted in a significant increase ($P<0.01$) in the total proportion of polyunsaturated fatty acids and a concomitant (significant) decrease in saturated fatty acids ($P<0.001$) and total n-6 PUFAs ($P<0.001$), as shown in Table 7.4. Furthermore, of all the fatty acids in the PE fraction, 44.6% of the total was due to the incorporation of the n-3 PUFAs, with PUFAs of the n-3 and n-6 classes totalling 56.9%. Therefore, n-3 PUFAs contributed 78% of all PUFAs in the PE phospholipid fraction. Table 7.5 demonstrates that there was no significant difference between all saturated fatty acids between the three dietary groups (42-43%) in the PC fraction.

The CLP fatty acid profile was quite distinct from that of the PE and PC fractions as shown in Table 7.6. Interestingly, DHA was significantly higher ($P<0.001$) in the CLP fraction from the FO group (22.4%) compared with the CON (3.1%) and SF (4.0%) groups, however, there was no significant difference in the proportions of AA between the three groups (all approximately 5%), as shown in Table 7.6. This also demonstrates that AA is not preferentially located in the CLP fraction as the proportion of this PUFA was low in all dietary groups. Nevertheless, this was offset by the very high proportions of OA; 18:1, n-9 and LA; 18:2, n-6 present in the CLP fraction, with OA being significantly higher, and LA being significantly lower in the FO group, in comparison to the REF and SF dietary groups.

The PI and PS phospholipid fraction was unique in that SA (18:0) and AA contributed more than 55% of the total fatty acids in all dietary groups (although the FO group had significantly lower AA) as shown in Table 7.7. The lower proportion of AA in the FO group was accompanied by significantly higher proportions of DHA ($P<0.001$) and total PUFAs ($P<0.001$) compared with the CON and SF groups.

Although the SPM phospholipid class represented only approximately 0.5% of all phospholipids examined (Table 7.3), the fatty acid profile of this lipid class was quite different to the other classes examined, particularly with regard to the proportion of DHA (Table 7.8). The percentage of DHA in the SPM phospholipid species was only 3.0%, 5.6% and 6.6% for CON, SF and FO, respectively, and these differences were not significantly different. Furthermore, the AA proportions were also not significantly different between the three dietary groups and were very much lower than the proportions

determined in the PE, PC and PI+PS classes. Additionally, there was no significant difference between the three dietary groups in either the total saturated fatty acids, total monounsaturated fatty acids, total PUFAs or total n-6 PUFAs in the SPM fraction. Although the proportion of total n-3 PUFAs was significantly higher in the FO group compared with the CON and SF groups, this was only due to a small but significant increase in the proportion of EPA (Table 7.8). Furthermore, EPA was significantly higher in the FO group compared with the CON and SF groups in all phospholipid fractions examined except the LPC fraction (Table 7.9). However, DHA was significantly higher in the FO group of the LPC fraction (2.4%) compared with the CON (0.6%) and SF (1.4%) groups, but the relative percentages were very much lower compared with all other phospholipid fractions examined. Indeed, the LPC fraction contained relatively less PUFAs and monounsaturated fatty acids with very high levels of total saturated fatty acids (84% and 77% in the CON and SF groups, and 75% in the FO group) with the majority of the saturated fatty acids being docosanoic acid (22:0). In the LPC fraction, the other major alteration of fatty acids by FO treatment was a significant increase in monounsaturated fatty acids ($P<0.01$), in particular nervonic acid (24:1, cis-Δ15-tetracosenoic acid).

7.3.6 Cardiac non-esterified fatty acids (NEFAs)

The total NEFA component was 102 ± 18 nmol/g heart, 141 ± 18 nmol/g heart and 162 ± 24 nmol/g heart, for CON, SF and FO, respectively (not significantly different) and was lower than that reported in pigs (3500 nmol/g heart) (Nair *et al.*, 1999) and 12 month old rats (513 nmol/g heart) (Charnock *et al.*, 1992). In this study, all samples were treated in an identical manner and prepared with the same solvents in order to minimise

methodological errors. Although the method of analysis used in this study permitted the values for each individual fatty acid in the NEFA fraction to be given in absolute terms (nmol/g heart), for comparative purposes the relative proportions (%) are given in Table 7.10. The absolute values (in nmol/g heart, wet wt) for the complete set of fatty acids analysed is shown in Appendix 1.

Of particular interest regarding the NEFA comparisons between the dietary groups is that of all the NEFAs examined, it was only the n-3 PUFAs, EPA, DPA and DHA that were significantly (P<0.01 to P<0.001) increased in the FO group compared with CON and SF dietary groups as shown in Figure 7.1.

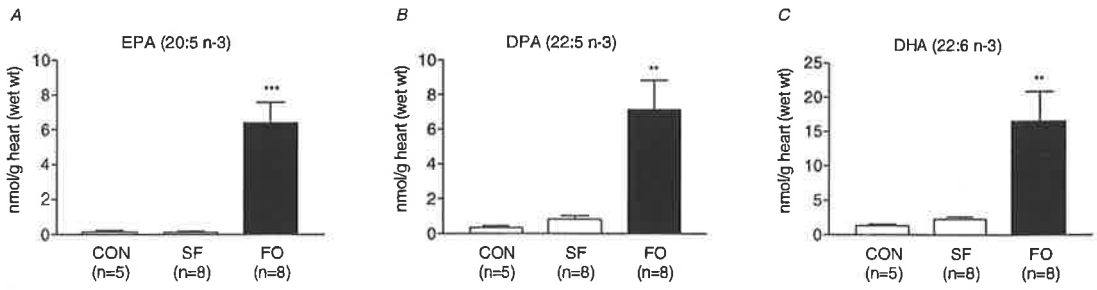


Figure 7.1 Amount of n-3 non-esterified fatty acids in rat myocardium.
The amounts of *A*, EPA, *B*, DPA or *C*, DHA (in nmol/g heart wet wt) are given. Data are means ± SEM for the number of animals indicated in parentheses per dietary group. Abbreviations, control (CON), saturated fat (SF) and fish oil (FO). **P<0.01, ***P<0.001 FO vs CON and SF.

None of the other fatty acid concentrations were changed by dietary treatment with FO. The levels of the NEFA, EPA was 0.15 nmol/g heart and 0.13 nmol/g heart in the CON and SF groups, respectively. However following dietary FO treatment this level was found to be approximately 40x higher (6.4 nmol/g heart, P<0.001). Furthermore the levels of the NEFA, DPA, was 8-20x higher in the FO group. Additionally, DHA was also

found to be at a significantly ($P<0.01$) higher concentration in the FO group (16.6 nmol/g heart) compared with CON (1.3 nmol/g heart) and SF (2.3 nmol/g heart) groups. Therefore the combination of these n-3 PUFAs provided a total of 31.1 nmol/g heart in the FO group compared with approximately 3-4 nmol/g heart for the CON and SF groups. This is further reflected in the n-6/n-3 ratios given in Appendix 1 and Table 7.10. Table 7.10 also demonstrates that the NEFA, AA (20:4, n-6) from the FO group was significantly decreased as a proportion of the total fatty acids compared with the SF group ($P<0.01$).

Table 7.4 Fatty acid composition (wt %) of the cardiac phosphatidylethanolamine phospholipids after dietary lipid supplementation

Major FAME[a]	CON (n = 6)	SF (n = 8)	FO (n = 8)
14:0	0.05 ± 0.01	0.07 ± 0.01	0.06 ± 0.02
14:1	n/d	n/d	n/d
16:0	5.45 ± 0.24	6.85 ± 0.22[c]	6.84 ± 0.40[f]
16:1	0.15 ± 0.02	0.16 ± 0.03	0.12 ± 0.01
18:0	29.79 ± 1.55	27.56 ± 0.61	27.78 ± 0.95
18:1[b]	11.84 ± 0.93	8.90 ± 0.26[d]	7.07 ± 0.50[g]
18:2 (n-6)	3.62 ± 0.34	2.94 ± 0.08	1.67 ± 0.28[g,i]
18:3 (n-3)	0.24 ± 0.15	0.06 ± 0.01	0.12 ± 0.04
20:0	0.09 ± 0.01	0.06 ± 0.01	0.12 ± 0.02[h]
20:1	0.21 ± 0.03	0.16 ± 0.02	0.26 ± 0.03[h]
20:2	0.41 ± 0.03	0.57 ± 0.04[d]	0.11 ± 0.01[g,j]
20:3 (n-6)	0.10 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
20:4 (n-6)	24.47 ± 4.12	26.24 ± 0.79	10.39 ± 0.34[g,j]
22:0	0.05 ± 0.02	0.06 ± 0.01	n/d
22:1	n/d	0.05 ± 0.02	0.09 ± 0.01
20:5 (n-3)	0.06 ± 0.01	0.11 ± 0.01	2.40 ± 0.05[g,j]
24:0	7.45 ± 1.92	8.51 ± 0.47	0.89 ± 0.09[g,j]
24:1	n/d	n/d	n/d
22:5 (n-3)	0.63 ± 0.05	1.21 ± 0.08[e]	2.98 ± 0.08[g,j]
22:6 (n-3)	15.63 ± 0.86	16.64 ± 0.72	39.10 ± 1.09[g,j]
Σ Sat.	42.81 ± 2.65	43.07 ± 0.77	35.65 ± 0.84[g,j]
Σ Mono.	12.22 ± 0.97	9.23 ± 0.26[d]	7.50 ± 0.52[g]
Σ Poly.	44.97 ± 3.43	47.70 ± 0.88	56.85 ± 0.85[g,i]
Σ n-6	28.19 ± 3.80	29.30 ± 0.81	12.17 ± 0.54[g,j]
Σ n-3	16.38 ± 0.89	17.97 ± 0.76	44.60 ± 1.17[g,j]
n-6/n-3	1.78 ± 0.26	1.66 ± 0.10	0.28 ± 0.02[g,j]

Data shown are mean ± SEM for 6 - 8 animals per dietary group. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. n/d, not detected. Superscripts indicate significant differences at [c]P<0.05, [d]P<0.01, [e]P<0.001, for CON vs SF; [f]P<0.05, [g]P<0.001, for CON vs FO; [h]P<0.05, [i]P<0.01, [j]P<0.001, for SF vs FO (by ANOVA). CON; control group, SF; saturated fat group, FO; fish oil group.

Table 7.5 Fatty acid composition (wt %) of the cardiac phosphatidylcholine phospholipids after dietary lipid supplementation

Major FAME[a]	CON (n = 6)	SF (n = 8)	FO (n = 8)
14:0	0.08 ± 0.00	0.20 ± 0.02	0.16 ± 0.02
14:1	n/d	n/d	n/d
16:0	12.89 ± 0.62	15.10 ± 0.26[d]	15.28 ± 0.28[g]
16:1	0.24 ± 0.05	0.14 ± 0.02	0.11 ± 0.02f
18:0	27.56 ± 0.59	26.61 ± 0.42	26.34 ± 0.58
18:1[b]	15.30 ± 0.54	13.85 ± 0.32[c]	9.34 ± 0.30[h,k]
18:2 (n-6)	5.78 ± 0.37	5.56 ± 0.36	2.73 ± 0.15[h,k]
18:3 (n-3)	0.03 ± 0.01	0.05 ± 0.02	0.07 ± 0.02
20:0	0.08 ± 0.01	0.08 ± 0.01	0.11 ± 0.01
20:1	0.17 ± 0.02	0.14 ± 0.02	0.25 ± 0.02[f,j]
20:2	0.48 ± 0.06	0.86 ± 0.02[e]	0.11 ± 0.01[h,k]
20:3 (n-6)	0.23 ± 0.11	0.26 ± 0.03	0.11 ± 0.01
20:4 (n-6)	32.42 ± 0.74	30.79 ± 0.48	24.89 ± 0.55[h,k]
22:0	0.05 ± 0.01	0.04 ± 0.01	0.22 ± 0.08[i]
22:1	0.04 ± 0.00	0.07 ± 0.01	0.14 ± 0.10
20:5 (n-3)	0.06 ± 0.02	0.09 ± 0.01	3.34 ± 0.13[h,k]
24:0	1.70 ± 0.23	1.63 ± 0.12	0.25 ± 0.03[h,k]
24:1	n/d	0.06 ± 0.01	0.24 ± 0.17
22:5 (n-3)	0.34 ± 0.03	0.90 ± 0.04	2.76 ± 0.31[h,k]
22:6 (n-3)	2.70 ± 0.20	3.75 ± 0.21	13.96 ± 0.72[h,k]
Σ Sat.	42.34 ± 0.96	43.64 ± 0.55	42.21 ± 0.78
Σ Mono.	15.74 ± 0.56	14.17 ± 0.34[c]	9.82 ± 0.31[h,k]
Σ Poly.	41.91 ± 0.65	42.19 ± 0.55	47.97 ± 0.99[h,k]
Σ n-6	38.43 ± 0.82	36.61 ± 0.38	27.74 ± 0.54[h,k]
Σ n-3	3.08 ± 0.21	4.73 ± 0.27	20.12 ± 1.11[h,k]
n-6/n-3	12.87 ± 1.23	7.91 ± 0.44[e]	1.41 ± 0.09e[h,k]

Data shown are mean ± SEM for 6 - 8 animals per dietary group. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. n/d, not detected. Superscripts indicate significant differences at [c]P<0.05, [d]P<0.01, [e]P<0.001, for CON vs SF; [f]P<0.05, [g]P<0.01, [h]P<0.001, for CON vs FO; [i]P<0.05, [j]P<0.01, [k]P<0.001, for SF vs FO (by ANOVA). CON; control group, SF; saturated fat group, FO; fish oil group.

Table 7.6 Fatty acid composition (wt %) of the cardiac cardiolipin phospholipids after dietary lipid supplementation

Major FAME[a]	CON (n = 6)	SF (n = 8)	FO (n = 8)
14:0	0.13 ± 0.02	0.27 ± 0.02[c]	0.40 ± 0.03[e,i]
14:1	n/d	n/d	n/d
16:0	3.86 ± 0.14	4.33 ± 0.12	4.32 ± 0.17
16:1	0.28 ± 0.04	1.01 ± 0.13[d]	1.99 ± 0.07[g,j]
18:0	2.50 ± 0.42	2.08 ± 0.16	2.21 ± 0.29
18:1[b]	21.62 ± 1.12	20.11 ± 1.11	25.18 ± 0.92[i]
18:2 (n-6)	59.16 ± 1.90	57.12 ± 2.21	31.80 ± 0.71[g,j]
18:3 (n-3)	0.11 ± 0.01	0.21 ± 0.01	0.27 ± 0.06[e]
20:0	0.05 ± 0.02	0.04 ± 0.01	0.05 ± 0.02
20:1	0.17 ± 0.03	0.12 ± 0.02	0.21 ± 0.06
20:2	0.26 ± 0.04	0.24 ± 0.03	0.42 ± 0.01[f,j]
20:3 (n-6)	1.01 ± 0.04	1.85 ± 0.03[d]	1.62 ± 0.07[g,h]
20:4 (n-6)	5.00 ± 0.36	4.90 ± 0.33	5.52 ± 0.23
22:0	0.04 ± 0.01	0.04 ± 0.02	n/d
22:1	0.03 ± 0.00	0.03 ± 0.01	0.30 ± 0.07[f,j]
20:5 (n-3)	n/d	0.05 ± 0.00	1.04 ± 0.04[j]
24:0	2.57 ± 0.58	2.90 ± 0.35	1.11 ± 0.16[e,i]
24:1	n/d	n/d	n/d
22:5 (n-3)	0.15 ± 0.02	0.73 ± 0.41	1.30 ± 0.17[e]
22:6 (n-3)	3.15 ± 0.37	4.04 ± 0.52	22.39 ± 0.77[g,j]
Σ Sat.	9.07 ± 0.86	9.62 ± 0.46	8.11 ± 0.43
Σ Mono.	22.09 ± 1.09	21.23 ± 1.10	27.54 ± 0.87[f,j]
Σ Poly.	68.85 ± 1.56	69.15 ± 1.45	64.35 ± 0.72
Σ n-6	65.17 ± 1.72	63.88 ± 1.92	38.94 ± 0.71[g,j]
Σ n-3	3.42 ± 0.36	5.04 ± 0.60	25.00 ± 0.96[g,j]
n-6/n-3	20.27 ± 2.31	14.27 ± 2.02	1.58 ± 0.08[g,j]

Data shown are mean ± SEM for 6 - 8 animals per dietary group. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. n/d, not detected. Superscripts indicate significant differences at [c]P<0.01, [d]P<0.001, for CON vs SF; [e]P<0.05, [f]P<0.01, [g]P<0.001 for CON vs FO; [h]P<0.05, [i]P<0.01, [j]P<0.001, for SF vs FO (by ANOVA). CON; control group, SF; saturated fat group, FO; fish oil group.

Table 7.7 Fatty acid composition (wt %) of the cardiac phosphatidylinositol and phosphatidylserine phospholipids after dietary lipid supplementation

Major FAME[a]	CON (n = 6)	SF (n = 8)	FO (n = 8)
14:0	0.12 ± 0.03	0.10 ± 0.02	0.12 ± 0.03
14:1	n/d	n/d	n/d
16:0	3.49 ± 0.39	4.87 ± 0.29	4.22 ± 0.39
16:1	0.26 ± 0.05	0.17 ± 0.05	0.18 ± 0.02
18:0	37.12 ± 0.44	39.28 ± 1.65	37.21 ± 0.81
18:1[b]	11.99 ± 0.34	9.63 ± 1.56	8.93 ± 0.51
18:2 (n-6)	3.01 ± 0.15	2.41 ± 0.15[c]	2.36 ± 0.17
18:3 (n-3)	0.10 ± 0.06	0.06 ± 0.02	0.08 ± 0.02
20:0	0.37 ± 0.05	0.20 ± 0.04	0.38 ± 0.06[g]
20:1	0.22 ± 0.02	0.20 ± 0.02	0.30 ± 0.04
20:2	1.21 ± 0.10	1.57 ± 0.18	0.28 ± 0.06[f,h]
20:3 (n-6)	0.34 ± 0.02	0.39 ± 0.04	0.47 ± 0.02[e]
20:4 (n-6)	26.45 ± 0.35	24.54 ± 0.52	19.26 ± 0.68[f,h]
22:0	0.29 ± 0.06	0.19 ± 0.04	0.24 ± 0.02
22:1	0.12 ± 0.03	0.08 ± 0.02	0.35 ± 0.04[f,h]
20:5 (n-3)	n/d	0.10 ± 0.01	1.87 ± 0.07[h]
24:0	6.99 ± 0.48	6.09 ± 0.20	0.60 ± 0.08[f,h]
24:1	0.09 ± 0.02	0.03 ± 0.01	0.07 ± 0.02
22:5 (n-3)	0.34 ± 0.05	0.80 ± 0.07[c]	3.94 ± 0.15[f,h]
22:6 (n-3)	7.75 ± 0.35	9.41 ± 0.58	19.47 ± 0.65[f,h]
Σ Sat.	48.28 ± 0.80	50.68 ± 1.64	42.63 ± 0.54[e,h]
Σ Mono.	12.58 ± 0.28	10.08 ± 1.58	9.80 ± 0.53
Σ Poly.	39.15 ± 0.55	39.24 ± 0.47	47.57 ± 0.57[f,h]
Σ n-6	29.80 ± 0.46	27.35 ± 0.55[c]	22.09 ± 0.62[f,h]
Σ n-3	8.14 ± 0.41	10.32 ± 0.64	25.30 ± 0.72[f,h]
n-6/n-3	3.71 ± 0.21	2.74 ± 0.21[d]	0.88 ± 0.05

Data shown are mean ± SEM for 6 - 8 animals per dietary group. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. n/d, not detected. Superscripts indicate significant differences at [c]P<0.05, [d]P<0.01, for CON vs SF; [e]P<0.05, [f]P<0.001, for CON vs FO; [g]P<0.05, [h]P<0.001, for SF vs FO (by ANOVA). CON; control group, SF; saturated fat group, FO; fish oil group.

Table 7.8 Fatty acid composition (wt %) of the cardiac sphyngomyelin phospholipids after dietary lipid supplementation

Major FAME[a]	CON (n = 6)	SF (n = 8)	FO (n = 8)
14:0	0.75 ± 0.11	0.57 ± 0.08	0.90 ± 0.12
14:1	n/d	n/d	n/d
16:0	16.73 ± 1.61	16.56 ± 1.38	22.61 ± 1.28[f]
16:1	1.49 ± 0.42	1.82 ± 0.68	1.51 ± 0.39
18:0	36.24 ± 1.08	36.57 ± 2.71	35.30 ± 1.21
18:1[b]	13.91 ± 1.99	13.01 ± 1.93	12.56 ± 1.71
18:2 (n-6)	10.59 ± 1.73	9.01 ± 2.02	5.50 ± 0.76
18:3 (n-3)	0.57 ± 0.14	0.94 ± 0.54	0.84 ± 0.28
20:0	1.54 ± 0.36	1.82 ± 0.48	1.83 ± 0.60
20:1	0.41 ± 0.11	1.26 ± 0.55	0.50 ± 0.15
20:2	1.82 ± 1.03	1.29 ± 0.24	1.22 ± 0.33
20:3 (n-6)	0.50 ± 0.07	0.74 ± 0.13	0.63 ± 0.08
20:4 (n-6)	8.16 ± 0.77	7.63 ± 1.11	6.44 ± 1.04
22:0	1.09 ± 0.36	0.69 ± 0.14	0.92 ± 0.15
22:1	n/d	0.94 ± 0.24	0.88 ± 0.12
20:5 (n-3)	0.46 ± 0.05	0.77 ± 0.26	1.36 ± 0.13[d]
24:0	2.97 ± 0.34	2.33 ± 0.20	1.84 ± 0.31[d]
24:1	0.71 ± 0.12	0.90 ± 0.23	1.06 ± 0.15
22:5 (n-3)	1.24 ± 0.25	1.11 ± 0.24	1.39 ± 0.16
22:6 (n-3)	3.02 ± 0.68	5.61 ± 1.32	6.57 ± 0.57
Σ Sat.	58.58 ± 1.75	57.81 ± 3.75	62.38 ± 2.11
Σ Mono.	16.51 ± 1.68	16.22 ± 2.04	15.37 ± 1.79
Σ Poly.	24.91 ± 2.55	25.97 ± 3.78	22.25 ± 1.54
Σ n-6	19.00 ± 1.77	17.19 ± 2.72	12.18 ± 1.00
Σ n-3	4.70 ± 0.50	7.66 ± 1.37	9.01 ± 0.50[d]
n-6/n-3	4.16 ± 0.45	2.53 ± 0.40[c]	1.34 ± 0.07[e,f]

Data shown are mean ± SEM for 6 - 8 animals per dietary group. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. n/d, not detected. Superscripts indicate significant differences at [c]P<0.01, for CON vs SF; [d]P<0.05, [e]P<0.001, for CON vs FO; [f]P<0.05, for SF vs FO (by ANOVA). CON; control group, SF; saturated fat group, FO; fish oil group.

Table 7.9 Fatty acid composition (wt %) of the cardiac lysophosphatidylcholine phospholipids after dietary lipid supplementation

Major FAME[a]	CON (n = 6)	SF (n = 8)	FO (n = 8)
14:0	0.17 ± 0.02	0.30 ± 0.04	0.42 ± 0.11
14:1	n/d	n/d	n/d
16:0	12.73 ± 0.55	14.31 ± 0.63	12.24 ± 0.68
16:1	0.49 ± 0.14	0.55 ± 0.21	0.42 ± 0.08
18:0	14.22 ± 1.09	20.00 ± 1.01[d]	11.13 ± 0.50[h]
18:1[b]	2.60 ± 0.42	3.41 ± 0.48	2.90 ± 0.68
18:2 (n-6)	1.79 ± 0.33	2.17 ± 0.20	1.96 ± 0.16
18:3 (n-3)	0.12 ± 0.04	0.97 ± 0.14[d]	0.73 ± 0.04[g]
20:0	10.05 ± 0.34	10.27 ± 0.60	11.45 ± 0.54
20:1	0.15 ± 0.04	0.19 ± 0.06	n/d
20:2	0.62 ± 0.11	1.23 ± 0.25	1.48 ± 0.18[f]
20:3 (n-6)	n/d	0.81 ± 0.49	0.71 ± 0.41
20:4 (n-6)	2.33 ± 0.21	4.85 ± 2.29	2.27 ± 0.36
22:0	36.15 ± 1.26	23.70 ± 1.26[e]	29.32 ± 0.87[g,h]
22:1	n/d	0.15 ± 0.01	n/d
20:5 (n-3)	n/d	0.41 ± 0.24	0.52 ± 0.10
24:0	10.86 ± 0.47	8.23 ± 1.01	10.32 ± 1.03
24:1	7.19 ± 0.44	8.33 ± 0.66	12.92 ± 1.35[g,h]
22:5 (n-3)	0.36 ± 0.12	0.68 ± 0.27	0.29 ± 0.09
22:6 (n-3)	0.59 ± 0.11	1.40 ± 0.34	2.43 ± 0.58[f]
Σ Sat.	84.13 ± 0.68	76.81 ± 2.41[c]	74.78 ± 1.71[g]
Σ Mono.	10.18 ± 0.57	12.53 ± 1.02	16.23 ± 1.46[g]
Σ Poly.	5.69 ± 0.48	10.65 ± 2.32	8.99 ± 0.86
Σ n-6	4.15 ± 0.38	7.36 ± 2.45	4.59 ± 0.60
Σ n-3	0.92 ± 0.23	2.24 ± 0.60	2.92 ± 0.55
n-6/n-3	6.96 ± 2.24	7.17 ± 4.16	2.15 ± 0.51

Data shown are mean ± SEM for 6 - 8 animals per dietary group. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. n/d, not detected. Superscripts indicate significant differences at [c]P<0.05, [d]P<0.01, [e]P<0.001, for CON vs SF; [f]P<0.05, [g]P<0.01, for CON vs FO; [h]P<0.01, for SF vs FO (by ANOVA). CON; control group, SF; saturated fat group, FO; fish oil group.

Table 7.10 Fatty acid composition (wt %) of the nonesterified fatty acids (NEFAs) in rat myocardium after dietary lipid supplementation

Major FAME[a]	CON (n = 5)	SF (n = 8)	FO (n = 8)
14:0	2.60 ± 0.15	2.48 ± 0.13	2.14 ± 0.14
14:1	0.45 ± 0.10	0.20 ± 0.02[d]	0.19 ± 0.03[f]
16:0	22.11 ± 1.40	21.40 ± 0.54	18.50 ± 0.52
16:1	0.96 ± 0.17	0.80 ± 0.07	0.61 ± 0.04
18:0	26.82 ± 0.79	20.84 ± 0.92	18.30 ± 1.55[e]
18:1[b]	22.25 ± 1.10	24.20 ± 1.69	20.91 ± 2.44
18:2 (n-6)	8.07 ± 1.01	10.58 ± 0.85	7.39 ± 1.53
18:3 (n-3)	1.04 ± 0.31	0.85 ± 0.09	0.61 ± 0.09
20:0	0.84 ± 0.29	0.99 ± 0.13	0.78 ± 0.15
20:1	0.86 ± 0.18	0.75 ± 0.05	0.93 ± 0.15
20:2	0.79 ± 0.31	0.99 ± 0.42	0.50 ± 0.23
20:3 (n-6)	0.43 ± 0.19	0.31 ± 0.03	0.38 ± 0.09
20:4 (n-6)	6.36 ± 1.09	8.33 ± 0.71	4.94 ± 0.53[i]
22:0	0.66 ± 0.11	1.22 ± 0.34	0.83 ± 0.13
22:1	2.64 ± 0.76	1.73 ± 0.45	1.62 ± 0.18
20:5 (n-3)	0.14 ± 0.09	0.08 ± 0.04	4.08 ± 0.38[i,f]
24:0	0.89 ± 0.23	1.43 ± 0.25	0.90 ± 0.14
24:1	0.40 ± 0.15	0.26 ± 0.09	0.37 ± 0.06
22:5 (n-3)	0.43 ± 0.11	0.66 ± 0.08	4.94 ± 0.67[g,j]
22:6 (n-3)	1.57 ± 0.17	1.88 ± 0.13	11.11 ± 1.66[g,j]
Σ Sat.	53.93 ± 2.66	48.37 ± 0.96	41.45 ± 2.14[f,h]
Σ Mono.	27.57 ± 1.50	27.95 ± 1.43	24.61 ± 2.48
Σ Poly.	18.51 ± 1.78	23.68 ± 0.98	33.95 ± 2.67[g,i]
Σ n-6	14.69 ± 1.78	19.22 ± 0.83	12.71 ± 1.79[h]
Σ n-3	3.18 ± 0.44	3.47 ± 0.25	20.73 ± 2.53[g,j]
n-6/n-3	4.76 ± 0.60	5.67 ± 0.32	0.69 ± 0.15[g,j]

Data shown are mean ± SEM for 6 - 8 animals per dietary group. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. n/d, not detected. Superscripts indicate significant differences at [c]P<0.05, [d]P<0.01, for CON vs SF; [e]P<0.05, [f]P<0.01, [g]P<0.001 for CON vs FO; [h]P<0.05, [i]P<0.01, [j]P<0.001 for SF vs FO (by ANOVA). CON; control group, SF; saturated fat group, FO; fish oil group.

7.4 DISCUSSION

This study demonstrates the selective incorporation of n-3 PUFAs into PE and PC phospholipids with concurrent decreases in the proportions of the potentially pro-arrhythmic fatty acid AA into these phospholipid species. The fatty acid profiles of other phospholipid species, including SPM, LPC, PI + PS, and CLP were also examined and found to contain distinct fatty acid profiles. Of particular interest in this study, was finding that dietary FO significantly increased the content of the n-3 non-esterified fatty acids EPA, DPA and DHA in the myocardium. Furthermore, there were very minor differences in the fatty acid composition within the various phospholipid classes examined, when comparing the SF or CON groups.

The distribution of phospholipids (and other components) of biological membranes is not random. The protein and lipid constitutents are distributed asymmetrically between the outer and inner leaflets of the lipid bilayer (Shinitzky, 1984; McMurchie, 1988). For example, in many cell systems PC and SPM are concentrated in the outer bilayer while PE, PI and PS are concentrated in the inner bilayer. There is also an unequal distribution of lipids based of fatty acyl chains with identical head groups that could be modified by changes in the dietary lipids (for example, as shown by the differences in the n-3 PUFAs EPA, DPA and DHA of the PC and PE fractions from the FO group compared with the CON and SF groups). Since IP_3 and diacylglycerol are generated from the inner surface of the bilayer where much of the n-3 PUFAs were incorporated in high proportions (i.e, into PE, PI + PS fractions), this asymmetric distribution of phospholipids (and fatty acids) may have various implications on IP_3 and diacylglycerol signalling mechanisms (e.g., Ca^{2+} cycling and other important biological processes).

Although the proportion of the PC phospholipid class (see Table 7.3) is similar to that obtained from neonatal cardiomyocytes i.e., about 45% of all phospholipids (Table 7.2 of (Lamers *et al.*, 1992)), the fatty acid profile as shown in this study was quite different in many respects to that of neonatal cardiomyocytes. For example, 16:0 was found to be in higher proprtions with 18:0 being lower, in neonatal cardiomyocytes (Lamers *et al.*, 1992). Furthermore, LA (18:2, n-6) in the PC fraction was 14% in neonatal cardiomyocytes and approximately 5-6% in this study using adult rat cardiomyocytes. AA was between 4-12% in neonatal cardiomyocytes and 25-32% in this study demonstrating large differences in the fatty acid composition of the PC phospholipid fraction between neonatal and adult ventricular tissue. In a similar manner, 16:0 and 24:0 were in higher proportions in adult rat SPM phospholipids whilst SA (18:0), LA (18:2n-6) and AA were in greater proportions compared with neonatal cardiomyocytes. Therefore this indicates that there may be differences in incorporation of specific fatty acids into cardiac phospholipid species that is dependent on the level of cardiac development and/or whether the cells are cultured. Furthermore, these differences in fatty acid profiles may have physiological relevance in that modification of membrane-associated protein functions may occur due to these specific changes in phospholipid fatty acid distribution.

In this study, the hearts were initially isolated and perfused with Tyrode solution containing digestive enzymes as well as BSA (for 20 min) to assist removal of unbound NEFAs. Therefore, it is unlikely that the myocardial tissue was contaminated with NEFAs originating from the blood or from the extracellular fluids. Although the total NEFA content of the hearts were similar in all dietary groups, there was a trend for a higher concentration in the FO group and this was associated with significant increases in

the NEFAs, EPA, DPA and DHA but not ALA. This result has particular importance and relevance to the previous acute and dietary studies presented in this thesis, since NEFAs (free fatty acids) that were used in the acute studies resulted in significant inhibition of electrically-induced contractile activity and voltage-dependent Na^+ currents. Additionally previous studies have shown that NEFAs applied directly to cardiomyocytes can block voltage-dependent L-type Ca^{2+} channels (Pepe *et al.*, 1994; Xiao *et al.*, 1997), K^+ channels (Macleod *et al.*, 1998) and cause negative inotropic and chronotropic effects (Kang & Leaf, 1994). This led to the observation that it was the NEFA itself that was antiarrhythmic not a metabolite or covalent incorporation of the NEFA into a membrane phospholipid. It could be speculated that the higher concentration of n-3 NEFAs in the myocardium of rats from the FO group, resulted from enhanced activity of phospholipase A_2, releasing fatty acids from the various phospholipid pools. Alternatively, higher circulating levels of n-3 PUFAs following FO consumption may have lead to increased uptake of the n-3 NEFAs. However, it is possible that incorporation of n-3 PUFAs into membrane phospholipids may act as a further pool for the formation of NEFAs to be released under appropriate conditions. Therefore, the availability of the n-3 PUFAs in both the NEFA and phospholipid forms following the consumption of fish oil as demonstrated here, may play an important role in the prevention of arrhythmias by the mechanisms already discussed in earlier sections.

CHAPTER 8

8 "Conclusions and future directions"

The aim of this study was to identify potential cardioprotective mechanisms of n-3 polyunsaturated fatty acid (PUFA) action at the level of the cardiomyocyte by comparing dietary and acute studies using adult rat cardiomyocytes.

In analysing specific effects of given interventions on the heart, isolated cardiomyocytes were used. These had the advantage that external influences such as the autonomic nervous system and humoral factors which may complicate the interpretation of results, were removed. Since the imposition of arrhythmic stimuli can induce normally quiescent cells to beat spontaneously, this indicates that single cardiomyocytes have the potential to develop automaticity in the whole heart. *In situ* this may give rise to regions of abnormal excitability and conductivity in the working myocardium, thus generating cardiac arrhythmias. Initial experiments in this thesis demonstrated that acute addition of non-esterified n-3 PUFAs (in particular, DHA) provided antiarrhythmic effects when using isoproterenol- and lysophosphatidylcholine-induced asynchronous contractile activity as a model of cardiac arrhythmias. However, other lipid forms (e.g., glycerides) containing DHA or saturated fatty acids, were either less potent or non-specific. Therefore agents (such as non-esterified n-3 PUFAs) that have been shown to protect isolated cells from displaying aberrant contractile behaviour may, in the above situation, work to protect these cells functioning abnormally in the working syncytia. These studies demonstrated that acute addition of DHA (and ALA) as well as benzyl alcohol (a membrane fluidising agent) prevented the development of isoproterenol-induced spontaneous contractile activity in isolated cardiomyocytes. Interestingly, at the same time these experiments

were being carried out at CSIRO, Rodrigo and colleagues at the University of Otago, New Zealand (Rodrigo *et al.*, 1999) reported that the n-3 PUFA, EPA, could prevent spontaneous contractile activity in saponin-permeabilised rat cardiomyocytes. They concluded, using their experimental model, that EPA prevented excessive release of Ca^{2+} from the SR, thus resulting in a reduction in the frequency of spontaneous contractions. Their results would indicate that the SR was a possible site of action, at least for the n-3 PUFA, EPA.

It is possible that isoproterenol-induced spontaneous contractile activity was prevented by acute n-3 PUFAs (and benzyl alcohol) by some effect related to increasing the sarcolemmal membrane fluidity. This may have altered the configuration of various sarcolemmal ion channel proteins, such as the Na^+ channel as shown in Chapter 3 (as well as inhibition of the L-type Ca^{2+} channel, reported by (Rodrigo *et al.*, 1999)), thus blocking the Na^+ ion movements. As a result, n-3 PUFA infusion into the lipid bilayer may change the physical environment (fluidity) in which the ion channel protein resides, and this may in turn shift the voltage-dependency of the ion channel. At resting membrane potentials for example, more Na^+ channels may reside in a "fluidised" environment that could cause the Na^+ channels to take up a partially inactivated or blocked state following acute addition of n-3 PUFAs or benzyl alcohol. Indeed, this is one of the mechanisms by which the clinically used Class I antiarrhythmic drugs, such as lidocaine (lignocaine) elicit their antiarrhythmic effects. Partially inactivating Na^+ channels in the presence of n-3 PUFAs would therefore likely prevent anomalous depolarising electrical (subthreshold) stimuli from generating action potentials which in turn would suppress the development of arrhythmias.

The rapid action of acutely added n-3 PUFAs on asynchronous contractile activity and on membrane fluidity would suggest that infusion of these lipids into the sarcolemmal membrane, as opposed to their incorporation into membrane phospholipids, is sufficient for them to exert their antiarrhythmic/antiasynchronous action. Given the above observation, and the finding from Chapter 4 in which antiarrhythmic (i.e., prevention of spontaneous contractility) actions of n-3 PUFAs were observed following dietary supplementation and concomitant incorporation of n-3 PUFAs into cardiac membrane phospholipids, it was necessary to determine whether incorporation of n-3 PUFAs into membrane phospholipids was associated with changes in membrane fluidity. Initially, it was anticipated that treatment of cardiomyocytes with isoproterenol would increase intracellular [Ca^{2+}], thus activating phospholipase A_2 activity and thereby liberating non-esterified (n-3) PUFAs indirectly (Hashizume *et al.*, 1997), thus modulating membrane fluidity. However, under basal conditions as well as in the presence of isoproterenol, there were no changes in cardiomyocyte membrane fluidity observed using the probe TMAP-DPH. However, there did appear to be a difference in the cardiomyocyte responsiveness to KCl depolarisation in both the CO and FO groups. This observation suggested that dietary incorporation of n-3 PUFAs into ventricular phospholipids modified the electrical activity of the cardiomyocytes following FO (and CO) treatment, and this may be related to the inhibition of the Na^+ channels.

Chapter 5 demonstrated that when sufficient n-3 PUFAs were incorporated into the ventricular phospholipids, following dietary fish oil supplementation, a small but significant shift in the voltage-dependence of inactivation of Na^+ channels occurred, compared with a saturated fat dietary supplement. Physiologically, this effect implies that at depolarising membrane potentials (such as that expected following myocardial ischaemia) in the presence

of dietary incorporated n-3 PUFAs, a higher proportion of Na^+ channels would exist in the "inactivated" state and therefore not be available to conduct Na^+ into the cytoplasm. In essence, this would result in the membrane being more resistant to depolarising stimuli (i.e., less "excitable"). Furthermore, it is possible that the increase in the concentration of the free, non-esterified n-3 PUFAs within the cardiomyocytes following dietary fish oil supplementation confers the antiarrhythmic and Na^+ channel blocking effects observed. Therefore, the high proportions of n-3 PUFAs incorporated into the various membrane phospholipid species (in particular the PE fraction), may simply act as a reservoir of n-3 PUFAs that can be liberated by the action of phospholipase A_2 to form the free non-esterified n-3 PUFAs which can then confer protective properties. Alternatively, the cardiomyocytes may selectively take up n-3 PUFAs circulating in the plasma which either remain as unesterified PUFAs within the cells, being associated with intracellular proteins, or become incorporated into the various phospholipid (and other lipids) fractions.

Previous investigators have recently demonstrated that acute addition of n-3 PUFAs to rat cardiomyocytes significantly alters the SR Ca^{2+} uptake, load and release mechanisms, as well as the sarcolemmal L-type Ca^{2+} channel (Negretti & O'Neill, 1997; Rodrigo *et al.*, 1999; Negretti *et al.*, 2000). Although these investigators propose these effects by n-3 PUFAs on the Ca^{2+} handling properties of cardiomyocytes as potential antiarrhythmic mechanisms, this study, demonstrated for the first time that the Ca^{2+} handling properties of cardiomyocytes from a fish oil supplemented group had only minor changes in Ca^{2+} handling, and this was not directly related to SR function. This highlights the distinct difference between the acute and dietary study results with respect to n-3 PUFA effects on rat cardiomyocyte Ca^{2+} handling. Therefore, future studies investigating the effects of free, unesterified n-3 PUFAs, should, ideally be supported by appropriate dietary

supplement studies using fish oils or purified EPA and/or DHA. Furthermore, it would also be appropriate to carry out studies to determine whether it is the activity, number of transporters or level of gene expression (e.g., the latter probed with appropriately configured cDNA arrays) of sarcolemmal Ca^{2+} transporters (and SR Ca^{2+} transporters) that may be altered by dietary fish oil supplementation.

Although these studies were carried out using rat myocardium, in order to allow extrapolation to the situation in the human heart, it would be advantageous to carry out similar patch-clamp studies using human cardiomyocytes. Preliminary studies carried out by the author as well as CSIRO Health Sciences and Nutrition and the Department of Physiology staff and students involved in these projects, attempted to isolate viable cardiac myocytes for such studies. Human atrial appendage tissue (taken from patients undergoing coronary artery bypass surgery at the Royal Adelaide Hospital) was digested under various conditions, however, very low cardiomyocyte viability and yields were routinely obtained. The viability and yield of human atrial myocytes was considered too low and therefore unsuitable for the experimental protocols that were to be used (i.e., patch-clamping of Na^{+} channels and determination of acute effects of n-3 PUFAs).

In comparing rat heart studies (such as those presented here) to that of human heart studies, careful consideration of the species differences, particularly with respect to the myocardial fatty acid distribution needs to be noted. A recent report by Blake (Blake *et al.*, 2000) demonstrated that 6 weeks of dietary fish oil supplementation to humans, prior to undergoing coronary artery bypass surgery, could significantly increase plasma EPA, DPA and DHA. However, it was only EPA that was significantly increased in the atrial tissue. This contrasts to that of the rat studies outlined in this thesis, whereby significant

increases in EPA, DPA and DHA were observed in myocardial tissue. Nevertheless, acute addition of EPA and DHA has been shown to be equally potent at preventing spontaneous contractility (Leifert, 1997) or blocking the whole-cell Na^+ currents in rat cardiomyocytes. Therefore, it would appear that although there are species differences with regard to fatty acid composition of the myocardium, the isolated rat cardiomyocyte model could provide useful information on such experiments, providing these differences in fatty acid incorporation into the myocardium are recognised. Future studies should also consider defining the active antiarrhythmic components of the fish oil concentrate. This may include carrying out dietary studies as described in this thesis using purified n-3 PUFAs such as EPA and/or DHA (as the dietary n-3 PUFA source) at various doses, and comparing with an appropriate control oil. This type of study design would then allow for the active components of fish and fish oils to be accurately identified as well as the threshold levels of the PUFAs required for their antiarrhythmic action.

Previous studies have reported that modifying the cholesterol/phospholipid ratio of cardiac membranes following dietary cholesterol supplementation results in changes in the catecholamine-stimulated adenylate cyclase activity in rat hearts (McMurchie *et al.*, 1987). Therefore, another potential mechanism of action not investigated in these studies, is that of the β-adrenergic receptor signalling pathway which may be altered in the fish oil group compared with the saturated fat group. To investigate this further, inotropic (contractile) responses to β-adrenergic receptor stimulation, for example by isoproterenol, would need to be measured following dietary fish oil supplementation. However, it would be expected that positive inotropic contractile responses would be associated with increases in the cellular $[Ca^{2+}]_i$ following isoproterenol stimulation. Indirectly, this study (Chapter 6) which measured the isoproterenol-induced Ca^{2+} transients following fish oil supplementation,

demonstrated that there was no significant difference in the level of diastolic or systolic $[Ca^{2+}]_i$ during steady-state contractions. The results in this thesis suggest that there was no difference in the β-adrenergic receptor signalling pathway following fish oil supplementation as measured by diastolic or systolic $[Ca^{2+}]_i$ levels. Therefore, it is unlikely that dietary fish oil supplementation (containing n-3 PUFAs) significantly modified the β-adrenergic receptor signalling pathway in rat cardiomyocytes.

In conclusion, these studies demonstrate antiarrhythmic effects of acute and dietary n-3 PUFAs in an isolated cardiomyocyte model of arrhythmia. The potential mechanisms of action of the n-3 PUFAs included a shift in the voltage-dependence of inactivation of Na^+ channels, as determined by patch clamp techniques and changes in Ca^{2+} efflux properties, possibly via the sarcolemmal Na^+/Ca^{2+}-exchanger. The antiarrhythmic effects were associated with significant incorporation of n-3 PUFAs (EPA, DPA and DHA) into the PE and PC fraction of phospholipids, compared with sphingomyelin and lysophosphatidylcholine. Importantly, significant increases were observed in the myocardial content (concentration) of the non-esterified n-3 PUFAs following dietary fish oil supplementation, which may partly explain the cardioprotective effects in humans.

Chapter 9

9 "Appendices"

Appendix 9.1 Fatty acid concentration of non-esterified fatty acids (NEFA) in rat myocardium after dietary lipid supplementation (nmol NEFA/g wet heart)

Major FAME[a]	CON (n=5)	SF (n=8)	FO (n=8)
14:0	3.35 ± 0.76	4.15 ± 0.39	4.45 ± 0.69
14:1	0.54 ± 0.15	0.33 ± 0.02	0.35 ± 0.02
16:0	25.44 ± 6.15	33.30 ± 4.75	34.93 ± 5.45
16:1	0.96 ± 0.10	1.20 ± 0.13	1.16 ± 0.21
18:0	27.26 ± 5.43	28.17 ± 2.78	29.27 ± 3.39
18:1[b]	22.15 ± 3.64	34.98 ± 6.15	32.07 ± 6.55
18:2 (n-6)	7.78 ± 1.14	14.70 ± 2.01	11.25 ± 2.30
18:3 (n-3)	1.04 ± 0.40	1.16 ± 0.14	0.94 ± 0.14
20:0	0.92 ± 0.49	1.14 ± 0.10	1.27 ± 0.39
20:1	0.90 ± 0.30	0.92 ± 0.09	1.65 ± 0.48
20:2	0.88 ± 0.54	1.25 ± 0.50	0.94 ± 0.56
20:3 (n-6)	0.33 ± 0.22	0.38 ± 0.04	0.66 ± 0.24
20:4 (n-6)	5.38 ± 0.79	11.04 ±1.79	7.68 ± 1.44
22:0	0.59 ± 0.20	1.68 ± 0.79	1.03 ± 0.14
22:1	2.00 ± 0.50	1.73 ± 0.32	2.28 ± 0.47
20:5 (n-3)	0.15 ± 0.09	0.13 ± 0.07	6.40 ± 1.17[d,f]
24:0	0.77 ± 0.33	1.36 ± 0.25	1.17 ± 0.26
24:1	0.31 ± 0.15	0.26 ± 0.10	0.46 ± 0.11
22:5 (n-3)	0.36 ± 0.09	0.83 ± 0.19	7.14 ± 1.68[c,e]
22:6 (n-3)	1.34 ± 0.21	2.27 ± 0.34	16.58 ± 4.31[c,e]
Total NEFAs	**102.3 ± 18.3**	**141.0 ± 18.2**	**161.6 ± 23.6**
Σ Sat.	58.34 ± 13.21	69.79 ± 8.35	72.12 ± 9.77
Σ Mono.	26.85 ± 4.07	39.42 ± 6.21	37.92 ± 7.50
Σ Poly.	17.08 ± 2.31	31.77 ± 4.23	51.60 ± 8.93[c]
Σ n-6	13.49 ± 1.84	26.12 ± 3.52	19.60 ± 3.15
Σ n-3	2.88 ± 0.56	4.40 ± 0.69	31.06 ± 7.11[c,e]
n-6/n-3	5.07 ± 0.62	6.12 ± 0.33	0.76 ± 0.16[d,f]

Data shown are mean ± SEM for 6 - 8 animals per dietary group. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-9 and n-7 isomers. n/d, not detected. Superscripts indicate significant differences at [c]P<0.01, [d]P<0.001, for CON vs FO; [e]P<0.01, [f]P<0.001, for SAT vs FO (by ANOVA). CON; control group, SF; saturated fat group, FO; fish oil group.

CHAPTER 10

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