Antiarrhythmic and Electrophysiologic Effects of Alpha-Linolenic Acid from Dietary Flaxseed

by

Bradley P. Ander

A thesis submitted to the Faculty of Graduate Studies of the

The University of Manitoba

in partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology

University of Manitoba

Winnipeg

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ABSTRACT

There is growing evidence that ω-3 polyunsaturated fatty acids (PUFA) may be beneficial against cardiovascular disease. Most of this work is data collected from studies investigating the fish oils, eicosapentaenoic acid and docosahexaenoic acid. Flaxseed is the richest source of the parent ω-3 PUFA, α-linolenic acid (ALA). Flaxseed oil is unique in that it can easily be incorporated into a variety of foods in order to increase the amount of dietary ω-3 PUFA that the general population ingests.

We investigated the antiarrhythmic and electrophysiologic effects of dietary flaxseed and its main fatty acid component, ALA. Following dietary supplementation with flaxseed, hearts from male New Zealand White rabbits were isolated and subjected to a global ischemia-reperfusion protocol. Hearts from these animals had a significantly lower occurrence of ventricular fibrillation even in the presence of a high cholesterol diet. Prior to ischemia, the QT intervals measured in the hearts of flaxseed fed rabbits were shortened, which is indicative of a shortening of action potential duration.

We also used cardiomyocytes to investigate the electrophysiologic changes that occur following either an acute exposure to ω-3 PUFA or dietary flaxseed supplementation. In acutely treated cardiomyocytes, application of ω-PUFA shortened the duration of the plateau phase and overall duration of the action potential, which is consistent with the shortened QT interval seen in the whole hearts. In cardiomyocytes from dietary flaxseed supplemented rabbits, current through delayed rectifier K^+ channels and L-type Ca^{2+} channels was augmented in cells compared to controls. There was also a greater transient increase in $[Ca^{2+}]_i$ during repeated stimulation in cells from the flaxseed-fed rabbits compared to controls.

We also examined the effects of ALA and other fatty acids on the sodium-calcium exchanger (NCX), an important ionic antiporter that is becoming an increasingly important target for not only antiarrhythmic drugs, but also therapies for hypertension and heart failure. ALA acutely applied to HEK293 cells expressing the cardiac or vascular isoform of the NCX resulted in significant inhibition of forward and reverse NCX activity. However, the IC_{50} values for inhibition of both modes of activity were ~6 times lower for the vascular isoforms compared to the cardiac isoforms.

In summary, this study shows that dietary flaxseed exerts a significant antiarrhythmic effect, particularly against reperfusion induced ventricular fibrillation. This effect is associated with a shortening of the QT interval at the whole heart level and with a shortening of the action potential duration at the cellular level. ALA, and ω-3 fatty acids may affect a number of cardiac currents to exert this effect. In particular, the augmented repolarizing K^+ currents may outweigh the increased Ca^{2+} currents. Reduced activity of the NCX may also explain the beneficial effects of ω-3 PUFA in various conditions of cardiovascular disease. Our results highlight potential mechanisms for the beneficial effects of dietary flaxseed on the heart and underscore the need for further basic and clinical investigations.

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For Shannon

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LIST OF ABBREVIATIONS

AA – arachidonic acid

ALA – α-linolenic acid

ANOVA – analysis of variance

APD – action potential duration

APD_{50} – action potential duration at 50% repolarization

APD_{90} – action potential duration at 90% repolarization

ATP – adenosine triphosphate

AV – atrioventricular

BCL – basic cycle length

BSA – bovine serum albumin

CAD – coronary artery disease

CAST – Cardiac Arrhythmia Suppression Trial

CHD – coronary heart disease

CHL – cholesterol

CHL/FLX – cholesterol/flaxseed

CVD – cardiovascular disease

DHA – docosahexaenoic acid

DMEM – Dulbecco's modified essential medium

ECG – electrocardiogram

EDP – end-diastolic pressure

EDTA – ethylenediaminetetraacetic acid

EFA – essential fatty acids

EGTA – ethylene glycol tetraacetic acid

EPA – eicosapentaenoic acid

FA – fatty acid

FAME – fatty acid methyl ester

FBS – fetal bovine serum

FLX – flaxseed

FRAP – fluorescent resonance after photobleaching

GC – gas chromatography

GLA – γ-linolenic acid

HBS – HEPES buffered saline

HEPES – hydroxyethylpiperazineethanesulfonic acid

hERG – human ether-a-go-go related gene

ICD – implantable cardiac defibrillator

K2P – two-pore domain K^+ channel

LA – linoleic acid

LTCC – L-type calcium channel

LVDP – left-ventricular developed pressure

MAP – monophasic action potential

MAPD – monophasic action potential duration

MI – myocardial infarction

MUFA – monounsaturated fatty acid

NAK – sodium-potassium ATPase

NCX – sodium-calcium exchanger

NHE – sodium-hydrogen exchanger

OA – oleic acid

PA – palmitic acid

PBS – phosphate buffered saline

PMSF – phenylmethylsulphonylfluoride

PPAR – peroxisome proliferator activated receptor

PPRE – PPAR response element

PUFA – polyunsaturated fatty acid

PVDF – polyvinylidene fluoride

REG – regular

RIPA – radioimmunoprecipitation assay

RVC – rat ventricular cardiomyocytes

RXR – retinoid X receptor

RyR – ryanodine receptor

SA – sinoatrial

SEM – standard error of the mean

SERCA – sarcoplasmic endoplasmic reticulum calcium ATPase

SL – sarcolemma

SR – sarcoplasmic reticulum

TMA-DPH - trimethylammonium diphenylhexatriene

Tris – trishydroxymethylaminomethane

TxA_2 – thromboxane A_2

VF – ventricular fibrillation

VGSC – voltage-gated sodium channel

VSMC – vascular smooth muscle cell

VT – ventricular tachycardia

XIP – exchanger inhibitory peptide

CHAPTER I: INTRODUCTION

Epidemiological surveys reveal an inverse relationship between consumption of ω-3 polyunsaturated fatty acids (PUFA) and the incidence of cardiovascular disease, and a growing body of evidence now suggests that these "good fats" can be used to help prevent or treat different aspects of disease. A growing number of clinical trials have overwhelmingly shown beneficial effects of ω-3 PUFA, but the mechanisms underlying these effects are still unclear.

Increased consumption of ω-3 PUFA has been linked to a lower incidence of sudden cardiac death. This may be due to a reduction in the factors that can lead to the triggering of fatal cardiac arrhythmias, or that these fatty acids exert specific antiarrhythmic effects. Drugs designed to treat arrhythmias often target and alter the function of ion channels in the heart. Evidence suggests that ω-3 PUFA affect several ionic currents, which may be the basis for this protective effect.

The most commonly studied ω-3 PUFA are the "fish oils", docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). These longer-hydrocarbon chain oils are metabolites of the parent ω-3 PUFA, α-linolenic acid (ALA). There is very little evidence to support or refute the possibility that ALA itself is cardioprotective. If ALA does provide a cardiovascular benefit, there could be a direct impact on many Canadians, especially in regards to farmers who grow flaxseed, since it is the richest terrestrial source of ALA.

Consumers are becoming increasingly aware of the potential health benefits of ω-3 PUFA, and flaxseed and purified flaxseed oil can be consumed or easily incorporated into other products to add extra value. If ω-3 PUFA are readily available in the diet of the population, it follows that the overall incidence of cardiovascular disease should decrease

accordingly.

In this study we examine the antiarrhythmic effects of dietary flaxseed, and explore the potential mechanisms for this effect. Experiments use dietary models to approximate the physiological setting, and acute models to gather mechanistic insight into how ALA and other ω-3 PUFA modulate cellular activity at the ionic level.

CHAPTER II: LITERATURE REVIEW

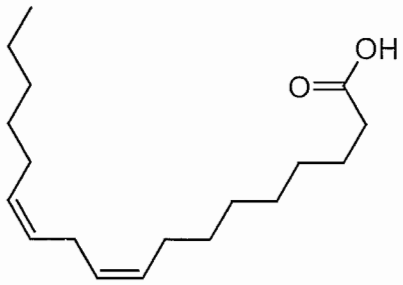
POLYUNSATURATED FATTY ACIDS

STRUCTURE

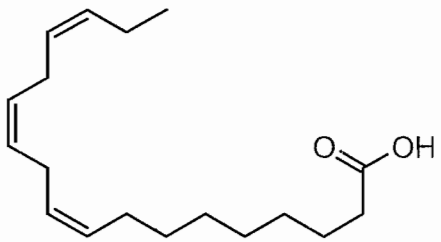
Fatty acids (FA) consist of a carboxylic acid head group on a hydrocarbon chain of varying length. They may be subcategorized based on the degree of saturation of the carbon chain. Saturated FA contain no double bonds, whereas monounsaturated FA (MUFA) and polyunsaturated fatty acids (PUFA) contain one, or two or more double bonds, respectively. Unsaturated FA can also be classified based on the location of the first double bond relative to the final "omega" carbon, or the methyl terminus of the carbon chain. For example, the ω-3 and ω-6 PUFA are two of the most biologically significant families of PUFA, and possess their first double bond on the third or sixth carbon from the chain terminus, respectively.

METABOLISM

Only plants are capable of *de novo* production of PUFA. However, animals, including humans, are able to metabolize these parent fatty acids, known as essential fatty acids (EFA) into longer chain ω-3 and ω-6 PUFA. The parent ω-3 PUFA is α-linolenic acid (18:3) and the parent ω-6 PUFA is linoleic acid (18:2) (Figure 1). Fatty acids are elongated and desaturated into other physiologically active PUFA by enzymes known as elongases and desaturases, respectively [1]. The delta-5 desaturase is believed to be the rate limiting step for the metabolism of PUFA [1]. Two important factors show how a dietary imbalance of the two classes can lead to an overabundance or a deficiency in either class of PUFA. First, humans and animals lack the ability to convert between ω-3 and ω-6 PUFA. Secondly, ω-3 and ω-6 PUFA share the same enzymes in the metabolic pathway.



Linoleic acid



α-Linolenic acid

Figure 1. The structure of two essential fatty acids – linoleic acid and α-linolenic acid.

Figure 2 shows the conversion of EFA to their downstream products through multiple elongation and desaturation steps. The ratio of ω-6 to ω-3 PUFA is very important to human health since the ω-6 and ω-3 pathways compete with one another for enzyme activity. An overabundance of FA from one family will limit the metabolic production of the longer chain products of the other. The typical Western diet provides ω-6 and ω-3 PUFA in a ratio ranging from 8:1 to 25:1 [2]. These values are in severe contrast with the recommendations of national health agencies of ~4:1 [3]. Lowering the ω-6:ω-3 ratio would reduce the competition for the enzymes and facilitate the metabolism of more downstream products of ALA.

EICOSANOIDS

While the activities of ω-3 or ω-6 PUFA may themselves be physiologically important, so are the actions of their eicosanoid metabolites. The metabolites of the 20-carbon PUFA, EPA and arachidonic acid (AA), are very important biologically with respect to inflammatory responses and blood clotting.

The 20-carbon ω-3 and ω-6 PUFA compete for the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. The 2- and 4-series eicosanoids derived from ω-6 PUFA are more biologically active than the 3- and 5-series eicosanoids derived from ω-3 PUFA [4]. Thromboxane A_2 (TxA_2), a metabolite of AA, is a potent vasoconstrictor and platelet aggregator. Fish oils inhibit TxA_2 production *in vitro* and *in vivo* [5]. ω-3 derivatives also decrease the affinity of the TxA_2 receptor for TxA_2, thus further inhibiting TxA_2-induced platelet aggregation [6]. Increased consumption of ω-3 PUFA results in greater incorporation of ω-3 PUFA into cell membrane phospholipids, ultimately leading to

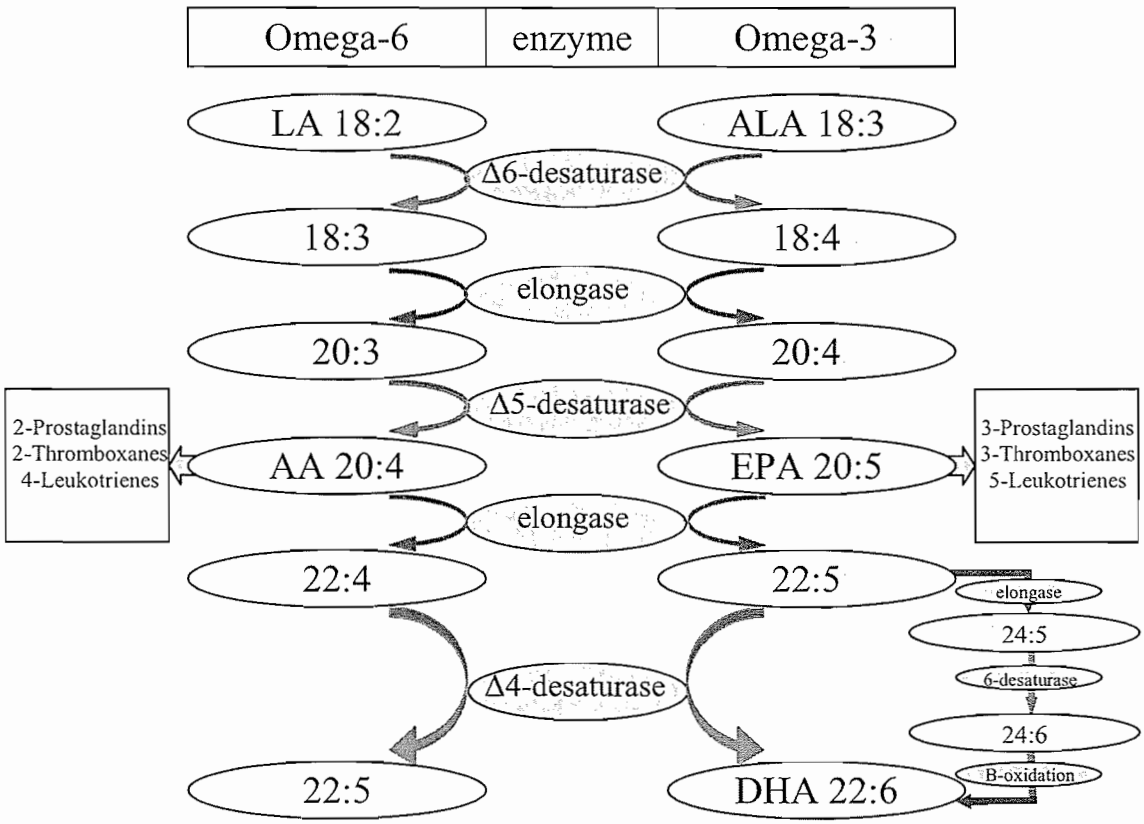


Figure 2. The metabolic pathways of ω-6 and ω-3 polyunsaturated fatty acids. Metabolism of the two fatty acid families requires competition for the same elongation and desaturation enzymes. The desaturation steps tend to be slow and rate limiting compared to the more rapid elongation steps. Eicosanoids, such as prostaglandins, leukotrienes and thromboxanes, can be derived from AA and EPA and can mediate different physiological actions. AA – arachidonic acid; ALA – α-linolenic acid; DHA – docosahexaenoic acid; EPA – eicosapentaenoic acid; LA – linoleic acid.

generation of more ω-3 derived eicosanoids. This results in simultaneous reduction of ω-6 PUFA-derived pro-inflammatory eicosanoids since ω-3 PUFA can competitively inhibit the conversion of AA to pro-inflammatory eicosanoids. ω-3 PUFA act as potential COX substrates, decrease the affinity of the COX enzyme for ω-6 PUFA, and suppress production of ω-6 eicosanoid inflammatory mediators [3, 7].

POLYUNSATURATED FATTY ACIDS IN THE FOOD SUPPLY

Most diets are already very rich in ω-6 PUFA, so a more conscious effort needs to be placed on incorporating ω-3 FA in the diet. Dietary sources of ω-3 PUFA are readily available, but in limited quantities. Many foods contain ALA including certain vegetable oils, dairy products, flaxseed, walnuts and vegetables [8]. Fatty fish, such as mackerel, herring and salmon, provide an excellent source of the long chain derivatives of ALA, EPA and DHA [3]. Estimated daily dietary intake of ω-3 PUFA is ~1.2 g for ALA and ~0.2 g for EPA and DHA, combined [9-11].

FLAXSEED IN CARDIOVASCULAR HEALTH

Flax (*Linum usitatissimum*) has been grown in many regions of the world for thousands of years. The straw of the plant has been used to produce linen since ancient Egyptian society [12], and the use of the seeds has ranged from dietary consumption to extraction of the oil for use in paints, varnishes, and linoleum [13]. Today, flax is commonly grown in the Canadian prairies. The plant can be recognized by its distinctive blue flower, and the seeds themselves resemble sesame seeds and are usually dark brown in colour (Figure 3). Flaxseed is the richest plant source of ω-3 fatty acids [14]. Up to 70% of the

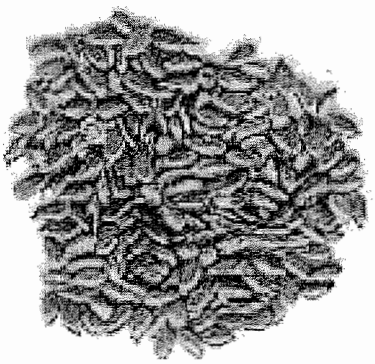


Figure 3. Flax and flaxseed

fatty acid content of flaxseed is ALA, representing 25% of the total weight of the seeds (Figure 4). Flaxseed has a palatable nutty taste and can easily be incorporated into a variety of foods, since it has a fairly long shelf life [15, 16] and shows very high stability when exposed to high baking temperatures [17-19]. Thus, it represents a potentially important means to increase ω-3 fatty acid consumption in the general population. There is growing evidence that flaxseed may be useful in the treatment of cancer and other diseases; however, data substantiating the effectiveness of ALA compared to fish oils with respect to a benefit in cardiovascular disease is lacking.

CARDIOVASCULAR DISEASE

Sudden death resulting from acute myocardial infarction (MI) accounts for the majority of deaths from cardiovascular disease in the developed world [20, 21]. Ischemia-induced arrhythmias, ventricular fibrillation (VF) in particular, are a serious and often fatal consequence of coronary heart disease (CHD). However, mortality and morbidity from cardiovascular disease have declined over the last decade, in part due to antiarrhythmic drugs, education of health care professionals, and increased public awareness of risk factors [21]. Dietary interventions, in particular, have recently received attention as effective antiarrhythmic strategies.

ISCHEMIC HEART DISEASE

Ischemic heart disease is the most common form of cardiovascular disease, which along with cancer, is the leading cause of death in the developed world [20, 21]. Ischemia, or a reduction in blood flow so that it no longer meets the demands of the tissue, occurs in the

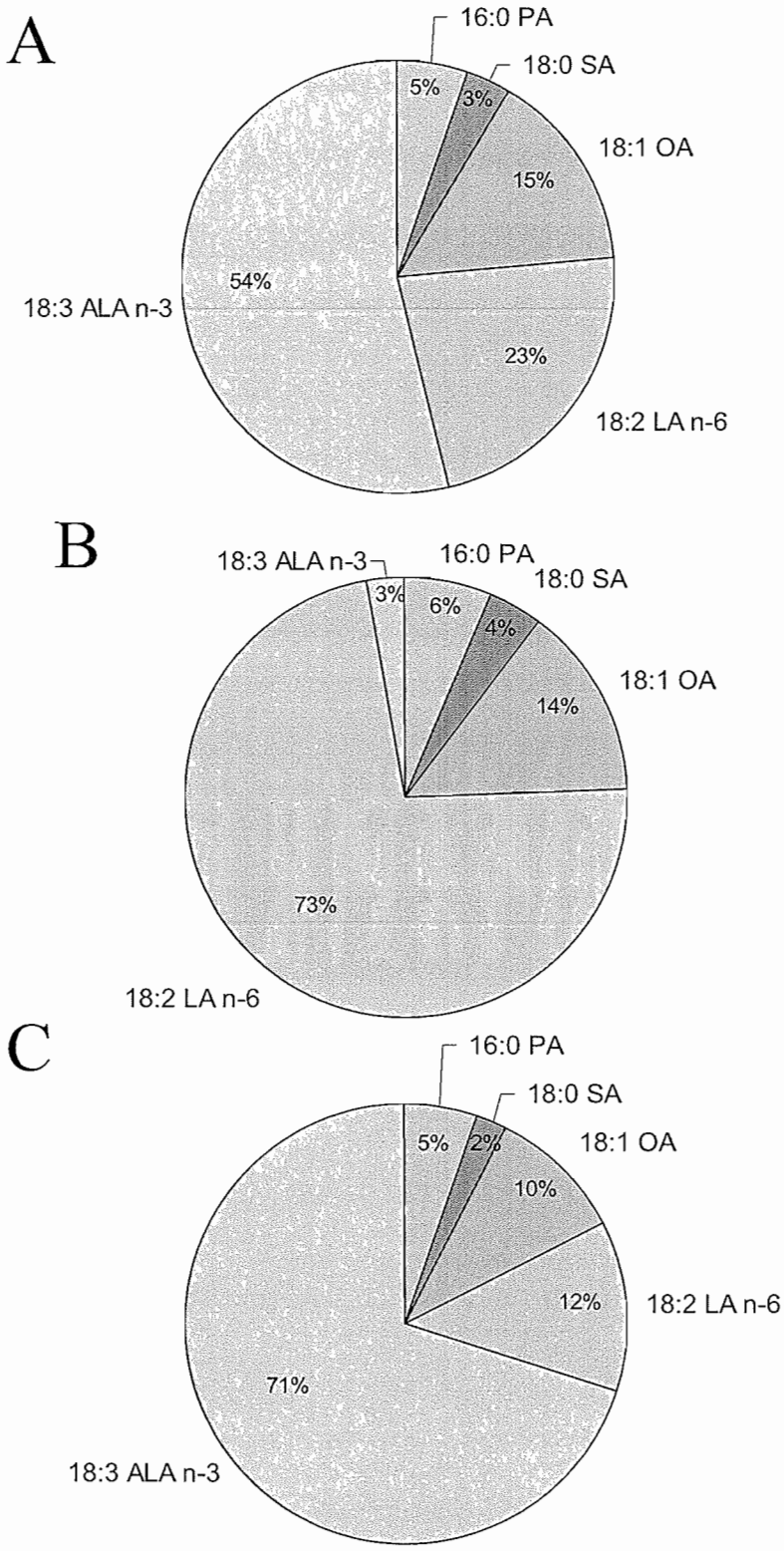


Figure 4. The fatty acid composition of three types of flaxseed – (A) standard flaxseed, (B) CDC flaxseed (low ALA), and (C) Promega flaxseed (high ALA, used in this study).

heart when flow though the coronary vessels is impaired. Usually this impairment is caused by a buildup of atherosclerotic plaque, fragments from ruptured plaques, formation of blood clots, vasospastic contractile event, or any combination of these factors (Figure 5). The narrower the lumen of the coronary vessels, the more likely a sudden complete blockage can occur and cause an MI. When blood supply is deprived in a region of the heart, metabolic waste products accumulate and cellular energy requirements cannot be met, thus resulting in dysfunction [22]. At first, the buildup on lactic acid from anaerobic metabolism will increase proton concentrations. This will stimulate removal via the Na^+-H^+ exchanger (NHE), which will cause a buildup of Na^+ in the cell [23]. Further elevation in intracellular Na^+ results from persistent I_{Na} passing through voltage-gated Na^+ channels [24, 25]. Together, the elevated intracellular Na^+ levels stimulate Ca^{2+} entry via reverse NCX activity (Figure 6).

Many types of arrhythmias are due to abnormal Ca^{2+} and changes in electrical excitability. The sarcoplasmic reticulum (SR) is a critical site within the cardiomyocyte for the regulation of intracellular Ca^{2+}, so it is also important to consider the effects of ω-3 PUFA on SR function. The SR stores calcium that is released during contraction. In response to trigger Ca^{2+} that enters through L-type Ca^{2+} channels (LTCC), Ca^{2+} is released into the cytosol through the ryanodine sensitive channels in the SR. Calcium is responsible for activation of many intracellular enzymes and signaling cascades and plays a pivotal role in muscle contraction. To maintain proper rhythm, Ca^{2+} must be cleared from the cytosol following contraction. Most of the Ca^{2+} is returned to the SR via the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA), and the rest is extruded from the cell via the NCX [26]. The SERCA pump is regulated by phospholamban, which must be phosphorylated in order to allow Ca^{2+} uptake into the SR via SERCA [27].

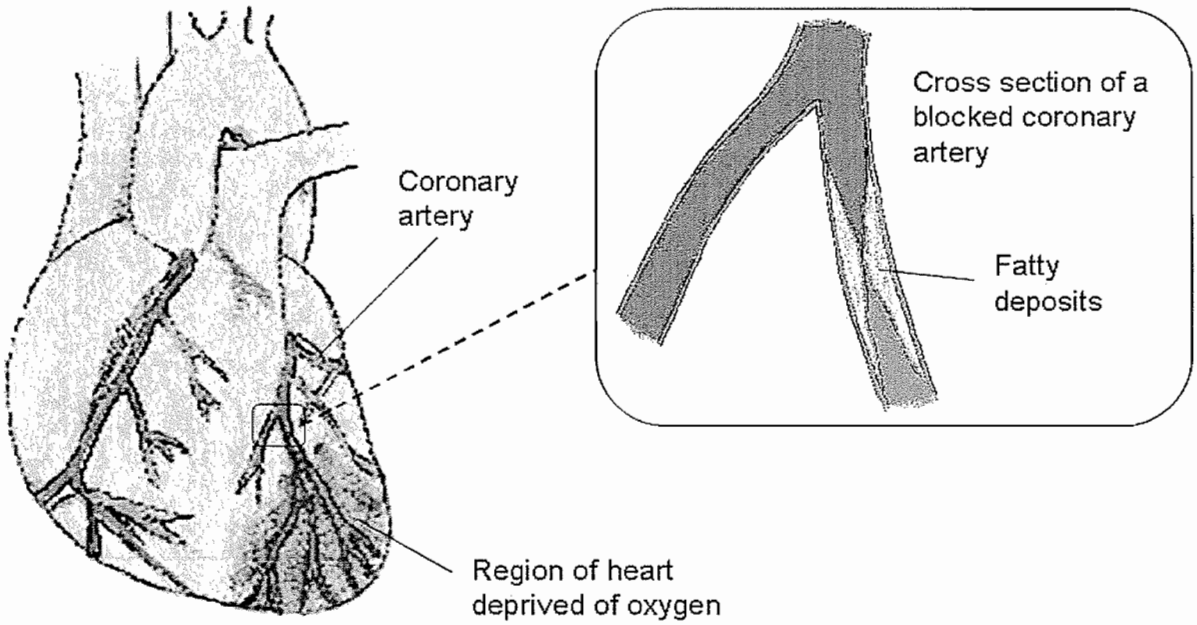


Figure 5. Regional ischemia in the heart caused by a blocked coronary artery. Luminal narrowing of a blood vessel can impair cardiac circulation and cause deprivation of oxygen and nutrients to regions of the heart, and allow buildup of metabolic by products. This can impair cardiac function and is often a factor in the onset of cardiac arrhythmias.

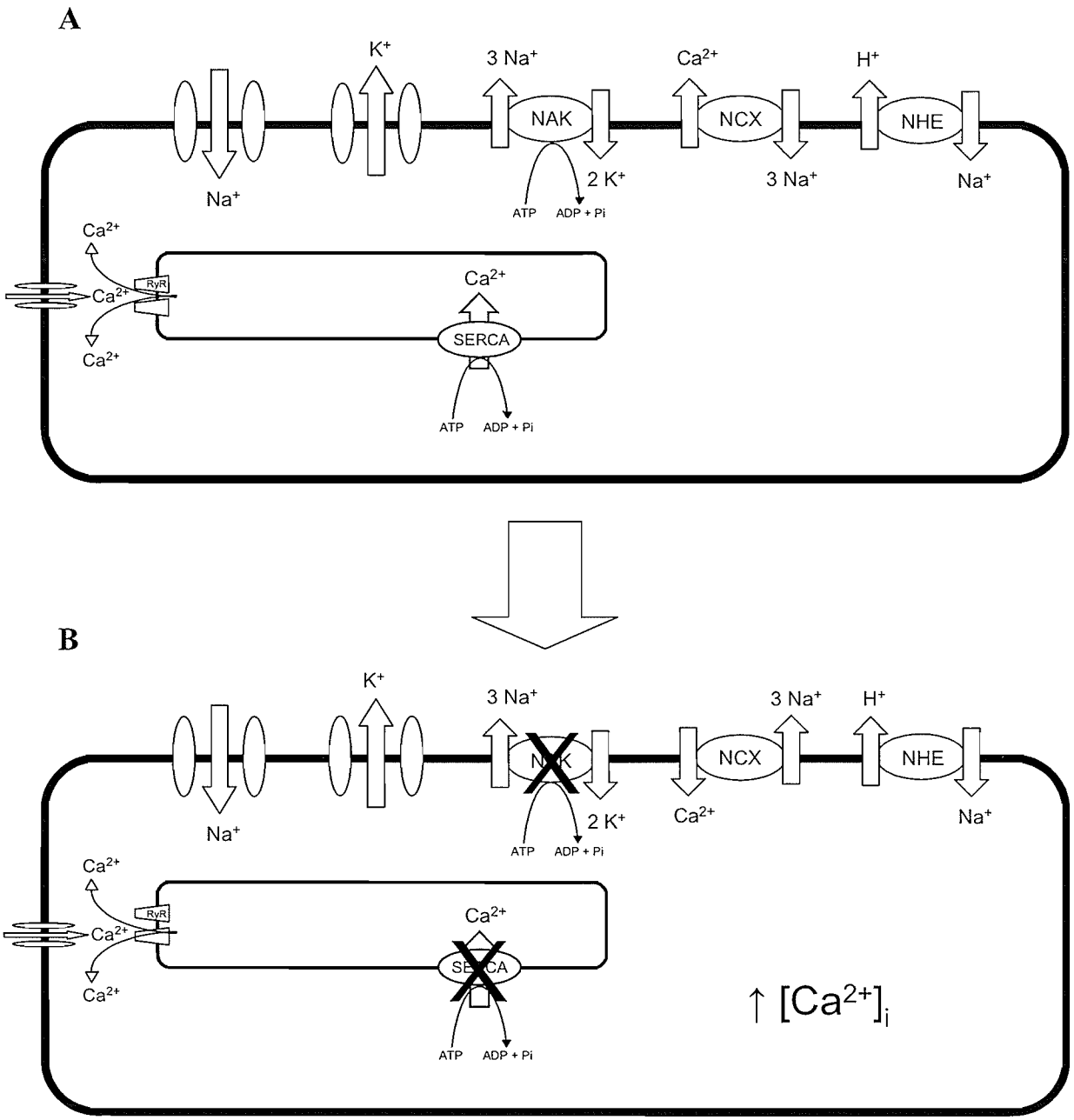


Figure 6. The primary ion channels and transporters in a myocardial cell. The NCX and SERCA are normally responsible for Ca^{2+} extrusion following each contraction (A), but during ischemia (B), lack of O_2 limits ATP production and thus, the function of the NAK and SERCA. Na^+ accumulates through persistent Na^+ entry and removal of protons by the NHE, so the NCX functions in reverse to compensate, bringing Ca^{2+} in to the cell. Combined with inhibited SERCA activity, Ca^{2+} overload results and subsequent contracture and/or potentially lethal arrhythmias occur. NAK – Na^+/K^+ ATPase; NCX – Na^+/Ca^{2+} exchanger; NHE – Na^+/H^+ exchanger; RyR – ryanodine receptor; SERCA – sarcoplasmic endoplasmic reticulum calcium ATPase.

ARRHYTHMIAS

Any disturbance to normal sinus rhythm and electrical activity of the heart is an arrhythmia. Cardiac arrhythmias can occur in atrial and ventricular tissue. The two most severe types of arrhythmias are tachycardia and fibrillation. Tachycardia is a rapid succession of four or more contractions that occur outside of the normal stimulation sequence [28]. Fewer than three successive early beats are termed premature beats or contractions. When the heart fails to follow its regular pumping pattern, the proper flow of blood may be disturbed. While the pumping of blood still can occur during tachycardia, the flow is inefficient and prolonged tachycardia, particularly in the ventricles, can lead to ischemia, heart failure or ventricular fibrillation.

Ventricular fibrillation has no discernible mechanical or electrical pattern and results in quivering heart tissue and negligible blood ejection from the ventricular or atrial chambers. Atrial fibrillation is the most common cardiac arrhythmia. Long standing atrial fibrillation can result in blood clot formation in the atrial appendages, and these clots can be expelled and cause ischemic stroke if regular rhythm is restored [29]. When the ventricles fibrillate, no blood is pumped through the body. The lack of sufficient delivery of oxygen and nutrients to tissues has lethal consequences. For example, the brain requires a constant supply of energy to function and within seconds of deprivation, an individual will have syncope [30]. For a fibrillating heart, the lack of nutrients will propagate a vicious circle of events that if not remedied, will result in death.

Mechanisms of ventricular arrhythmias

Any electrical impulse that occurs in the heart has the potential to travel through the

heart and initiate a contraction. The two broad categories that describe irregular initiation of electrical stimulation in the heart are abnormal automaticity and re-entry.

Abnormal automaticity

Usually, the pace of the heart is driven by the spontaneous activity of the SA node, which overrides any intrinsically slower pacemaker activity in the different regions of the heart. This phenomenon is known as capture. When cardiac tissue is repeatedly captured by the wave of depolarization generated from a faster pacemaker, the internal pacemaker of that tissue will become less active due to a phenomenon termed overdrive suppression, which results in a hyperpolarization of that tissue [31]. If cells within an area of the heart ultimately fail to receive a stimulus from the SA node, a formerly quiescent pacemaker could regain activity. If a region of the heart becomes ischemic, it could also spontaneously depolarize and trigger a stimulus. Depending on the timing, this depolarization can propagate throughout the heart and cause an abnormal contraction. If this ectopic focus is unable to be captured by the pace of the SA node, it may become the new dominant pacemaker of the heart, or stimulate along with the SA node under pathophysiological conditions [32]. In either case, unless the ectopic focus is located near the SA node or atria, the proper mechanical sequence of cardiac chambers can be disturbed, with pathological consequences.

Triggered activity is another mechanism for spontaneous electrical activity in the heart. When cellular diastolic Ca^{2+} levels become elevated, as during ischemia-reperfusion injury or exposure to digitalis glycosides, the NCX must increase its activity to remove Ca^{2+} [33]. This can result in a localized depolarization depending on the level of Ca^{2+} overload, which may be large enough to trigger an extra systole and cause premature contraction [34].

Re-entry

Abnormal movement of a wave of electrical activity around an anatomical or functional obstacle can be arrhythmogenic in cardiac tissue and can lead to re-entry [35]. Normally, the path of the electrical signal is in a single direction due to the refractoriness of the recently stimulated tissue. If a region of the heart has a unidirectional block of conduction, caused by ischemia-induced depolarization, for example, wave of impulse can propagate back towards its origin and prematurely re-stimulate tissue outside of the normal conduction sequence. So long as the tissue is not refractory, a self-propagating loop of electrical stimulation can result and stimulate a region of the heart to beat with an abnormal rhythm [35].

Pharmacological Treatment of Arrhythmias

The high morbidity and mortality associated with ventricular arrhythmias has led to an intense search for pharmacological treatments, over the last several decades. However, development of an effective antiarrhythmic drug has proven to be a difficult task. Potential antiarrhythmic drugs that block the fast sodium channel (I_{Na}), such as encainide, flecainide, and moricizine, or blockers of the potassium channels, like d-sotalol and quinidine, have been tested in clinical trials. However, the results of these trials revealed a higher mortality rate in the treated individuals compared to placebo [36-39], which suggests that the strategy to block single ion channels to suppress or prevent arrhythmias may be too simplistic*. The best antiarrhythmic drug in use today is amiodarone, which is a relatively non-specific

* While the prophylactic use of these drugs has proven ineffective, some antiarrhythmic drugs, such as lidocaine, may be useful in clinical settings when used under direct supervision of a physician [40].

blocker of sodium, potassium (I_{Kr} and I_{Ks}) and calcium (L-type) currents [41]. Clinical trials with amiodarone show positive results, yet the potential for a more effective antiarrhythmic drug exists, due to the side effects inherent with the use of amiodarone.

POLYUNSATURATED FATTY ACIDS AND CARDIOVASCULAR DISEASE

ω-3 PUFA may provide beneficial effects against ischemic heart disease (IHD) by limiting the atherosclerotic process, reducing the likelihood of blood clot formation, or having a direct antiarrhythmic effect. While highly controlled studies are required to identify the precise mechanisms for the protective effects, a number of studies have examined different aspects of the effect of PUFA on CVD.

EPIDEMIOLOGICAL STUDIES

A diet rich in fish has been touted as one of the key ways to promote good heart health. This conclusion is primarily based on observations that the Greenland Inuit population have a significantly lower incidence of heart disease compared to the immigrant population [42]. Another study comparing island inhabiting Japanese to the mainland population also found an inverse relationship between higher fish consumption and cardiovascular disease [43]. Blood samples showed that those who ate large amounts of fish had highly elevated EPA levels. Fatty fish and other marine foods are a rich source of ω-3 PUFA. A survey of the Inuit population of Nunavik, who consume a traditional diet of ω-3 PUFA from marine sources, found that they had a lower mortality rate from ischemic heart disease, possibly in part due to elevated HDL-cholesterol and reduced triglyceride concentrations in the plasma [44]. Recent studies have shown that ω-3 PUFA can provide

beneficial effects against platelet aggregation, dyslipidemia, atherosclerosis, and arrhythmias [45-55]. Numerous studies have investigated the mechanisms for the cardioprotective effects of EPA and DHA, the fatty acids found in fish oils. However, evidence is lacking whether the parent ω-3 fatty acid, ALA, is beneficial as well.

Analysis of the Finnish, Dutch, and Italian cohorts of the Seven Countries Study [56] revealed an inverse relationship between fatty fish consumption and 20-year CHD mortality, specifically relating to coronary artery disease (CAD) [57]. The Health Professionals Study found no relationship at all between fish consumption and CAD, but it did find a beneficial effect of dietary ALA on CAD [58, 59], whereas this link was not observed in the Dutch cohort of the Seven Countries Study [3]. The only study to show a negative effect of fish consumption on CAD was conducted in Finland, but it may have been influenced by mercury contamination of the fish [58].

CLINICAL TRIALS

Several clinical trials have examined dietary supplementation with ω-3 PUFA on various cardiovascular parameters. The GISSI-Prevenzione trial has been the largest and most controlled study to date and found that 1 g/day ω-3 PUFA (1 to 2 ratio of EPA to DHA) treatment over 3.5 years resulted in a 30% decrease in all mortality. There was a also a 30% decrease in cardiovascular deaths and a 45% reduction in sudden cardiac death [60].

Studies investigating dietary fish consumption have found beneficial effects similar to those observed in studies with ω-3 PUFA supplements. The US Physicians' Health Study followed male physicians who maintained a dietary record for 11 years and had no prior history of MI, cerebrovascular disease or cancer [61]. At least one fish meal per week

significantly reduced the occurrence of sudden cardiac death, while nonfatal MI levels were unchanged [62]. More recent studies have pointed out that the positive cardiovascular effects of fish are only associated with broiled or baked fish, rather than fish that is fried [63].

The Indian Experiment of Infarct Survival provided patients with a suspected MI, diagnosed by electrocardiogram abnormalities, enzyme levels, and reports of chest pain, with a dietary supplement of either 2 g/day of ω-3 PUFA (EPA and DHA), mustard seed oil (containing 2.9 g/day of ALA), or a placebo [64]. After one year, the number of cardiac events in the fish oil and mustard seed oil groups were lower and the incidence of nonfatal MI was significantly reduced [64]. However, current investigations put the validity of this study into question [65].

The Nurses' Health Study followed female nurses with no prior CVD or cancer for 16 years and identified a significant inverse relationship between fish consumption and CHD [66]. Dietary ALA appeared to be the most protective PUFA [62], especially against sudden cardiac death [67]. Yuan *et al.* examined fish and shellfish consumption in Chinese men and found ingestion of over 200 g/week reduced fatal MI risk by 59% over those consuming less than 50 g/week [68]. Secondary cardiovascular complications were greatly reduced by adopting a "Mediterranean" style diet in the Lyon Heart Study [69].

Perhaps the most direct evidence showing the efficacy of ω-3 PUFA as antiarrhythmic in humans comes from a small, well controlled acute study in which tachycardia was induced in patients with implanted defibrillators. In 9/10 cases, the intravenous injection of fish oils immediately restored normal cardiac rhythm [70]. While studies such as this directly show the antiarrhythmic potential of ω-3 PUFA, obvious ethical concerns limit the number of such studies.

Not all trials have found a positive correlation between intake of ω-3 PUFA and cardiovascular disease. In patients who recently survived an MI, capsules providing both ~575 mg/day of EPA and ~1150mg/day DHA had no beneficial effect on the incidence of cardiac events when compared to a corn oil (ω-6 PUFA) control [71]. However, this may reflect the relatively small sample size or the possibility that the ω-3 rich regular Norwegian diet did not allow for a true "control" population [72].

Recent studies in patients with implantable cardiac defibrillators who are at high risk for cardiac events generally fail to show any significant benefits from fish oil supplements [73, 74]. The Health Professionals Follow-up Study found no reduction in risk of CHD in men without established CVD by increasing the number of fish meals per week beyond one or two [75].

Increased fish intake and ω-3 PUFA supplementation are not without potential adverse effects. Grossly elevated intake of fish oil may cause an increased risk of bleeding by inhibiting clot formation [3, 58, 76]. Fish oil capsules can also cause minor gastrointestinal disturbances and eructation [3]. Recent studies also report that fish may contain methyl mercury (and other contaminants) that can negatively affect CHD [76]. Finally, unsaturated FA, including ω-3 PUFA, are prone to oxidation. An antioxidant may need to be added to concentrated ω-3 PUFA supplements to prevent the production of damaging lipid peroxides [77].

Ultimately, ω-3 PUFA may protect against cardiovascular disease by several mechanisms. These include an anti-atherogenic effect [2, 78], lowering serum triglycerides [2], lowering blood pressure [76, 79], improving endothelial function [76], reducing inflammatory responses [76], inhibiting platelet aggregation and thrombosis [2, 76],

improving vascular relaxation [80] and decreasing the incidence of arrhythmias [2, 55, 76].

ANTI-ATHEROGENIC EFFECTS

Atherosclerosis can impair blood flow around the body and in the heart, and compromise cardiac function. Dietary factors play a significant role in the development of atherosclerosis. Long chain ω-3 PUFA provide anti-atherogenic effects in experimental and epidemiological studies [81-83]. The Study on Prevention of Coronary Atherosclerosis by Intervention with Marine Omega-3 fatty acids demonstrated that consumption of 1.65 g/day of a fish oil supplement over 2 years by patients with CAD reduced the progression and increased regression of coronary atherosclerosis [83], but not in the carotid arteries, suggesting that ω-3 fatty acids may have different effects in different vascular beds [83].

The anti-atherogenic effects of ω-3 PUFA may occur through one or more of several mechanisms such as through an anti-inflammatory action [84, 85], and/or enhancing the circulating lipid profile and limiting plaque formation. They may also modulate vascular smooth muscle cell proliferation [84] and migration. By altering the composition of cell membranes, they may alter metabolic processes such as eicosanoid biosynthesis, cell signaling and gene expression, which could also have limiting effects on plaque formation.

ANTI-AGGREGATORY EFFECTS

Narrowing of the blood vessels due to an atherosclerotic plaque can provide a setting in which a thrombus can more readily block blood flow and cause an MI. As described above, ω-3 fatty acids compete for the same elongation and desaturation enzymes as the ω-6 fatty acids. While eicosanoids derived from both parent fatty acids are pro-aggregatory,

those derived from AA (2- and 4-series) are 100-fold greater in activity than those derived from EPA (3- and 5-series) [4]. Consequently, the EPA metabolites are generally considered as anti-aggregatory. An improved balance between AA and EPA could reduce the likelihood of clot formation.

A variety of models have been used to test the effects of enriching the diet with ω-3 fatty acids. Experiments in which saturated fat or PUFA (either ω-3 or ω-6) was added to the diet obtained striking results. In response to ADP, platelet aggregation was significantly increased in plasma obtained from rabbits consuming an ω-6 rich diet compared to the ω-3 fed groups consuming fish oil (EPA and DHA) or flaxseed oil (ALA) [50]. This increased aggregation was even greater than that observed in the coconut oil group (saturated fat). The same trends applied when platelet activation was initiated by collagen, but not thrombin [50]. The inhibitory effects on platelet aggregation were directly related to the fatty acid composition of the platelet lipids. It was also hypothesized that a PUFA effect on membrane viscosity could affect the activity of the proteins in the platelet membranes that are involved in aggregation as receptors or enzymes.

Although beneficial in preventing the potential blocking of a blood vessel by a thrombus, extreme inhibition of clotting mechanisms could have side effects. Increases in bleeding times have been reported in humans with increased intake of fish oils [86, 87] but not flaxseed oil [88]. There is anecdotal evidence of people taking aspirin and ω-3 supplements experiencing hematuria and spontaneous nosebleeds likely resulting from severe effects on platelet aggregation [89].

ANIMAL STUDIES

Initial experiments performed on isolated hearts in the early 1980s found that PUFA raise the electrical threshold required to induce ventricular arrhythmias and offset the hypoxia-induced reduction of threshold. [90]. Animals consuming ω-3 fatty acids exhibited significant reductions, or even abolition, of arrhythmias compared to control groups [54, 91]. Direct intravenous injection of an emulsion of concentrated fish oil proved effective at preventing fatal ventricular fibrillation in dogs subjected to exercise stress tests following a coronary artery ligation [53]. Follow-up studies showed injections of purified EPA and DHA, as well as the parent ω-3, ALA, were equally protective against VF [45]. The anti-arrhythmic effects of ω-3 fish oils have also been demonstrated in non-human primates [92]. Some studies report a reduced incidence of cardiac arrhythmias in groups receiving ω-6 PUFA supplementation [54, 93], whereas others show no protective effect [91]. The difference in findings may be due to the duration of the feeding trials, as no effect was observable after 4 weeks of feeding compared to feeding for 10 or 12 weeks. However, the protective effects of ω-3 PUFA are consistently greater than the ω-6 PUFA. Subsequently, much work has focused on the ω-3 PUFA found in fish oils, EPA and DHA, due to the overwhelming epidemiological evidence relating the intake of fatty fish to the decrease in cardiovascular disease (see above).

CARDIAC ACTION POTENTIAL

The cell membrane is permeable to different ions in varying degrees. Due to the unequal distribution of ions and maintenance of these gradients by ionic pumps, an electrical potential exists across the membrane. For example, ventricular myocytes have a resting

potential of –85 mV. While many cell types in the heart have some degree of pacemaker activity, the cells located in the sinoatrial (SA) node drive the overall heart rate. Ion channels reside in all cardiac cell membranes and these channels are ligand or voltage sensitive. Cells of the SA node spontaneously depolarize via the entrance of Na^+ and K^+ ions (known as I_f or "funny" current) and eventually the cells reach threshold and a cascade of channel openings and closings results [33]. Cardiac cells are connected by gap junctions, which electrically couple cells in each region of the heart to one another [94]. The resulting pattern of membrane potentials that occur due to the movement of ions through the rapidly opening and closing channels in the heart is termed the action potential. Action potentials in the heart have five characteristic phases, termed phase 0-4 (Figure 7).

Phase 0, the rapid depolarization of the cell, is characterized by the sharp upstroke of the action potential. This depolarization is due to the rapid influx of Na^+ through voltage-gated Na^+ channels (I_{Na}) that occurs when cells reach a threshold potential of –70 mV. This depolarizing upstroke can bring cells up to a peak potential of +30 mV. L-type Ca^{2+} channels are activated as cells reach –40 mV, producing further inward current but with a slower time course [95].

Phase 1 is characterized by a notch in the action potential. A strong outward current is produced by the efflux of K^+ from the cell via activated transient outward potassium channels (I_{to1}) and Cl^- entry via Ca^{2+}-dependent outward Cl^- channels (I_{to2}). Due to the heterogeneous distribution of some ion channels across the ventricular myocardium, not all ventricular cells will exhibit the notch characteristic. Endocardial tissues have little I_{to1}, and therefore do not exhibit the same notch characteristic as action potentials in the epicardial tissue [96].

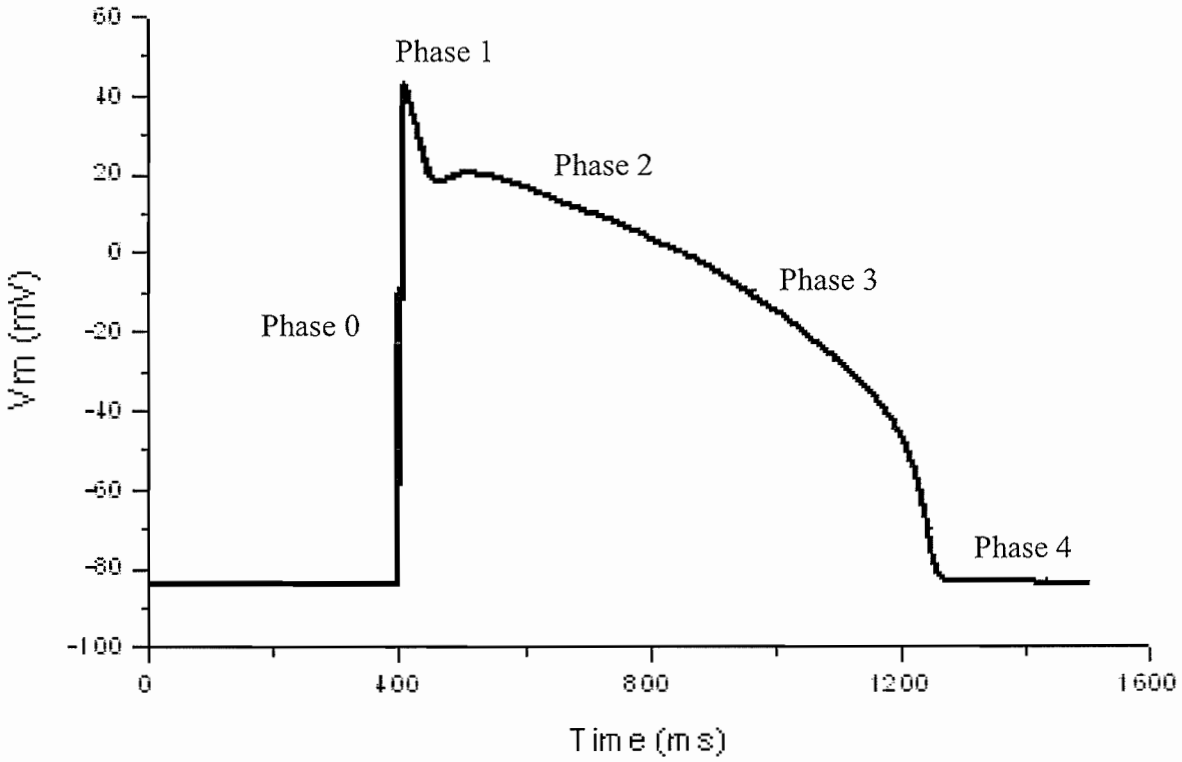


Figure 7. The five phases of the cardiac action potential (0-4).

Phase 2 of the action potential is observed as a plateau in current. The plateau in membrane potential is sustained by Ca^{2+} entry through voltage activated L-type Ca^{2+} channels and K^+ exit through the activated I_{to} and newly activated delayed rectifier channels (I_K). Systole, or contraction of the heart, is coupled to this rise in intracellular Ca^{2+}. Two components comprise the delayed rectifier current: the early activating, rapidly declining component, I_{Kr}, and the slowly activating, slowly declining component, I_{Ks}. I_{Kr} is heterogeneously expressed across the ventricle, with a greater abundance in the epicardium [97]. L-type Ca^{2+} channels close near the end of phase 2.

Phase 3 is the repolarization of cardiac cells. The continued efflux of K^+ through the rapid and slow delayed rectifier channels combined with the closure of Ca^{2+} channels returns cells towards their resting potential. The repolarization of epicardial tissue is more rapid than endocardial tissue due to the heterogeneity previously discussed which causes the action potentials to be shorter in the epicardium compared to the endocardium. Enhancing the repolarization, inward rectifying K^+ channels (I_{k1}) are opened during phase 3 as well, passing K^+ out of the cell

Phase 4 is the resting phase of the cardiac cell. The resting potential is maintained by the background efflux of K^+ through I_{k1} channels. This phase corresponds to diastole, or the cardiac relaxation phase.

Different species exhibit distinct patterns of cardiac ion channel expression. The human action potential described above pertains to primates and other species such as rabbit, dog and pig. Rats have very high expression of I_{to1} in the heart and thus lack a characteristic plateau and abruptly repolarize [98]. Conversely, guinea pig cardiomyocytes lack I_{to} expression and do not show a notch corresponding to Phase 1 [98]. These interspecies

differences prove useful for the study of individual ion channel characteristics.

Action potentials propagate through the heart starting at the SA node, then move through the atria, atrioventricular (AV) node, bundle of His and Purkinje fibres to the ventricular myocardium [33]. Conduction through the AV node is slow and this delay is critical to allow ventricular filling time before their stimulation and contraction. Normal conduction of the electrical signal through the heart is required to maintain proper mechanical function. Should aberrant conduction through the normal cardiac electrical circuit occur, disturbances to the mechanical rhythm of the heart can produce damaging or even fatal effects.

SODIUM-CALCIUM EXCHANGER

STRUCTURE

The NCX is a membrane spanning protein that consists of nine transmembrane segments with a large intracellular loop existing between the 5^{th} and 6^{th} domains [99, 100]. As an antiporter, the NCX will transport 3 Na^+ in exchange for a single Ca^{2+} [101]. Since this charge or the ions transported does not balance, operation of the NCX is electrogenic and produces a net current in the direction of Na^+ movement. The NCX operates bidirectionally depending on the electrochemical gradients of Na^+ and Ca^{2+}. When Ca^{2+} is being removed from the cell, the NCX is said to be operating in "forward mode", while it is operating in "reverse mode" when it brings Ca^{2+} into the cell (Figure 8). No energy source is required for the NCX to operate.

Different isoforms of the NCX are found throughout the body and differ in their expression pattern and/or regulatory characteristics. The NCX is encoded by three different

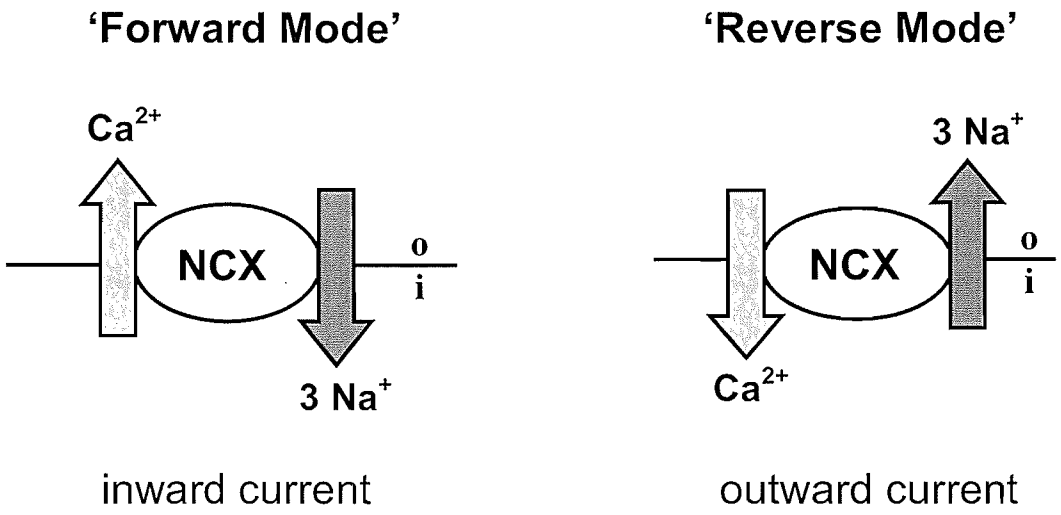


Figure 8. Illustration of forward (Ca^{2+} removal) and reverse mode (Ca^{2+} entry) sodium-calcium exchanger activity. Due to the unbalanced charge carried across the membrane, forward mode NCX activity produces an inward (depolarizing) current while reverse mode NCX activity produces an outward (repolarizing) current.

genes [100]. The most common isoform is NCX1, which is expressed in several tissues of the body. A region of the protein near the carboxyl terminus of the intracellular loop may undergo alternative splicing to produce a number of splice variants that are differentially expressed and regulated. Each splice variant is composed of 2-5 of six exons, designated A-F [100]. Each isoform of the NCX is encoded by one of two mutually exclusive exons (A or B), and at least one additional exon (C-F). For example, the cardiac isoform, NCX1.1, is made up of ACDEF, whereas the vascular isoform, NCX1.3, is composed of BD. Expression levels of NCX can even vary within the same tissue. For example, the amounts of NCX expressed across the ventricular wall are greater in the epicardial layer of tissue than the endocardial layer [102]. This distribution can be disrupted by NCX upregulation in heart failure, leading to increased susceptibility to arrhythmias.

The intracellular loop of the NCX contains many of the regulatory domains of the exchanger. The NCX is regulated by both intracellular Na^+ and Ca^{2+}. When intracellular Na^+ levels are high, activity of the NCX will be limited, through a process called I_1 inactivation [103]. Small levels of regulatory Ca^{2+} are required in order for the exchanger to operate. This is known as I_2 regulation [104]. The exchanger inhibitory peptide (XIP) is a 20 amino acid peptide that matches a sequence in the intracellular loop of the exchanger. Inhibition of the exchanger results when XIP is incubated with the NCX [105]. How this inhibition occurs is not understood.

FUNCTION

The primary role of the NCX is to maintain cellular Ca^{2+} homeostasis [106]. In the heart, the NCX is responsible for extruding the Ca^{2+} that enters through the L-type Ca^{2+}

channels on a beat-to-beat basis during excitation-contraction coupling. In the vasculature, the NCX helps restore resting Ca^{2+} levels and relax tissue following an agonistic response. The cardiac glycosides, such as digitalis, produce their inotropic effect via an indirect effect on the NCX. Ouabain, for example, targets the Na^{+}-K^{+} ATPase and when the intracellular Na^{+} concentrations rise, reverse activity of the NCX will ensue to remove the Na^{+}, but at the same time increasing the intracellular Ca^{2+} concentrations [107].

INHIBITORS OF THE SODIUM-CALCIUM EXCHANGER

Several pharmacological inhibitors of the NCX have been developed over the last several years, yet there are no results published showing efficacy beyond the basic laboratory setting. The ideal NCX inhibitor would not affect forward NCX activity, but would preferentially inhibit the NCX when reverse mode activity was induced. Nickel is not transported by the NCX and can be used to block NCX activity [108], but it is only useful in a laboratory setting. KB-R7943 was the first selective NCX inhibitor developed [109, 110]. This drug binds preferably to the NCX when it is in the I_1 state, which is related to reverse mode activity [111]. KB-R7943 exhibited a significant antiarrhythmic effect [112], but it also acts on other channels at higher concentrations [113-115]. SEA0400 was developed subsequently and has a much lower IC_{50} value and is more specific for the NCX than KB-R7943 [116]. Again, SEA0400 binds preferably to the I_1 state of the exchanger and exhibits antiarrhythmic effects in the laboratory setting [117]. The most recently produced inhibitor of the NCX is SN-6 [111]. This new drug binds to the exchanger under ATP-depleted conditions and may be useful in ischemia-reperfusion injury [118].

EFFECTS OF POLYUNSATURATED FATTY ACIDS ON ION CHANNELS

IN VITRO EXPERIMENTS

The sarcolemmal membrane contains a variety of ion channels, exchangers and pumps important to the conduction of action potentials and the maintenance of ionic gradients. Voltage-gated sodium channels (VGSC), potassium channels, and calcium channels are responsible for the initiation, duration and propagation of the action potentials. Effects of ω-3 PUFA on these proteins are summarized in Table 1. Generally, in cultured neonatal cardiomyocytes, perfusion with ω-3 and ω-6 PUFA raises the threshold potential required for action potential initiation, polarizes the resting membrane potential, and shortens action potential duration [119]. All of these effects could help the heart maintain electrical stability during ischemia and reduce the likelihood of arrhythmogenesis.

Sodium channels

Treatment of neonatal cardiac myocytes with PUFA increases the voltage threshold required for opening of the Na^+ channel [119]. Raising the threshold of Na^+ channel activation would make the cell less excitable and therefore less prone to spontaneous stimulations that could induce arrhythmias. The peak Na^+ current is also significantly decreased by ω-3 fatty acids [120, 121].

Acute *in vitro* experiments have predominantly found inhibitory effects of ω-3 PUFA on cardiac Na^+ currents [120, 122-124]. Investigators have even determined specific sites on the Na^+ channel that, when blocked or mutated, abolish the inhibitory interaction with ω-3 PUFA [125, 126]. Interestingly, two reports have studied the effects of unsaturated fatty acids on human Na^+ channels and found that internal application of AA augmented Na^+

Table 1: Summary of effects of ω-3 PUFA on ion channels and transporters.

Channel/ transporter	ω-3 PUFA tested	Effect on ionic current/activity	References
SL			
VGSC	ALA	↓	[120, 121, 129]
	EPA	↓	[120, 121, 126, 129]
	DHA	↓	[121, 124, 129]
$Ca^{2+}_{L\text{-type}}$	ALA	↑*, ↓'	[130]* [131]'
	EPA	None*, ↓'	[132, 133]* [121]'
	DHA	None*, ↓'	[132, 133]* [121]'
I_{to}	ALA	↓	[134]
	EPA	↓	[121, 134]
	DHA	↓	[121, 134]
I_K	ALA	↓	[134]
	EPA	↓	[134]
	DHA	↓	[134, 135]
I_{k1}	ALA	None*	[134]*
	EPA	None*, ↓'	[134, 136]* [121]'
	DHA	None*, ↓'	[134],[136]* [121]'
I_{sus}	EPA	↓	[136]
	DHA	↓	[136]
K_{ATP}	ALA	↓	[137]
TRAAK	EPA	↑	[138]
	DHA	↑	[138]
TREK	EPA	↑	[138]
	DHA	↑	[138]
NHE	EPA	↓	[139]
	DHA	↓	[139]
NCX	ALA	↑	[140]
	EPA	↓	[141]
	DHA	↓	[141]
SR			
RyR	EPA	↓ open state	[142, 143]
SERCA	EPA	↓	[144]
	DHA	↓	[144]

ALA - α-linolenic acid; DHA – docosahexaenoic acid; EPA – eicosapentaenoic acid; NCX – Na^+/Ca^{2+} exchanger; NHE – Na^+/H^+ exchanger; PUFA – polyunsaturated fatty acids; RyR – ryanodine receptor; SERCA – sarcoplasmic endoplasmic reticulum calcium ATPase; SL – sarcolemma; SR – sarcoplasmic reticulum; VGSC – voltage-gated sodium channel.

current, but external application reduced Na^+ current [127, 128]. While no ω-3 PUFA were tested, these studies do suggest that there may be at least two distinct mechanisms by which fatty acids interact with ion channels.

On its own, inhibition of Na^+ currents would slow conduction velocities and allow arrhythmogenic re-entrant circuits to develop. This, of course, is potentially dangerous. In theory, the targeting and inhibition of VGSC was a viable strategy in the design of antiarrhythmic drugs. However, the CAST trials demonstrated a greater incidence of death in patients treated with Class I drugs compared to placebo [37]. Despite this apparently undesirable effect, it is readily apparent that ω-3 PUFA are safe. Like amiodarone, which is currently the most effective drug for treatment of cardiac arrhythmias, the ω-3 fatty acids have blocking effects not only on cardiac Na^+ channels, but also on Ca^{2+} and K^+ channels, which are discussed below. This broad effect may be part of the reason why ω-3 PUFA have such a potent antiarrhythmic effect.

Calcium channels

L-type Ca^{2+} current plays an important role in the plateau of the cardiac action potential (phase 2) and influences its duration. Typically, Ca^{2+} enters the cell through voltage-gated Ca^{2+} channels and triggers the release of Ca^{2+} from the SR, which is required for contraction of the heart. The reported effects of ω-3 PUFA on the L-type Ca^{2+} channel are varied, but show either an inhibition or no effect at all on Ca^{2+} current. For example, some evidence suggests ω-3 PUFA directly inhibit the Ca^{2+} current through L-type Ca^{2+} channels [131, 145]. This inhibition could reduce the incidence of Ca^{2+} release from the SR and act to limit Ca^{2+} overload. Other studies that observed no direct effects of ω-3 PUFA on

L-type Ca^{2+} current have still discovered an interaction with the channel, related to the maintenance of normal function in the presence of channel agonists or antagonists. In neonatal cardiomyocytes, DHA was able to block the effects of the L-type Ca^{2+} channel agonist, Bay K8664, and the antagonist, nitrendipine [132, 133]. It did not block effects of the L-type Ca^{2+} channel antagonists verapamil and diltiazem [132]. Since the latter two drugs block the channel at sites other than the dihydropyridine binding site [146], this evidence suggests that ω-3 PUFA interact with the L-type Ca^{2+} channel at a functionally associated, but different site, which can affect binding to the dihydropyridine site. Similarly, in adult cells, DHA almost completely prevented the effects of isoproterenol [147]. Interestingly, in this study, administration of DHA alone did not result in a blockade of Ca^{2+} current. Rather, the channels were regulated so as to provide the proper influx of Ca^{2+} required for normal release of Ca^{2+} from the SR.

Potassium channels

Potassium channels are largely responsible for determining the duration of the cardiac action potential and maintaining the cellular resting potential. The delayed rectifier channels (I_K) are primarily responsible for repolarization of the cells in the later phases of the cardiac action potential. PUFA can inhibit I_K channels [134, 135], but it seems this effect can only be achieved with application of the fatty acid to the external surface of the cell [148]. Blocking the repolarizing current would result in a prolongation of the action potential and increased refractoriness of the heart. This would aid in the prevention of re-entry mechanisms of arrhythmia, since impulses emerging from areas of slow conduction would encounter refractory tissue. However, the PUFA concentrations required to have similar

inhibitory effects on I_K (IC_{50} = 20 μM) are about 4 times greater compared to I_{Na} (IC_{50} = 5 μM) and 20 times greater compared to Ca^{2+} (IC_{50} = 2 μM) [120, 131, 134]. This would suggest that PUFA exert the greatest antiarrhythmic effect through an action on Ca^{2+} channels, followed by Na^+ channels, and lastly by K^+ channels.

Transient outward K^+ channels (I_{to}) are also blocked by PUFA [121, 134, 136]. These K^+ channels are responsible for the very rapid and large outward K^+ current that opposes the inward flow of Ca^{2+} and Na^+ that depolarize the cell. The notch observed at phase 1 of the action potential characterizes the current through this channel. Another K^+ channel that is more sustained than I_{to} and is also activated upon depolarization is known as I_{sus}. It is also inhibited by DHA [136]. Both these outward currents play a key role in the repolarization of the cell, and their blockade results in a prolongation of the action potential. As mentioned previously, the increased refractoriness of the tissue would inhibit the creation of re-entrant circuits.

The ATP-sensitive K^+ channel (K_{ATP}) is normally inactive when intracellular [ATP] is normal, but under conditions that reduce the cytosolic [ATP], such as ischemia, the channels are opened and pass an outward current. These channels are also blocked by ω-3 PUFA [137]. However, not all of the K^+ channels are affected by ω-3 fatty acids. The inward rectifier K^+ channel (I_{K1}) does not appear to be influenced by ω-3 PUFA [134, 136]. Since these channels are activated by repolarization, the normal or slightly depolarized membrane potential is maintained even though other K^+ channels are blocked.

The two-pore domain K^+ channels are a class of channels that have drawn recent attention because of several unique characteristics. One of the first of these channels to be discovered and characterized was the TRAAK channel. This outward current K^+ channel is

stimulated by AA as well as an acidic pH [149], which would be of aid in an ischemic environment. A more recently discovered member of this two-pore family is TREK-1. It also is stimulated by PUFA and decreased pH, but appears to be activated by an even wider range of stimuli. The net effect of the activation of these channels is shortening of the action potential or hyperpolarization. This may help to reduce the excitability of the heart and limit the release of Ca^{2+}.

Sarcolemmal ion pumps and transporters

During ischemia, intracellular H^+ accumulates under anaerobic metabolism. This stimulates the Na^+/H^+ exchanger (NHE) to remove H^+ from the cell in exchange for Na^+. The concomitant rise in intracellular Na^+ stimulates the reverse mode operation of the NCX whereby three Na^+ ions are removed from the cell in exchange for the entry of a single Ca^{2+} ion. This can result in arrhythmias and/or cell death (see Figure 3). The forward mode of the NCX (Ca^{2+} outward) results in a net flux of inward charge. When the intracellular $[Na^+]$ decreases and the intracellular $[Ca^{2+}]$ increases, the exchanger will function in forward mode. The electrogenic operation of the NCX can result in transient depolarizations, which are a mechanism for delayed after-depolarizations that can produce premature beats or ventricular tachycardia [150].

Although studies are limited, ω-3 PUFA appear to inhibit the NHE. This inhibitory effect, however, is limited to the longer ω-3 fatty acids, as ALA does not affect the exchanger [139]. Data regarding the effects of ω-3 PUFA on the NCX are conflicting. Early work showed that NCX is significantly stimulated by ALA [140]. The authors speculated that the negative charge of the free fatty acids facilitates binding of Ca^{2+} to the exchanger and

increases exchange rates. The effects of the longer ω-3 fatty acids were not studied. However, EPA was recently shown to inhibit NCX current measured in HEK293 cells transiently expressing the exchanger [141]. The effects of ALA were not examined in this study.

PUFA inhibit Na^+/K^+ ATPase activity [151]. This effect would not be beneficial under normal physiological conditions. Intracellular Na^+ levels would rise with Na^+ pump inhibition and this would stimulate reverse NCX and elevate the intracellular $[Ca^{2+}]$. However, compounds such as digitalis, which inhibit the Na^+/K^+ ATPase, are used for patients with heart failure in order to strengthen the force of contraction [152]. This positive inotropic effect may be beneficial when cardiac function is already impaired.

Cardiac sarcoplasmic reticulum

Uptake of Ca^{2+} via SERCA is more efficient in the presence of ω-3 PUFA. In rabbit cardiac SR isolated from rabbits consuming flaxseed, Ca^{2+} is sequestered faster and requires less energy [153]. This enhanced uptake is in direct contrast to the depression in SR function that accompanies increased cholesterol incorporation into the SR [154-157].

During ischemia, damage to cardiomyocytes can result in cellular instability due to a change in the regulation of Ca^{2+}-induced Ca^{2+} release from the SR. Increases in cytosolic Ca^{2+} can activate phospholipases that cleave phospholipids from the cell membrane [158]. Depending on the fatty acid content of the membrane, ω-3 PUFA could be released to exert their effects on nearby channels, potentially regaining some electrical stability in the cell. PUFA also directly inhibit the Ca^{2+} release channel of the SR, the ryanodine receptor (RyR) [142]. This is important, particularly in post-ischemic settings in which the SR is overloaded

with Ca^{2+}. In a model of elevated intracellular Ca^{2+}, addition of EPA to cardiomyocytes reduced the frequency of spontaneous Ca^{2+} waves from the SR, but slightly increased the total Ca^{2+} that was released with each wave [142, 143]. The net effect was a decrease in intracellular Ca^{2+} released from the SR over the same time period compared to controls. The inhibition of the RyR by EPA was apparent when the ω-3 PUFA were removed from solution and the frequency of spontaneous Ca^{2+} waves increased above control levels [143]. This indicates that the SR contained an elevated amount of Ca^{2+} and that EPA directly inhibited its release via the RyR. The reason for the higher than normal stores of Ca^{2+} could be either due to increased uptake of Ca^{2+} through the SERCA and/or a reduced leak of Ca^{2+} from the SR.

CHAPTER III: HYPOTHESIS

The central hypothesis for this thesis is that dietary flaxseed is cardioprotective and that this protection is due to the effects of its α-linolenic acid content. Moreover, we hypothesize that dietary flaxseed exerts its antiarrhythmic effect by altering electrophysiologic characteristics of cardiac cells.

CHAPTER IV: MATERIALS AND METHODS

DIETARY STUDIES

DIET AND FEEDING

All experiments conform to the guidelines of the Canadian Council on Animal Care concerning the "Care and Use of Experimental Animals" [159]. Male New Zealand White rabbits were housed in individual cages at constant room temperature and kept on a 12-h light-dark cycle. Rabbits (2.8 ± 0.1 kg) were randomly assigned to receive one of four prepared diets; regular (REG) – standard rabbit ration (Co-op Complete Rabbit Ration, Federated Co-operatives Limited, Saskatoon, SK, Canada), flaxseed (FLX) – standard ration containing 10% ground flaxseed (wt/wt, ALA comprises 70% of total fatty acids compared to 55% in regular seed, Promega Flax from Polar Foods Inc., Fisher Branch, MB, Canada), cholesterol (CHL) – standard ration containing 0.5% cholesterol (wt/wt), and cholesterol plus flaxseed (CHL/FLX) – standard ration containing both 0.5% cholesterol and 10% ground flaxseed.

Diets were prepared by grinding the required amount of standard ration, and then mixing in the appropriate dietary components. Flaxseed was ground prior to mixing it into the diet. Once mixed, the diets were moistened, repelleted, and then fan-dried (Figure 9). All experimental diets were kept refrigerated and protected from light. The concentration of flaxseed used in this study (10%) is similar to that used in other animal studies to show health-related benefits [160-163]. Nutritional composition of the diets differed only in the total fat content resulting from inclusion of flaxseed (Table 2). The specific fatty acid composition of the diets is shown in Table 3. Each rabbit was fed 125 g/day of diet for 8 or

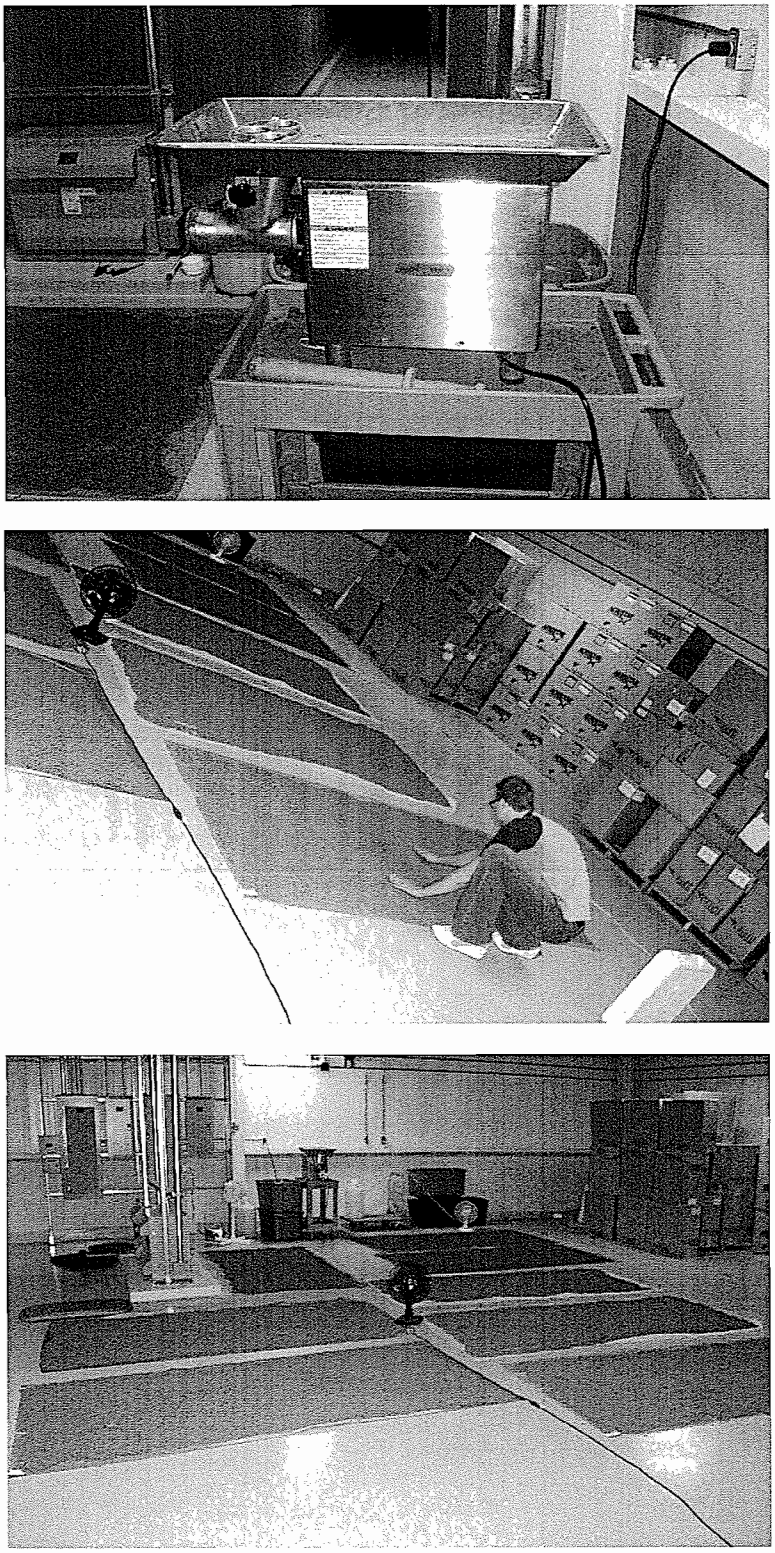


Figure 9. Commercial rabbit chow was moistened and ground using a large meat grinder and mixed with the appropriate additives, repelleted using the grinder, spread out and fan dried.

Table 2. Nutritional composition of the diets.[1]

	REG	FLX	CHL	CHL/FLX
		g/kg dry diet		
Crude Protein	21.3	20.5	20.4	20.5
Carbohydrates	51.4	51.7	52.5	50.6
Crude Fat	5.4	8.1	5.2	8.9
Crude Fibre	13.5	11.7	13.6	12.4
Ash	8.4	8.1	8.1	7.7

[1] Abbreviations: REG, regular chow; FLX, 10% flaxseed chow; CHL, 0.5% cholesterol chow; CHL/FLX, 0.5% cholesterol plus 10% flaxseed chow.

Table 3. Fatty acid composition of the diets.[1]

Fatty Acid	Diet			
	REG	FLX	CHL	CHL/FLX
	g/100 g fatty acids			
14:0	0.8	0.6	0.7	0.5
16:0	18.3	14.2	17.6	13.4
18:0	6.2	5.9	5.7	5.2
16:1 (ω-9)	1.1	0.8	1.0	0.6
18:1 (ω-9)	30.0	25.4	28.6	23.4
18:1 (ω-7)	4.8	4.1	4.6	3.7
22:1 (ω-9)	0.1	0.4	0.3	0.1
18:2 (ω-6)	31.4	17.5	34.3	19.6
18:3 (ω-3)	5.6	29.9	5.9	32.2

[1] Fatty acids not shown represent less than 0.5 g / 100 g fatty acids in the diet. Abbreviations: REG, regular chow; FLX, 10% flaxseed chow; CHL, 0.5% cholesterol chow; CHL/FLX, 0.5% cholesterol plus 10% flaxseed chow.

16 weeks. This amount was determined based on the daily food consumed in a pilot study and the recommended ration to meet nutritional requirements.

TISSUE SAMPLING AND ANALYSIS

Blood collection

Blood was drawn from the left marginal ear vein of fasted rabbits at 0 and 8 or 16 weeks, and collected into Vacutainer tubes containing EDTA (Becton Dickinson, Oakville, ON, Canada). Blood samples were centrifuged at 4500 × g for 10 min at room temperature and plasma was stored at -80°C until analyzed. Prior to analysis, plasma samples were thawed and centrifuged at 6800 × g and chemistry test slides were brought to room temperature. Plasma levels of cholesterol and triglycerides were analyzed using the VetTest 8008 blood chemistry analyzer (IDEXX Laboratories Inc., Westbrook, ME, USA).

Extraction and derivatization of fatty acids from plasma

Fatty acids were extracted from plasma and derivatized using the method of Lepage and Roy [164]. Briefly, 100 µl of plasma was added to 2 ml of methanol-benzene (4:1) in a test tube. While vortexing, 200 µl of acetyl chloride was added to the tube. The tubes were sealed and heated to 90°C for one hour. Five ml 6% K_2CO_3 was then added to neutralize the solution and the upper benzene layer was removed for analysis.

Extraction and derivatization of fatty acids from tissue

A separate set of rabbits (n=5 per group) were fed the four diets described above for 8 weeks. The heart, aorta, gastrocnemius muscle, liver, kidney, and brain were isolated from

the rabbits, flushed with phosphate buffered saline (PBS) containing (mM): KCl 2.7, KH_2PO_4 1.5, NaCl 136.9, Na_2HPO_4 4.3 adjusted to pH 7.4, and quick frozen in liquid nitrogen before storage at -80°C. Lipids were extracted from the tissues and derivatized using the method of Folch *et al.* [165]. One gram of each tissue was minced and homogenized by Polytron on ice in 20 ml of chloroform-methanol (2:1). The homogenate was combined with 4.2 ml 0.73% NaCl in a separatory funnel, mixed vigorously and allowed to separate overnight. The chloroform layer was then dried with Na_2SO_4, filtered and evaporated by rotary evaporation. The lipids were then resuspended and diluted to 1.0 mg/ml in chloroform. Nitrogen gas was used to evaporate 100 µl of this extract in a test tube to which 1.0 ml 7% boron trifluoride-methanol was added. The tube was sealed with a Teflon cap and heated to 90°C for one hour [166]. The tube and contents were cooled to room temperature and the methylation was terminated by the addition of 1.0 ml H_2O. The fatty acid methyl esters were then extracted into hexane, washed with water, dried with Na_2SO_4, filtered, concentrated under nitrogen gas and finally resuspended in hexane.

Extraction and derivatization of fatty acids from isolated cardiomyocytes

Fatty acids were extracted from the diet-treated rabbit cardiomyocytes and derivatized using the same one-step method described earlier for plasma in which 100 µl of cell suspension was used [164]. In order to quantitatively determine the fatty acid composition of the cardiomyocytes, cell aliquots were centrifuged in a microcentrifuge at 3500 × g prior to the extraction/derivatization. The volume of each pellet was recorded and then the pellets were resuspended in a known volume of PBS in order to obtain a comparable dilution factor.

Gas chromatographic separation of fatty acid methyl esters

A Varian CP-3800 gas chromatograph (Mississauga, ON, Canada) equipped with a flame ionization detector and Varian CP-Sil 88 capillary column (50 m × 0.25 mm × 0.20 μm) was used to analyze 0.5 μl of each extract, which were injected with a CP-8400 autosampler at a split ratio of 1:100. Flow rate of the helium carrier gas was 1 ml/min. The initial oven temperature was held at 80°C for 1 min, raised to 140°C at 30°C/min, and then raised to 225°C at 5°C/min and held for 10 min. The total run time for each sample was 30 min. Components were identified by comparison with authentic standards (Nu-Chek Prep, Elysian, MN, USA).

ISCHEMIA-REPERFUSION OF RABBIT HEARTS

Rabbits were anaesthetized with isoflurane and hearts were rapidly excised and placed into Tyrode's solution of pH 7.4 containing (mM): NaCl 115.0, $NaHCO_3$ 28.0, NaH_2PO_4 0.5, glucose 20.0, KCl 4.0, $CaCl_2$ 2.0, and $MgCl_2$ 0.7. The aorta was tied onto the cannula of the perfusion apparatus (Figure 10). Retrograde perfusion of the hearts was initiated within 60 s of excision. Hearts were perfused at a flow rate of 20 ml/min with Tyrode's solution maintained at 37.0 ± 0.5°C and bubbled with 95% O_2/5% CO_2.

INSTRUMENTATION OF THE HEART

Hearts were instrumented as previously described [167]. Briefly, the right atrium was removed, and the atrioventricular node was crushed to allow pacing of the heart. Electrodes were placed high into the right ventricle and hearts were paced at 2 Hz for the duration of the experiment (Figure 10). Temperature of the heart was maintained at 37.0 ± 0.5°C and

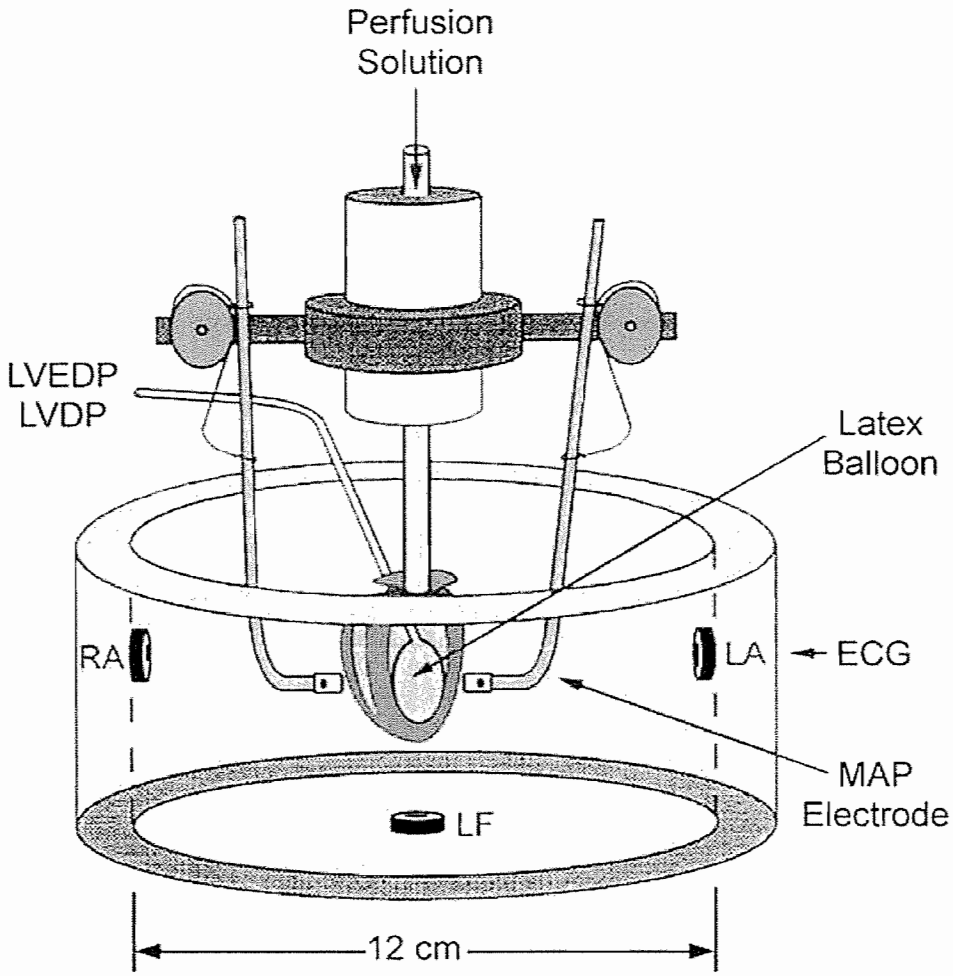


Figure 10. Diagram of the modified Langendorff Setup used for global ischemia-reperfusion of the isolated rabbit hearts. Abbreviations: LVEDP, left-ventricular end-diastolic pressure; LVDP, left-ventricular developed pressure; MAP, monophasic action potential; RA, right arm; LA, left arm; LF, left foot.

measured with a 421 thermistor probe (YSI Inc., Yellow Springs, OH, USA) inserted through the tricuspid valve into the right ventricle.

Monophasic action potentials (MAP) were recorded on the endocardial and epicardial surfaces of the left ventricle as previously described [167]. The instrumented heart was immersed in a circular acrylic bath (15 cm diameter) containing Tyrode's solution bubbled with 95% O_2/5% CO_2. Three Ag/AgCl electrodes, mounted in the bath in a simulated Einthoven configuration, were used to record "true" volume-conducted electrocardiograms (ECG) [167, 168], which were used to distinguish between normal rhythm, ventricular tachycardia, and ventricular fibrillation (Figure 11).

ISCHEMIA-REPERFUSION PROTOCOL OF THE ISOLATED RABBIT HEARTS

Global ischemia was initiated 50 min following excision of the heart by bypassing the flow to the heart and returning it to the buffer reservoir. Also, the solution in the acrylic bath was bubbled with 95% N_2/5% CO_2 during ischemia. Following 30 min ischemia, hearts were reperfused for 45 min by re-establishment of flow with oxygenated Tyrode's and the immersion bath was again bubbled with 95% O_2/5% CO_2. An MP100 data acquisition system using AcqKnowledge 3.0 software (Biopac Systems Inc., Goleta, CA, USA) was used to amplify and acquire the left-ventricular developed pressure, EDP, MAP and ECG signals at 2 kHz. Electrocardiogram traces were used to classify spontaneous arrhythmias that persisted for greater than 30 s as either VT or VF according to the Lambeth Conventions [28], and QT intervals were measured in pre-ischemic traces. Electrical stimulation was not stopped during arrhythmic episodes.

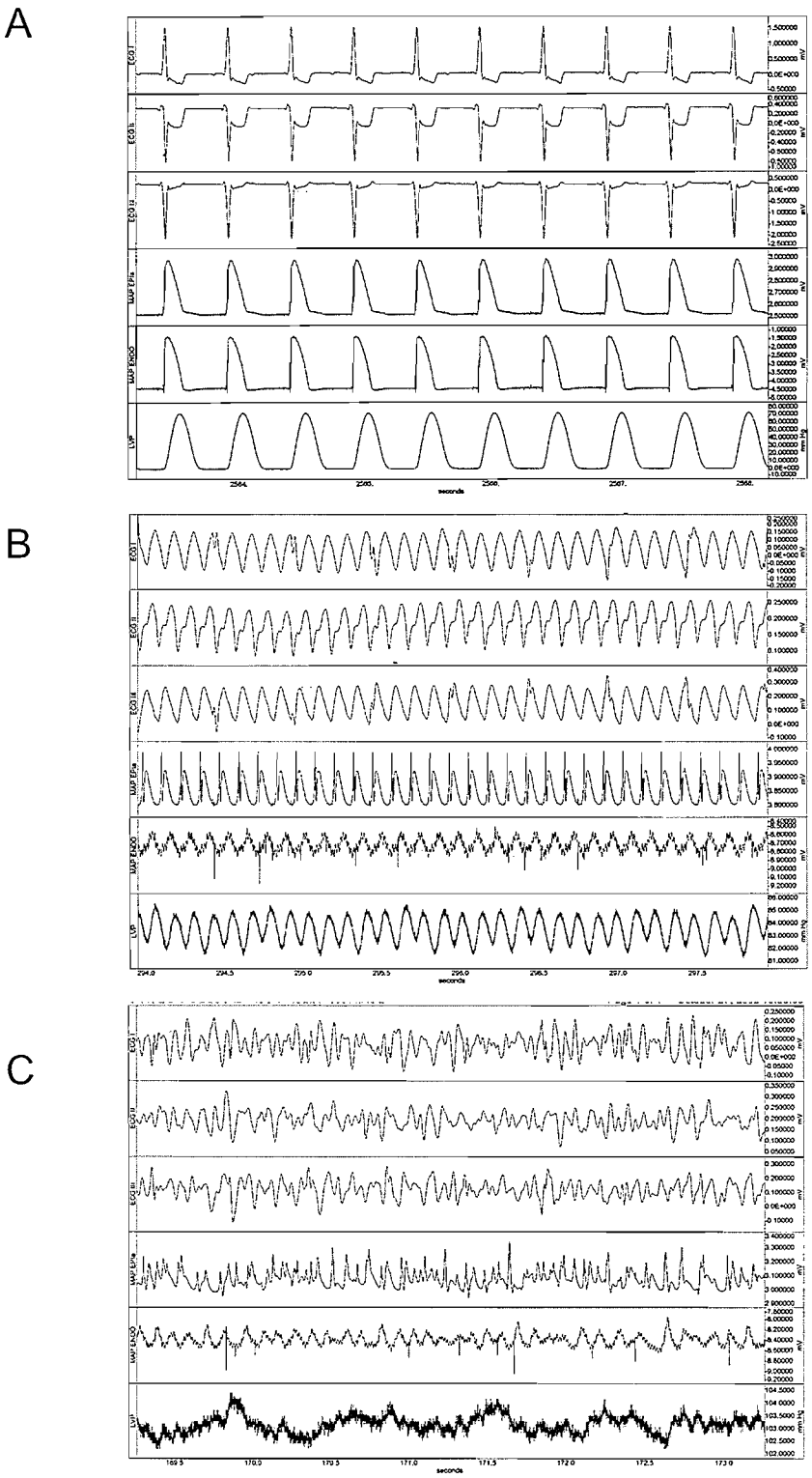


Figure 11. Electrocardiogram, monophasic action potential and left-ventricular pressure recordings from isolated rabbit hearts showing normal paced rhythm at 2 Hz (A), ventricular tachycardia (B) and ventricular fibrillation (C).

MEASUREMENT OF CURRENTS IN DIET TREATED RABBIT CARDIOMYOCYTES

DELAYED RECTIFYING POTASSIUM CURRENT

Cells from the left ventricle of New Zealand White rabbits were isolated using the technique described under *Cardiomyocyte Isolation*, with the exception that the cardiomyocytes were kept in Ca^{2+}-free KB solution following isolation rather than slowly re-introducing them to Ca^{2+}. This method resulted in a greater number of robust cells for current recordings. The delayed rectifying K^+ current (I_{Kr}) was measured in these cardiomyocytes using a novel whole-cell patch clamp technique [169]. Briefly, the pipette solution contained (mM): CsCl 130, $MgCl_2$ 1, MgATP 2, HEPES 10, and EGTA 10 (pH 7.2 with CsOH). The bath solution contained CsCl 130, $MgCl_2$ 1, glucose 10 and HEPES 10 (pH 7.4 with CsOH). Nifedipine (10 μM) was added to block $Ca^{2+}_{L\text{-type}}$ channels. Cell capacitance was measured and corrected following whole cell access. Borosilicate glass pipettes (2-5 MΩ) were connected to the headstage of an Axopatch-1D amplifier and grounded via a Ag-AgCl wire in an agar bridge. Cells were held at -80 mV and subjected to 10 mV incremental depolarizations lasting 4 s and then stepped back to -80 mV. Voltages up to +60 mV were examined in these cells. I_{Kr} was determined as the maximum inward current measured at the "hook" of the trace. Currents were normalized to cell capacitance. All experiments were performed at 23 ± 2°C and recorded using pClamp 9.2 software (Axon Instruments).

L-TYPE CALCIUM CURRENT

L-type Ca^{2+} currents were measured using the whole-cell patch clamp technique

[170]. The pipette solution contained (mM): $CaCl_2$ 0.5, $MgCl_2$ 1, DL-aspartic acid (free acid) 145, and HEPES 5 (pH 7.3 with CsOH). The bath solution contained (mM): TEA(Cl) 140, $CaCl_2$ 5, $MgCl_2$ 1, glucose 11.1, and HEPES 10 (pH 7.3 with TEA(OH)). 4-aminopyridine (3 mM) was added to block the transient outward K^+ current. Cell capacitance was measured and corrected following whole cell access. Borosilicate glass pipettes (2-5 MΩ) were connected to the headstage of an Axopatch-1D amplifier and grounded via a Ag-AgCl wire in an agar bridge. Cells were held at -80 mV and stepped to -40 mV for 200 msec in order to activate the Na^+ current. L-type Ca^{2+} currents were activated by a 2.5 s test pulse between -30 mV and +80 mV. $I_{Ca\text{-}L}$ was measured as the peak inward current and normalized to cell capacitance. Recovery of Ca^{2+} channels was also measured by following a Ca^{2+} current triggered at 0 mV with a second pulse at increasing intervals. The relative size of the second current was compared to the initial current to calculate the rate of recovery. All experiments were performed at 23 ± 2°C and recorded using pClamp 9.2 software (Axon Instruments).

INTRACELLULAR CALCIUM MEASUREMENTS

Intracellular $[Ca^{2+}]$ was measured in cardiomyocytes isolated from diet-treated animals using the fluorescent Ca^{2+} indicator fura-2. Cells were incubated with the cell permeant aceto-methyl-ester of fura-2 for 15 minutes before being placed on a coverslip in the chamber of a Nikon inverted microscope. Individual cells were selected and exposed to dual excitation wavelengths (340 and 380 nm) by a Photon Technology International spectrofluorometer. Single emission was recorded at 505 nm. The ratio of Ca^{2+}-saturated (340 nm) to Ca^{2+}-free (380 nm) fura-2 represented the change in $[Ca^{2+}]_i$. Absolute $[Ca^{2+}]_i$

was not required, so no calibration procedure was used. Cells were perfused with HEPES buffer at 1 ml/min and stimulated at frequencies ranging from 0.2-1 Hz to record Ca^{2+} transients. The sizes of the Ca^{2+} transients were calculated, along with systolic and diastolic Ca^{2+} levels, half-relaxation time, and time to peak transient.

ISOLATION OF SARCOLEMMAL MEMBRANE FROM RABBIT HEARTS

Cardiac sarcolemma was isolated using a modification of Pitts' method [171]. Hearts were quickly excised from anaesthetized rabbits and placed in cold Tyrode's solution. Once the hearts were cleared of blood, the left ventricle was dissected, weighed, and minced with fine scissors in ~10 ml of 0.6 M sucrose, 10 mM imidazole, pH 7.0 with HCl ("solution 1"). The minced heart and all solution was transferred to an ice cold 50 ml centrifuge tube and homogenized by Polytron at setting 13.6 for 6 × 15 s with 20 s intervals kept on ice. In a second ice cold centrifuge tube, 20 ml of solution 1 was used to clean of the homogenizing probe. The contents of the tubes was pooled and then balanced between the two tubes. The homogenate was centrifuged for 30 min at 12,000 × g. The supernatant was collected into a 50 ml graduated cylinder and mixed with 3 parts 20 mM MOPS/KOH, 160 mM KCl, pH 7.4 with KOH ("solution 2"). The solution was poured to fill four 40 ml centrifuge tubes ~2/3 full which were then ultracentrifuged for 60 min at 100,000 × g. Samples of the cytosol-containing supernatant were collected and frozen in liquid nitrogen. The pellets were suspended in 2-3 ml of solution 2/g left ventricle and homogenized in a glass homogenizer (20 ×) on ice. The homogenate was layered gently onto 100mM TRIS-HCl, 50 mM Na_2pyrophosphate, 300 mM KCl, 0.88 M sucrose, pH 8.3 with NaOH ("solution 3") and balanced by solution 2 then ultracentrifuged for 90 min at 100,000 × g in a swing out rotor.

The white layer at the interface (sarcolemma) was collected by eyedropper and combined with solution 2 to fill centrifuge tubes 2/3 full before centrifugation at 100,000 × g for 30 min. Samples of the cytosol were collected from the remaining layers of the sucrose gradient. The final pellet was suspended in 1.5 ml 250 mM sucrose and 10 mM histidine (pH 7.4) using a small glass homogenizer. Sarcolemmal samples were quick frozen in liquid nitrogen.

WESTERN BLOTS

Protein was collected from sarcolemma using radioimmunoprecipitation assay (RIPA) buffer (mM): NaCl 150, Tris 50, EDTA 1, EGTA 1, PMSF 1, benzamidine 1, protease inhibitor cocktail 1 μg/ml, 1% Triton-X-100 and 0.5% deoxycholate, pH 7.5. Protein samples (200 μg) were loaded into wide lanes of a 7.5% polyacrylamide gel and transferred to a PVDF membrane. Separate membranes were incubated with antibodies for Kv1.4, Kv1.5, Kv2.1, Kv4.2, Kv4.3, IsK, HERG, TWIK2, TREK1, and Ca_L-α1c (Alomone, Jerusalem, Israel). Secondary antibody (1:10000) conjugated to horseradish peroxidase was detected using an ECL Plus chemiluminescence kit and captured on X-ray film.

ACUTE STUDIES

PREPARATION OF FATTY ACID SOLUTIONS

Fatty acids were conjugated to BSA to increase their solubility in the aqueous bath [172]. Briefly, a 3% BSA solution (fatty acid-free) was prepared in perfusion solution and the desired fatty acid was added to produce a final concentration of 25 mM. An equivalent amount of Na_2CO_3 was added along with 95% ethanol and double distilled H_2O. Ethanol

was evaporated and the solution was dialyzed at 4°C overnight in the perfusion solution. The following day, the solution was brought up to final volume, aliquoted, and stored at -20°C under N_2. The solutions were thawed and added to the bath solution to achieve the desired final concentration.

CARDIOMYOCYTE ISOLATION

Adult rabbit cardiomyocyte isolation

Hearts were excised from anaesthetized male New Zealand White rabbits (2.5-3.0 kg) and mounted on a Langendorff apparatus. Hearts were flushed at 20 ml/min with Ca^{2+}-free HEPES-buffered saline (HBS) containing (mM): NaCl 132.0, HEPES (free acid) 10.0, $MgCl_2$ 1.2, glucose 10.0, KCl 4.0, taurine 60.0, and 0.25% BSA adjusted to pH 7.4, and then perfused with the same solution containing collagenase (180 U/ml, Worthington, USA) and protease (0.5 U/ml, Sigma, USA). The digestion was stopped when biopsy samples from the left ventricle contained many quiescent, rod-shaped single cells (~10-15 min). The heart was perfused with Ca^{2+}-free HBS for 5 additional min. The atria and right ventricle were removed and the left ventricle and septum were minced in a Petri dish with 100 mM Ca^{2+} HBS. Cells were shaken for 20 min in 100 mM Ca^{2+}-free HBS to further dissociate cells, filtered through cheese cloth and centrifuged at 70 × g for 2 min. The pellet was suspended in 200 mM Ca^{2+} HBS for 10 min, centrifuged at 70 × g for 2 min, and then resuspended in 1.8 mM Ca^{2+} HBS.

Adult rat cardiomyocyte isolation

Hearts were removed from anaesthetized adult male Sprague-Dawley rats (250-300 g) and attached via the aorta to a cannula on a Langendorff apparatus. The hearts were perfused at 10 ml/min with Ca^{2+}-free isolation buffer prepared in Dulbecco's modified essential medium (DMEM) warmed to 37°C. Atria were removed before switching to isolation buffer containing 216 U/mg collagenase type II and 0.6 mg/ml hyaluronidase for 15 min. Once digested, the heart was removed from the cannula and minced with fine scissors in IB3. Gentle pipetting of the solution aided digestion of the heart before being filtered through a double layer of gauze and then centrifuged at 70 × g. Cells were resuspended in IB3 and concentrated $CaCl_2$ was slowly added to bring up $[Ca^{2+}]$ to 1.25 mM. Cells were then suspended in Media 199, seeded on laminin coated coverslips and maintained in an incubator until used for experiments.

Neonatal rat cardiomyocyte isolation and culture

Cardiomyocytes were isolated from the ventricles of Sprague-Dawley rat pups (0-24 hr) by enzymatic digestion using collagenase and trypsin [173]. Briefly, hearts were collected from neonatal rats, minced, and digested in PBS containing collagenase, trypsin and DNase. Digestion was stopped by the addition of fetal bovine serum (FBS). Cells were filtered and centrifuged before being purified using a Percoll gradient. Cells were seeded at a low density onto culture dishes in F-10 medium containing 10% FBS, 10% horse serum, and 100 U/ml penicillin and 100 μg/ml streptomycin. After one day, the medium was changed to DMEM containing 0.5% FBS, 20 nM selenium, 10 μg/ml insulin, 10 μg/ml transferrin, 20 μg/ml ascorbic acid, and 0.2% BSA, which was changed every two days.

ACTION POTENTIAL RECORDINGS

Rabbit cells were placed in a bath mounted on an inverted microscope (Nikon, Tokyo, Japan) and perfused with Tyrode's solution containing (mM): NaCl 132.0, HEPES (sodium salt) 20.0, $MgSO_4$ 1.2, glucose 11.1, KCl 4.0 and $CaCl_2$ 2.0 adjusted to pH 7.4. Action potentials were recorded in current clamp mode using the perforated patch-clamp technique [174]. Borosilicate glass pipettes (World Precision Instruments, Inc., Sarasota, FL, USA) were pulled on a P-87 micropipette puller (Sutter Instrument Co., Novato, CA, USA) to a resistance of 2-5 MΩ. Immediately prior to use, pipette tips were filled with antibiotic-free solution containing (mM): L-aspartic acid (monopotassium salt) 130.0, KCl 15.0, HEPES (free acid) 5.0, NaCl 10.0, $CaCl_2$ 0.5, and $MgCl_2$ 1.0 (pH 7.3). The rest of the pipette was back-filled with pipette solution containing Amphotericin B (Sigma Chemical Co., St. Louis, MO, USA), which had been prepared as a stock of 30 mg/ml in dimethyl sulfoxide and diluted to 120 μg/ml. Pipettes were connected to the headstage of an Axopatch-1D amplifier (Axon Instruments, Foster City, CA, USA) and grounded via a Ag-AgCl wire inserted through an agar bridge seated in the bath.

Upon gigaseal formation, Amphotericin B was allowed to perforate the cell membrane and membrane potential was recorded in current clamp mode. No holding potential was applied to the cells. Cells were stimulated by injection of 3 ms depolarizing pulses at 0.5 Hz using a Pulsar 6i stimulator (FHC, Brunswick, ME, USA). Action potentials were recorded before and after addition of 25 μM DHA, ALA or OA directly to the bath. All experiments were performed at 23 ± 2°C. Action potentials were recorded using pClamp 7.0 software (Axon Instruments). Action potential durations were calculated for 50% (APD_{50}) and 90% (APD_{90}) repolarization.

GENE MANIPULATION

Vascular smooth muscle cells

Cultured vascular smooth muscle cells adenovirally transfected with the vascular isoform of the NCX (NCX1.3) were also used to test the effects of ALA on the NCX in intact cells. Rabbit aortic vascular smooth muscle cells were grown in culture using the explant technique [175]. The recombinant adenovirus expressing canine NCX1.3 [176] was used to infect cultured vascular smooth muscle cells (VSMC) using a multiplicity of infection (MOI) of 150 viral particles/cell after 2 days incubation in DMEM-5% FBS [177]. An empty vector was used for control. Cells were infected for 2 days before experimental use. For the rat cardiomyocytes, VSMC were incubated with fura-2 in order to measure $[Ca^{2+}]_i$ as described below. The effects of ALA and KB-R7943 treated groups were compared to controls.

Western blot of HEK293 cells stably expressing NCX1.1 or NCX1.3

The cardiac (NCX1.1) and vascular (NCX1.3) isoforms of the Na^+-Ca^{2+} exchanger were stably expressed in HEK293 cells as previously described [178]. Control nontransfected HEK293 cells, or cell lines expressing NCX1.1 or NCX1.3, were grown to 70% confluency in 175 cm^2 flasks. Protein was collected from cells lysed in RIPA buffer (mM): NaCl 150, Tris 50, EDTA 1, EGTA 1, PMSF 1, benzamidine 1, protease inhibitor cocktail 1 μg/ml, 1% Triton-X-100 and 0.5% deoxycholate, pH 7.5. Protein samples (200 μg) were loaded on a 7.5% polyacrylamide gel and transferred at 35 V overnight to a nitrocellulose membrane. Membranes were incubated with antibody (1:500) directed at the intracellular loop of NCX1 (R3F1, Swant, Bellinzona, Switzerland) overnight at 4°C.

Secondary antibody (1:10000) conjugated to horseradish peroxidase was detected using an ECL Plus chemiluminescence kit and captured on X-ray film.

Neonatal rat cardiomyocytes

Silencing and overexpression of NCX1.1

Short hairpin RNA sequences were transfected by adenovirus into one day old neonatal rat cardiomyocytes [179]. Briefly, an insert containing either a sequence encoding a region specific to the NCX (NCX-KD) or a scrambled control sequence (CTRL) was placed between the human U6 RNA promoter and a sequence for a short RNA hairpin and poly T transcription signal. Adenovirus containing the sequence for NCX1.1 was also used to infect control neonatal rat cardiomyocytes to increase the expression of the NCX.

Generation of stably transfected mutant NCX HEK293 cell lines

Canine sequences of NCX1.1 with either the Δ238-735 ("loop deletion") or K229Q mutations were kindly provided by Dr. Larry Hryshko , Department of Physiology, University of Manitoba [180]. Briefly, linearized cDNA was subcloned into pCDNA3.1(-)/hygro plasmids (Invitrogen), which contain a sequence conferring hygromycin resistance. Recombinant plasmids were transformed into competent *E. coli* cells (DH5α), amplified and purified using a QIAprep spin kit. The plasmids were linearized with the restriction enzyme, *Xho1* (Invitrogen), and cleaned using a QIAquick PCR purification kit. HEK293 cells were seeded on culture dishes and a Ca^{2+} phosphate/DNA precipitate solution was added dropwise to the cells and incubated for 24 hours. The medium was removed from the plates, and the cells were washed before adding fresh medium. Hygromycin was added to the medium 24

hours later and replaced every 3-4 days until all the cells on the non-hygromycin resistant control plates died. Stable cell lines were chosen by screening cell growth in a multiwell culture dish. The best clones were selected to grow in large flasks, and levels of expression were determined by Western blot in order to select cell lines of similar expression levels.

BIOCHEMICAL AND ELECTROPHYSIOLOGICAL ASSAYS

Western blot of stable cell lines

Protein was collected from cells lysed in RIPA buffer. Fifty μg of protein isolated from the cells were electrophoresed through a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane overnight at 35 V. The membrane was probed with an antibody (1:500) for the intracellular loop of NCX1 (R3F1, Swant, Bellinzona, Switzerland) overnight in the cold room. The secondary antibody (1:10000) conjugated to horseradish peroxidase was detected using a chemiluminescence kit (Pierce) and measured by BioRad FluorSMax and densitometrically quantified with Quantity One software.

Measurement of intracellular Ca^{2+} in vascular smooth muscle cells and cardiomyocytes

Intracellular $[Ca^{2+}]$ was measured using the fluorescent Ca^{2+} indicator fura-2. Cells were incubated with the cell permeant aceto-methyl-ester of fura-2 for 15 minutes before being placed on a coverslip in the chamber of a Nikon inverted microscope. Individual cells were selected and exposed to dual excitation wavelengths (340 and 380 nm) by a Photon Technology International spectrofluorometer. Single emission was recorded at 505 nm. The ratio of Ca^{2+}-saturated (340 nm) to Ca^{2+}-free (380 nm) fura-2 represented the change in $[Ca^{2+}]_i$. Absolute $[Ca^{2+}]_i$ were not required, so no calibration procedure was used. Following

incubation with fura-2, cells were washed and incubated in 25 μM ALA for 30 minutes. Stimulation of reverse NCX activity was achieved by substituting Na^+ in the bathing solution with Li^+. Li^+-stimulated reverse NCX activity was compared between control, ALA and KB-R7943 treated groups.

Action potential recordings in neonatal rat cardiomyocytes

Two days following transfection with the adenovirus, cells were dissociated from the plates with collagenase and trypsin and plated at low density on collagen coated glass cover slips. Cells transfected for 3-6 days were placed in a bath mounted on an inverted microscope and perfused with Tyrode's solution containing (mM) NaCl 132.0, HEPES (sodium salt) 20.0, $MgSO_4$ 1.2, glucose 11.1, KCl 4.0 and $CaCl_2$ 2.0 adjusted to pH 7.4. Action potentials were recorded in current clamp mode using the perforated patch clamp technique [174]. Borosilicate glass pipettes were pulled to a resistance of 2-5 MΩ. Immediately prior to use, pipette tips were filled with antibiotic-free solution containing (mM) L-aspartic acid (monopotassium salt) 130.0, KCl 15.0, HEPES (free acid) 5.0, NaCl 10.0, $CaCl_2$ 0.5, and $MgCl_2$ 1.0 (pH 7.3). The rest of the pipette was back-filled with pipette solution containing Amphotericin B (Sigma), which had been prepared as a stock of 30 mg/ml in dimethyl sulfoxide and diluted to 120 μg/ml. Pipettes were connected to the headstage of an Axopatch-1D amplifier and grounded via a Ag-AgCl wire inserted through an agar bridge seated in the bath. Upon gigaseal formation, Amphotericin B was allowed to perforate the cell membrane and membrane potential was recorded in current clamp mode. No holding potential was applied to the cells. Cells were stimulated by injection of 3 ms depolarizing pulses at cycle lengths ranging from 500 to 4000 ms using a Pulsar 6i stimulator. All

experiments were performed at room temperature of 23 ± 2 °C. Action potentials were recorded and analyzed using pClamp 7.0 software (Axon Instruments). Action potential durations were calculated for 50% (APD_{50}) and 90% (APD_{90}) repolarization.

Effects of ω-3 PUFA on spontaneous neonatal cardiomyocyte activity

Action potentials were recorded from small groups (2-3 cells) of neonatal rat cardiomyocytes that showed spontaneous contractile activity using the above method. Activity was recorded for five minutes before 25 μM of ALA was applied to the cells via the bathing solution. Following 3 minutes, spontaneous activity was measured for an additional five minutes (via recording of action potentials). The average number of beats per minute for control and treatment were obtained and compared.

Measurement of NCX current in HEK293 cells

HEK293 cells were enzymatically removed from culture dishes with 0.25% Trypsin-EDTA and placed in a chamber on an inverted microscope. Cells were perfused with (mM): NaCl 137, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.5, HEPES 10, d-glucose 10, pH 7.4. Glass pipettes (1.8-2.2 MΩ) were filled with (mM): NaCl 5, CsOH 100, KCl 5, $MgCl_2$ 2, TEA-Cl 20, HEPES 10, d-glucose 8, Na_2ATP 1, EGTA 5, $CaCl_2$ 4.94, pH 7.2. Pipette capacitance was corrected following gigaseal formation and cell capacitance was measured and corrected following whole cell access. Pipettes were connected to the headstage of an Axopatch-1D amplifier and grounded via a Ag-AgCl wire in an agar bridge. Cells were held at -40 mV and then ramped from +60 mV to -100 mV over 1600 ms. Dose-response curves were obtained for ALA in NCX1.1 and NCX1.3 cell lines at concentrations up to 100 μM. To compare effects

of different classes of fatty acids, currents were recorded before and after addition of 25 μM oleic acid (OA, 18:1ω-9), linoleic acid (LA, 18:2ω-6), ALA (18:3ω-3) or EPA (20:5ω-3), introduced from a separate reservoir. $NiCl_2$ (5 mM) was used to block the total NCX current. All experiments were performed at 23 ± 2°C. Current recordings were acquired and analyzed using pClamp 9.2 software (Axon Instruments).

Measurement of delayed rectifying potassium current in HEK293 cells

HEK293 cells expressing the human ether-a-go-go gene (hERG), which encodes the pore-forming subunit of I_{Kr} channels [181, 182], were kindly provided by Dr. Shetuan Zhang, Department of Physiology, University of Manitoba [183]. Cells were patch clamped in the whole cell configuration using a pipette solution of (mM): KCl 130, EGTA 5, $MgCl_2$ 1, Na_2ATP 5, and HEPES 10 (pH 7.2 with KOH). The bath solution contained (mM): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, and HEPES 10 (pH 7.4 with NaOH). Borosilicate glass pipettes (2-5 MΩ) were connected to the headstage of an Axopatch-1D amplifier and grounded via a Ag-AgCl wire in an agar bridge. Cell capacitance was measured and corrected following whole cell access. Cells were held at -80 mV and depolarized in 10 mV increments lasting 3 s before clamping to -50 mV. Voltage steps up to +50 mV were examined. Currents were recorded in the absence or presence of 25 μM ALA. Currents were normalized to cell capacitance. All experiments were performed at 23 ± 2°C and recorded using pClamp 9.2 software (Axon Instruments).

STATISTICAL ANALYSIS

Statistical analysis of data was performed using SigmaStat software (version 2.03, SPSS Inc., Chicago, IL, USA). A Student's t-test was used to compare reversal potentials and IC_{50} values for ALA inhibition of the forward and reverse modes of NCX activity and percent changes in APD. Interactions in the CHL/FLX group were evaluated by a two-way analysis of variance (ANOVA). An ANOVA on ranks was used if variances were not equalized by other transformations. The association between cardiac [ALA] and incidence of arrhythmias was assessed by Pearson product moment correlation. Differences in the incidence of arrhythmias between groups were assessed using the Fisher Exact test. One-way ANOVA and Student-Newman-Keuls *post hoc* tests were used to compare all other data. $P < 0.05$ was considered significant. All results are expressed as mean ± SEM.

CHAPTER V: RESULTS

DIETARY EFFECTS OF FLAXSEED

PILOT FEEDING STUDIES

Animals were fed for 8 weeks *ad libitum* with the four prepared diets. On average, rabbits would consume 177 g/day diet (range between 123-218 g/day). At the final endpoint of 8 weeks, the rabbit weights were significantly different. Rabbits from the flax group weighed significantly more than the rabbits in the other groups (3.8 ± 0.1 kg vs. 3.5 ± 0.1 kg). Based on these results, rabbits for the study were subsequently placed on a restricted diet of 125 g/day of chow, which did keep the weights of the rabbits the same between groups (Figure 12). This amount was sufficient to satisfy the daily nutritional requirements of the animals.

Rabbits were fed 10% flaxseed diets and the health was monitored daily to observe for any potential side effects of the diet. Feeding periods as long as 16 weeks were conducted. No apparent complications were observed in the animals by the investigators or animal care technicians and weights of animals remained the same between the groups (3.7 ± 0.1 kg).

CHANGES IN TISSUE FATTY ACIDS

Plasma

By 8 weeks, plasma ALA levels increased by 10-fold in the FLX group compared to the REG diet, whereas all other fatty acids were similar between these two groups (Table 4). In contrast, consumption of diets containing 0.5% cholesterol (CHL and CHL/FLX) caused a significant elevation in nearly all fatty acid species compared to REG and FLX groups.

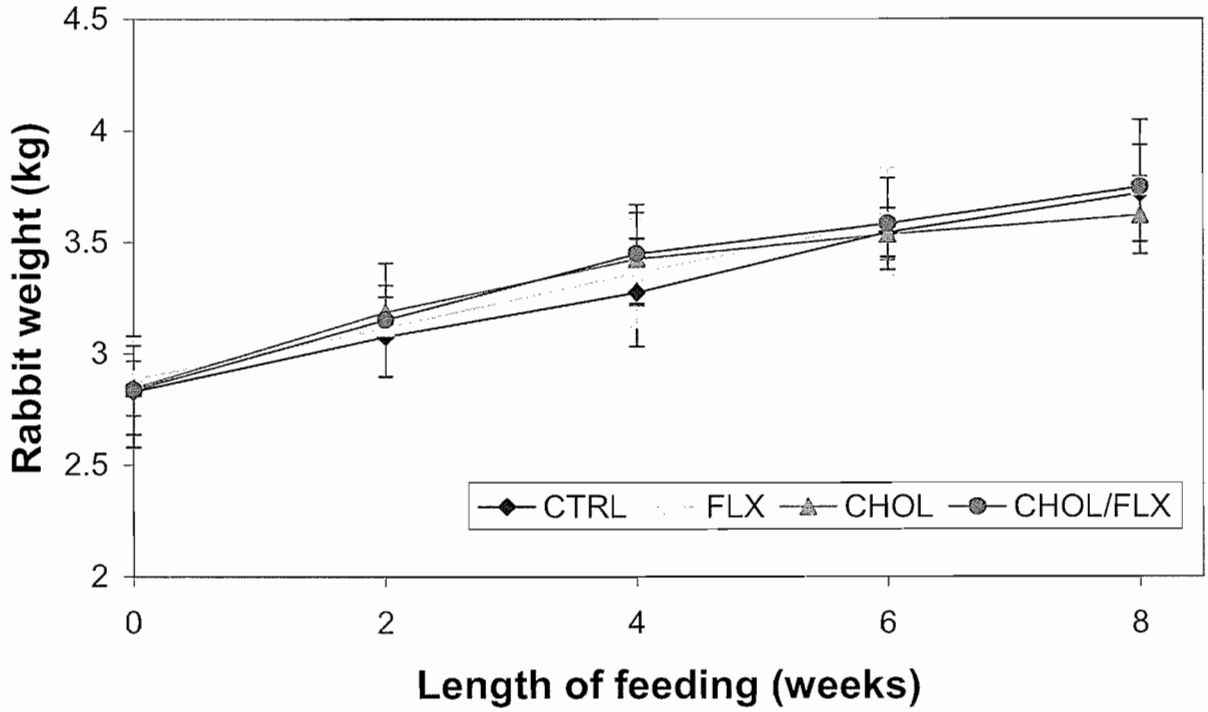


Figure 12. Rabbit weight following restricted feeding (125 g/day) of a regular diet (CTRL), or regular diet containing 10% flaxseed (FLX), 0.5% cholesterol (CHOL), or 10% flaxseed and 10% cholesterol (CHOL/FLX). P > 0.05 amongst groups.

Table 4. Concentration (mg/dl) of plasma fatty acids before and after feeding trials.[1]

Fatty Acid	0 weeks				8 weeks				16 weeks			
	REG	FLX	CHL	CHL/FLX	REG	FLX	CHL	CHL/FLX	REG	FLX	CHL	CHL/FLX
C14:0	1.2 (0.3)	3.4 (0.1)	0.6 (0.2)	0.5 (0.2)	0.5 (0.1)	0.1 (0.1)	1.7†‡§ (0.2)	1.1‡§¶ (0.2)	1.0 (0.3)	1.8 (1.1)	9.8†‡§ (1.7)	4.4†‡§¶ (0.7)
C16:0	26.7 (5.5)	14.1 (1.1)	17.1 (1.7)	18.7 (2.5)	14.8 (1.6)	9.4 (1.0)	81.7†‡§ (7.4)	80.1†‡§ (5.9)	38.1 (4.6)	35.1 (10.8)	442.8†‡§ (34.8)	316.6†‡§¶ (34.2)
C18:0	17.0 (1.1)	12.5 (0.8)	13.9 (0.7)	16.1 (1.4)	14.5 (1.1)	10.7 (0.8)	28.3†‡§ (2.4)	33.7†‡§¶ (2.8)	15.9 (1.7)	14.4 (3.3)	148.9†‡§ (11.7)	113.2†‡§¶ (11.2)
C20:0	*t*	*t*	*t*	*t*	*t*	*t*	0.8†‡§ (0.1)	1.0†‡§ (0.1)	*t*	*t*	1.9†‡§ (0.3)	1.0†‡§¶ (0.2)
C22:0	*t*	*t*	*t*	*t*	*t*	*t*	0.2 (0.1)	0.3†‡§ (0.1)	*t*	*t*	4.0†‡§ (0.8)	4.6†‡§ (0.4)
C16:1 ω-9	2.4 (0.6)	1.2 (0.2)	1.3 (0.2)	1.2 (0.3)	1.9 (0.3)	0.8 (0.1)	19.9†‡§ (1.9)	13.9†‡§¶ (1.3)	2.4 (0.8)	2.4 (1.6)	91.3†‡§ (9.5)	48.9†‡§¶ (5.1)
C18:1 ω-9	25.9 (3.9)	15.4 (1.7)	20.5 (2.6)	18.6 (2.7)	17.4 (2.5)	10.2 (1.0)	142.8†‡§ (10.4)	142.8†‡§ (12.3)	57.8 (8.7)	47.1 (14.6)	875.4†‡§ (58.9)	643.4†‡§¶ (33.4)
C20:1 ω-9	*t*	*t*	*t*	*t*	*t*	*t*	1.2†‡§ (0.1)	*t*	*t*	*t*	1.9†‡§ (0.5)	0.4 (0.3)
C18:2 ω-6	23.4 (3.6)	14.2 (1.3)	18.5 (1.7)	18.5 (2.0)	12.5 (2.3)	8.6 (1.2)	76.8†‡§ (8.6)	81.2†‡§ (7.1)	39.6 (4.0)	32.4 (7.7)	458.2†‡§ (42.5)	318.2†‡§¶ (26.2)
C20:2 ω-6	*t*	*t*	*t*	*t*	*t*	*t*	1.2†‡§ (0.1)	1.1†‡§ (0.3)	*t*	*t*	2.3†‡§ (0.5)	1.1†‡§¶ (0.3)
C18:3 ω-3	2.0 (0.7)	0.3 (0.1)	1.0 (0.2)	0.9 (0.2)	0.9 (0.3)	5.9†‡ (1.0)	16.6†‡§ (1.9)	108.0†‡§¶ (13.9)	3.1 (0.6)	23.4†‡ (7.3)	60.9†‡§ (7.4)	256.2†‡§¶ (15.4)
C20:3 ω-6	*t*	*t*	*t*	*t*	*t*	*t*	1.0†‡§ (0.1)	0.8†‡§¶ (0.1)	*t*	*t*	*t*	1.1 (0.7)
C20:4 ω-6	2.2 (0.1)	2.0 (0.1)	2.2 (0.1)	2.1 (0.1)	1.9 (0.1)	1.2 (0.3)	3.7†‡§ (0.2)	3.3†‡§ (0.1)	1.8 (0.3)	1.0 (0.5)	26.6†‡§ (1.9)	15.6 (1.7)†‡§¶
C20:5 ω-3	*t*	*t*	*t*	*t*	*t*	*t*	2.7†‡§ (0.1)	0.9†‡§¶ (0.1)	*t*	*t*	2.4†‡§ (0.3)	3.7†‡§¶ (0.3)
C22:6 ω-3	*t*	*t*	*t*	*t*	*t*	*t*	*t*	*t*	*t*	*t*	0.2†‡§ (0.1)	0.9†‡§¶ (0.3)
ω-6/ω-3	12.8 ± 4.8	54.0 ± 18.5	20.7 ± 4.5	22.9 ± 5.1	16.0 ± 5.91	1.75 ±0.36†‡	4.28 ±0.61†‡	0.80 ±0.12†‡	13.4 ± 2.9	1.42 ± 0.6†‡	7.62 ± 1.1†‡§	1.28 ± 0.2†‡¶
Total Fatty Acids	106.3 (15.0)	60.5 (5.1)	79.8 (7.0)	80.9 (8.9)	69.2 (7.8)	50.1 (5.2)	393.3†‡§ (32.3)	484.0†‡¶ (44.0)	159.9 (21.0)	157.7 (47.1)	2127.4†‡§ (171.2)	1632.5†‡§¶ (125.6)

[1] Abbreviations and symbols: *t*, trace amounts present (< 0.1 mg/dL); REG, regular fed; FLX, flax fed; CHL, cholesterol fed; CHL/FLX, cholesterol plus flax fed; † $P < 0.05$ versus pre-feeding at 0 weeks; ‡ $P < 0.05$ versus REG at respective time; § $P < 0.05$ versus FLX at respective time; ¶ $P < 0.05$ versus CHL at respective time. SE shown in parentheses.

Plasma ALA in CHL animals was significantly higher than in the FLX group. Plasma ALA increased by ~100-fold in the CHL/FLX group compared to controls. The concentration of the longer chain ω-3 PUFA, EPA also increased in both the CHL and CHL/FLX groups. Only very small amounts of DHA were detected in plasma from CHL and CHL/FLX rabbits at 16 weeks.

None of the dietary interventions significantly affected triglyceride concentrations in this study (Table 5). Total plasma cholesterol levels also were unchanged in the REG and FLX groups, but were significantly higher in the CHL and CHL/FLX groups as expected (Table 5).

Tissue

Figures 13-15A summarize the fatty acids present in liver, kidney, brain, smooth muscle (aorta), and skeletal muscle (gastrocnemius) from rabbits fed the different dietary treatments for 8 weeks. In all tissues, the flaxseed diets significantly elevated ALA content of the tissues and effectively lowered the ratio of ω-6:ω-3 fatty acids.

Figure 15B summarizes the fatty acids present in cardiac muscle, and thus, in the hearts used for the Langendorff experiments at 8 and 16 weeks. Dietary flaxseed increased cardiac ALA levels by 6-fold in the FLX and CHL/FLX groups. Levels of EPA also were nearly 3-fold greater than controls in these groups, but DHA levels only rose slightly. The ω-6 PUFA arachidonic acid decreased significantly in all groups compared to control. The overall ratios of ω-6:ω-3 fatty acids were also lowered in the flax supplemented groups.

Table 5. Concentrations of cholesterol and triglycerides in plasma from rabbits before and after 8 or 16 weeks feeding.

Lipid	Group	Time (weeks)		
		0	8	16
			mg/dL	
Cholesterol	REG	71.0 (4.5)	47.3 (4.0)	11.3 (1.8)
	FLX	72.8 (8.7)	32.8 (13.6)	11.8 (3.9)
	CHL	60.4 (4.7)	601.6 (117.5)*	681.6 (53.7)*
	CHL/FLX	55.4 (6.9)	737.9 (68.9)*	615.2 (26.5)*
Triglycerides	REG	166.1 (28.5)	101.7 (22.9)	102.0 (3.8)
	FLX	100.3 (13.6)	90.8 (13.9)	72.4 (4.2)
	CHL	108.7 (12.9)	60.2 (11.1)	144.5 (14.9)
	CHL/FLX	108.3 (17.4)	98.1 (32.3)	138.6 (19.1)

* $P < 0.05$ versus control and flaxseed groups at same time point. Abbreviations: REG, regular chow; FLX, 10% flaxseed chow; CHL, 0.5% cholesterol chow; CHL/FLX, 0.5% cholesterol plus 10% flaxseed chow. SE shown in parentheses.

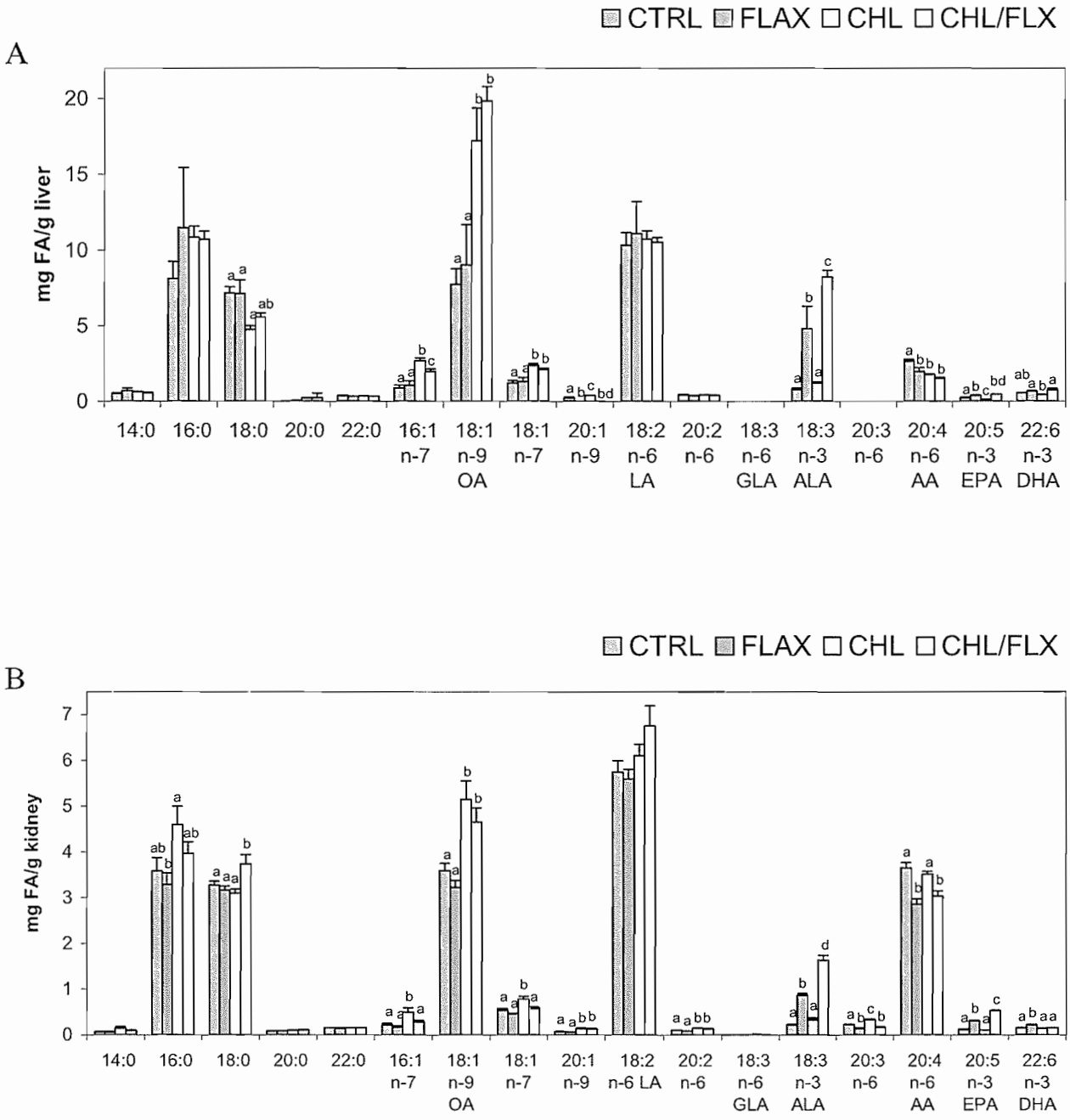


Figure 13. Fatty acid profiles of the rabbit liver (A) and kidney (B) following 8 weeks of feeding with either a regular, 10% flaxseed, 0.5% cholesterol, or 0.5% cholesterol/10% flaxseed diet. For each fatty acid, values not sharing a common letter are significantly different ($P < 0.05$).

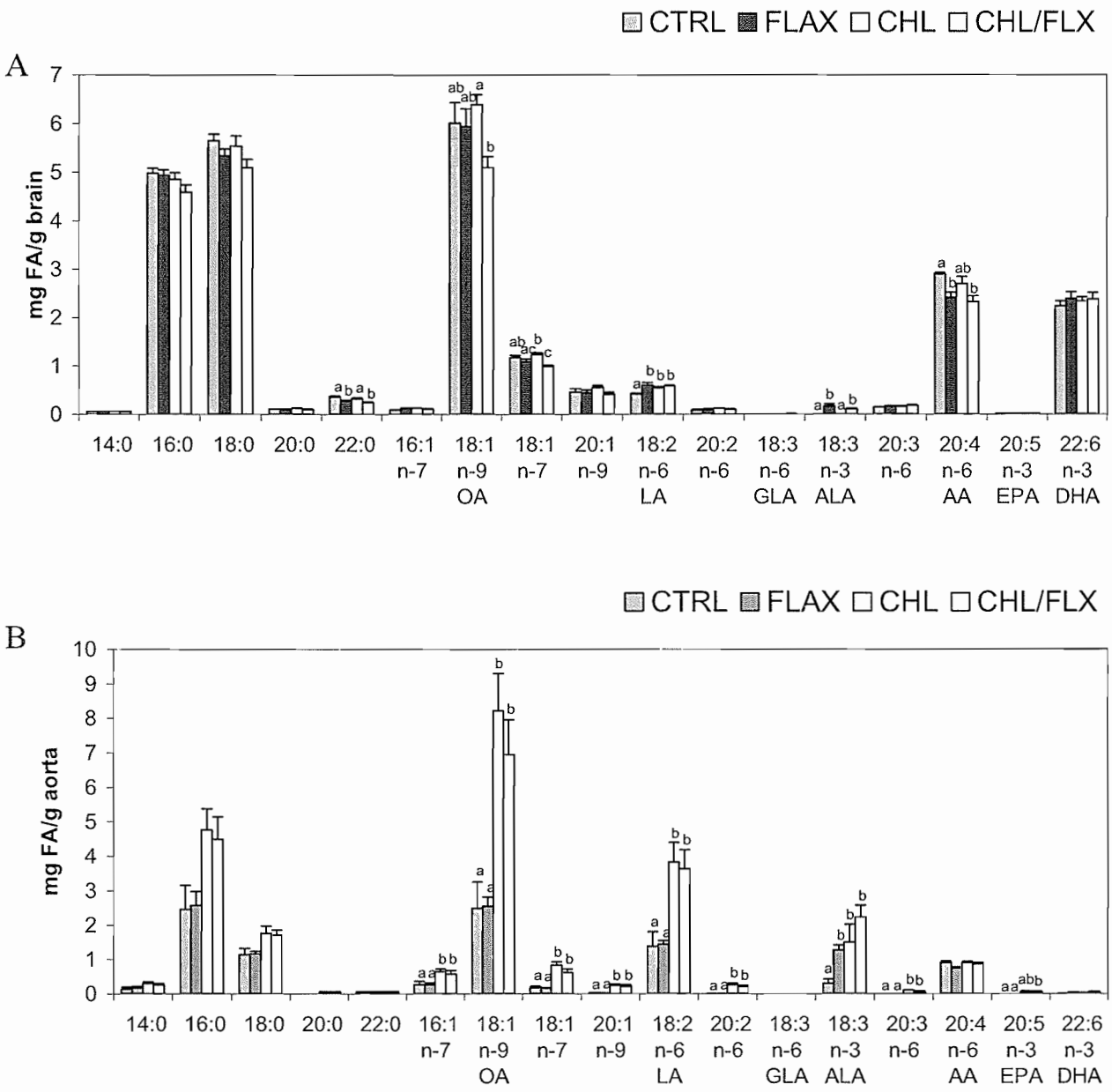


Figure 14. Fatty acid profiles of the rabbit brain (A) and aorta (B) following 8 weeks of feeding with either a regular, 10% flaxseed, 0.5% cholesterol, or 0.5% cholesterol/10% flaxseed diet. For each fatty acid, values not sharing a common letter are significantly different ($P < 0.05$).

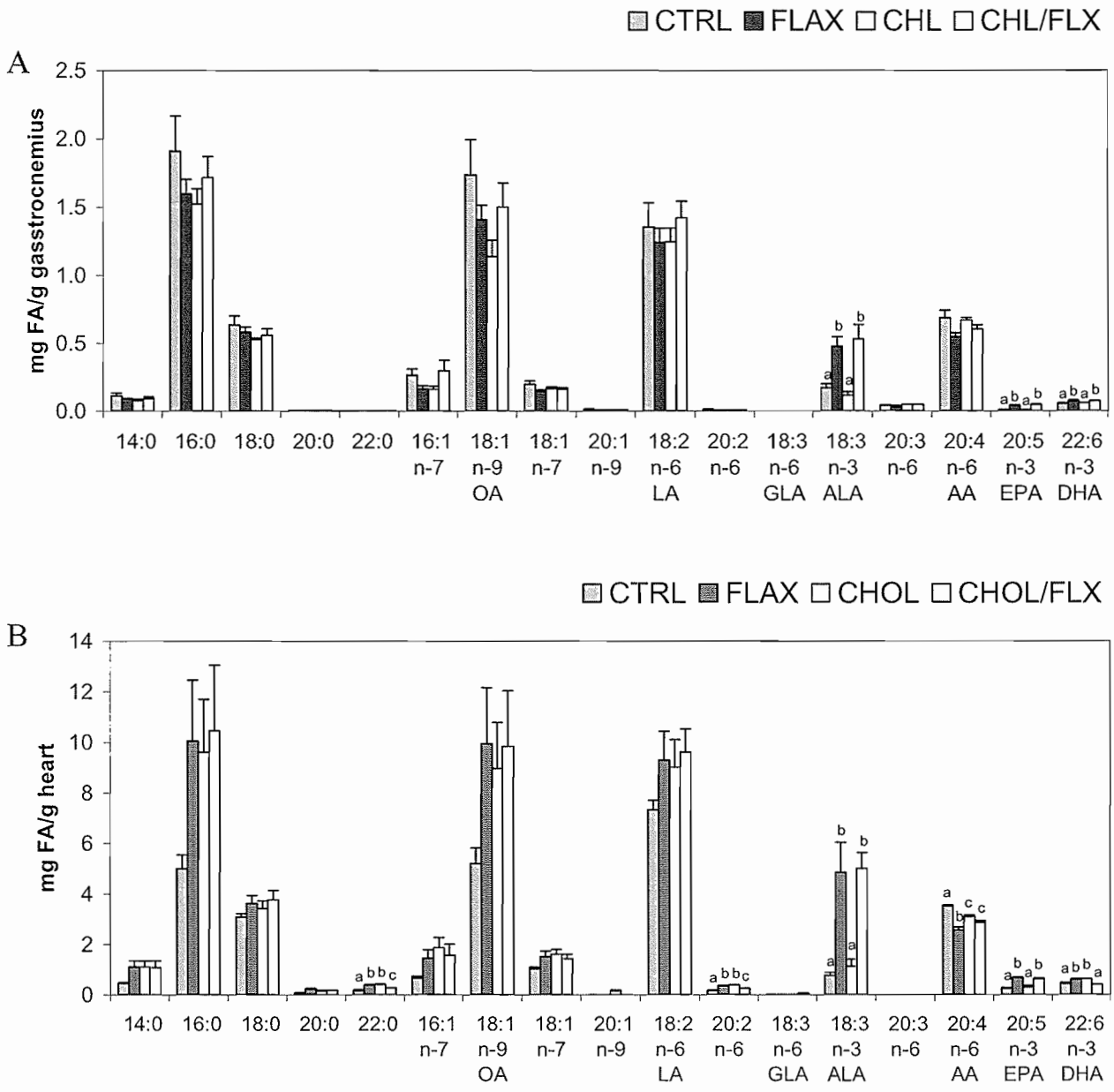


Figure 15. Fatty acid profiles of the rabbit skeletal muscle (A) and heart (B) following 8 weeks of feeding with either a regular, 10% flaxseed, 0.5% cholesterol, or 0.5% cholesterol/10% flaxseed diet. For each fatty acid, values not sharing a common letter are significantly different ($P < 0.05$).

Cardiomyocytes

We also examined the fatty acid profile in rabbit left-ventricular cardiomyocytes. As with the profile obtained in cardiac tissue, isolated cardiomyocytes also showed significantly elevated amounts of ALA following a dietary flaxseed regimen, with or without cholesterol compared to controls and cholesterol alone groups (Figure 16). The only other fatty acid to show a change was EPA, which was also elevated in cardiomyocytes from flaxseed fed animals.

ISCHEMIA-REPERFUSION

The health of the animals was monitored throughout the study. One cholesterol fed rabbit was removed from each of the 8 and 16 week trials due to complications resulting from loss of appetite. One rabbit fed a control diet was removed from the 8 week study for unrelated health reasons. The final group sizes for the 8 week trial were 7, 8, 7, and 8 for REG, FLX, CHL, and CHL/FLX, respectively, and 8, 8, 7, and 8 for the respective groups in the 16 week trial. Results from 8 and 16 weeks were similar so data from the two groups were pooled. Food was consumed daily in its entirety and rabbits gained weight at the same rate in all four groups. Weights did not differ significantly amongst the four groups of rabbits at any single time point of the feeding trial. Body weight after feeding was (kg) 3.7 ± 0.1, 3.7 ± 0.1, 3.6 ± 0.1, and 3.7 ± 0.1 for the REG, FLX, CHL, and CHL/FLX rabbits. This result would suggest that any small differences among the diets with respect to their caloric content were not large enough to induce weight differences in the animals.

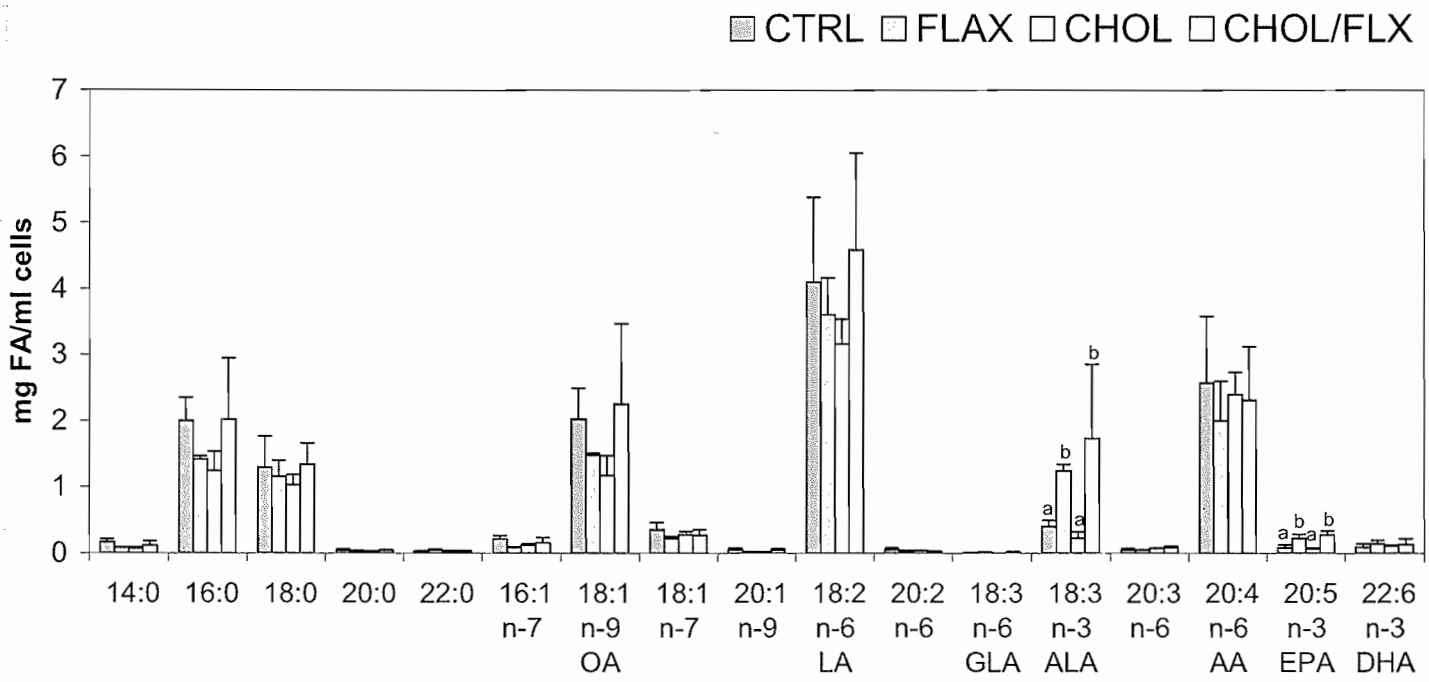


Figure 16. Fatty acid profile of cardiomyocytes isolated from rabbits consuming one of the four experimental diets for 8 weeks. For each fatty acid, values not sharing a common letter are significantly different ($P < 0.05$).

Effects on arrhythmogenesis

Time elapsed before first arrhythmia during ischemia

Table 6 shows the average time until onset of any type of arrhythmia in the hearts during ischemia. At 8 weeks, the onset of arrhythmia was delayed by ~5 min in hearts from rabbits receiving dietary flax (FLX and CHL/FLX) relative to controls. Onset of arrhythmia in hearts from cholesterol fed rabbits occurred at about the same time as controls. Following 16 weeks feeding, hearts from cholesterol-only supplemented rabbits entered arrhythmia ~2 min earlier than hearts from control rabbits. Inclusion of flaxseed in the diets of these cholesterol-fed rabbits returned the values to control levels. Hearts from the flaxseed fed group were remarkably resistant and did not exhibit any kind of arrhythmia during ischemia.

Frequency of arrhythmias following 8 weeks feeding

Figure 17 summarizes the incidences of sustained VT (A) and VF (B) occurring during ischemia and reperfusion in hearts isolated after 8 weeks feeding. During ischemia, VT occurred in 29% of hearts from both control and cholesterol fed rabbits, and 38% of hearts from flaxseed and cholesterol-flaxseed fed rabbits. Fibrillation was observed in 29% of hearts from control rabbits and 14% of hearts from cholesterol fed rabbits during ischemia. VF did not occur in any hearts from flaxseed or cholesterol plus flaxseed supplemented rabbits (0%).

In general, reperfusion induced arrhythmias more frequently than ischemia. Upon reperfusion, VT occurred in 57% of control rabbits, followed by flax (50%), cholesterol (43%), and then cholesterol plus flax fed (38%). Control hearts exhibited a 43% incidence of VF and this was similar to that of flax alone (38%). The greatest incidence of VF (57%)

Table 6: Time until onset of first arrhythmia during ischemia.

Group	Time of first arrhythmia (min)	
	8 weeks	16 weeks
Regular	20.3 ± 1.3	25.1 ± 2.5
Flax	24.7 ± 1.2	>30.0*
Cholesterol	21.6 ± 1.3	22.9 ± 2.2
Cholesterol/Flax	25.2 ± 2.0	24.5 ± 1.1

*Did not exhibit arrhythmia during 30 minute ischemia.

occurred in hearts from cholesterol fed rabbits. In contrast, VF was completely suppressed in hearts from rabbits fed flax plus cholesterol (0%, $P < 0.05$ versus OL).

Frequency of arrhythmias following 16 weeks feeding

Incidences of VT and VF during ischemia and reperfusion in isolated hearts after feeding for 16 weeks are also shown in Figure 17. During ischemia, VT occurred in 38% of hearts from control or cholesterol-flax fed rabbits. Hearts from cholesterol fed rabbits exhibited a slightly lower incidence of VT (29%). In contrast, none of the hearts from the flax fed rabbits exhibited VT (0%) during global ischemia. Fibrillation occurred in 25% of control hearts. Cholesterol diet increased VF to 43%. In contrast, none of the hearts from flax fed rabbits fibrillated (0%), and VF was only 13% in hearts from cholesterol-flax fed rabbits.

Hearts from control, flax, and cholesterol plus flax fed rabbits all exhibited a 50% incidence of VT during reperfusion, whereas cholesterol fed animals had an increased incidence of 71%. Hearts from cholesterol fed animals also exhibited significantly greater VF (71%, $P < 0.05$) than hearts from regular fed (13%) or flax fed animals (13%). Including flax with cholesterol in the diet reduced the incidence of VF by nearly one-half to 38%.

Frequency of arrhythmias following 8 and 16 weeks feeding combined

Hearts isolated from rabbits fed different diets exhibited a 19-38% incidence of VT during ischemia, compared to 33% in controls (Figure 18). Fibrillation was observed during ischemia in 27% of REG hearts and this was similar to the CHL group (29%). In contrast,

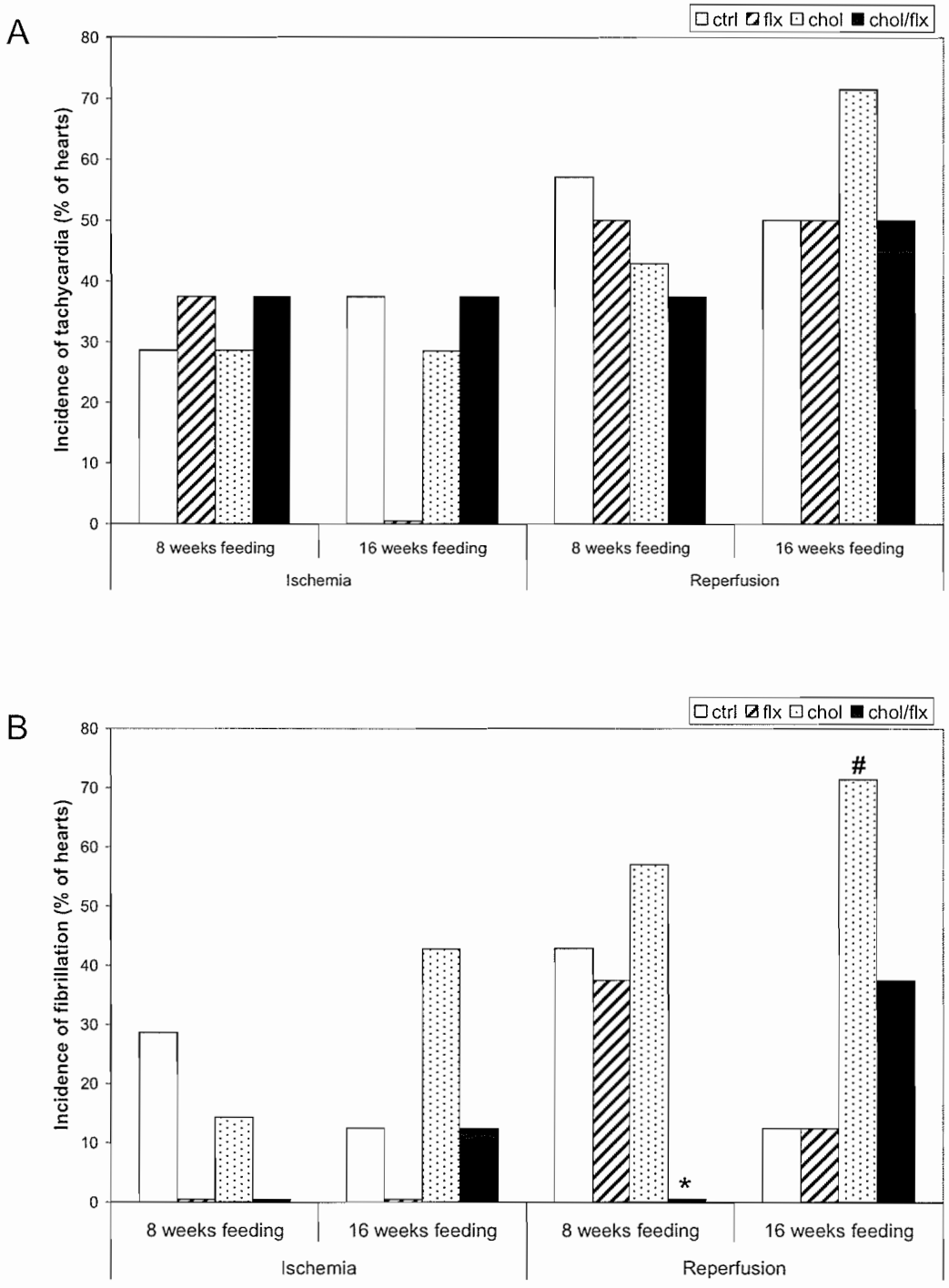


Figure 17. Incidence of ventricular tachycardia (A) and fibrillation (B) during 30 min ischemia and 45 min reperfusion in hearts isolated from rabbits fed either regular (ctrl), 10% flaxseed supplemented (flx), 0.5% cholesterol supplemented (chol), or 0.5% cholesterol and 10% flaxseed supplemented diets (chol/flx) for 8 weeks or 16 weeks. * $P < 0.05$ versus chol; # $P < 0.05$ versus ctrl and flx.

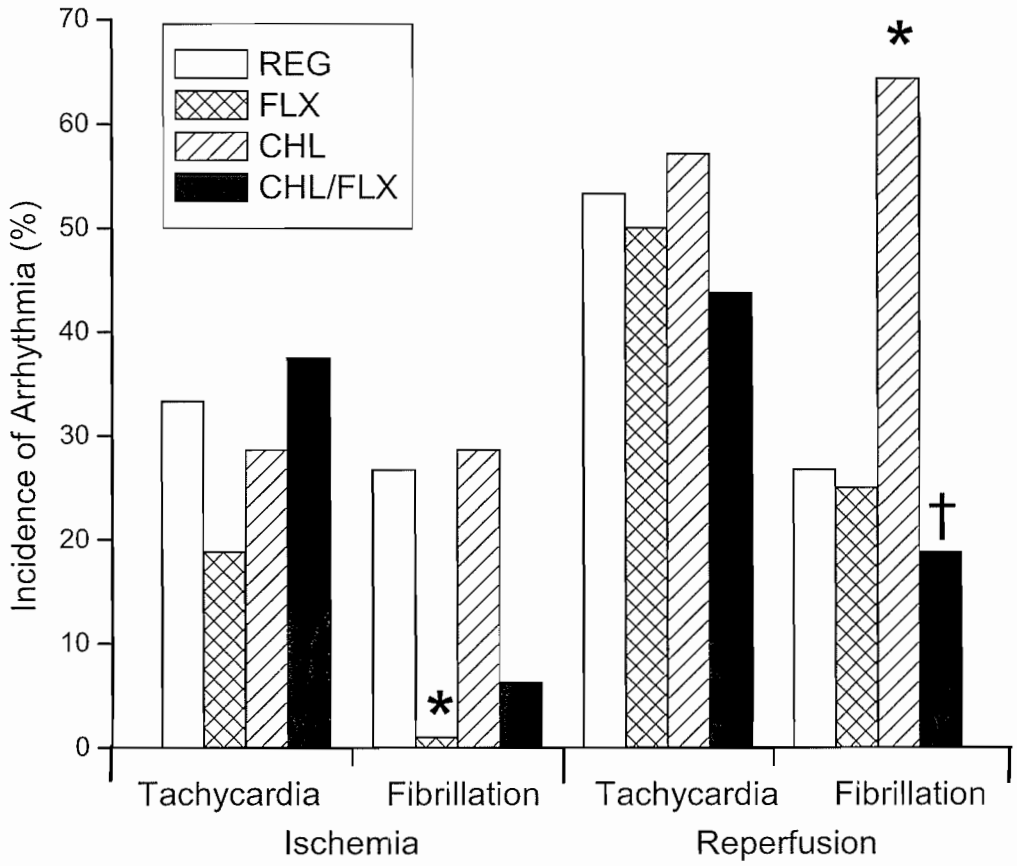


Figure 18. Incidence of spontaneous ventricular tachycardia and fibrillation during 30 min of global ischemia and 45 min of reperfusion in hearts isolated from rabbits fed either regular (REG), 10% flaxseed supplemented (FLX), 0.5% cholesterol supplemented (CHL), or 0.5% cholesterol and 10% flaxseed supplemented diets (CHL/FLX) for up to 16 weeks. * $P < 0.05$ versus REG, † $P < 0.05$ versus CHL.

the flaxseed diet significantly suppressed VF when given alone (0%) and also reduced VF in the CHL/FLX group (6%), although the latter was not statistically significant. Reperfusion induced a greater incidence of tachyarrhythmias than ischemia. Upon reperfusion, VT occurred in 53% of control hearts and this was unaffected by diet. Reperfusion resulted in VF in 27% of control hearts and this value was not reduced by flaxseed. However, CHL hearts exhibited a significantly greater incidence of VF (64%) and this was significantly decreased by inclusion of flaxseed in the diet (19%).

Duration of arrhythmias following 8 weeks feeding

Hearts exhibiting VT during ischemia did not differ significantly in their duration as shown in Figure 19A. Of those 8 week hearts which did enter VT, the longest average duration was observed in those from the control group (7.5 ± 0.8 min), whereas hearts from cholesterol fed rabbits had the shortest (1.1 ± 0.4 min). Average durations of VT during ischemia in the hearts from flax and cholesterol-flax fed groups fell between (2.0 ± 1.25 and 3.5 ± 2.0 min, respectively). The onset of reperfusion either continued or initialized arrhythmia in many of the hearts. The majority of VT episodes after 8 weeks of dietary intervention were less than 2-3 min in duration, although some hearts, from all groups but the cholesterol-flax fed, persisted for 15 min or longer, increasing the average durations for their group.

A greater duration of VF was observed in the hearts from the cholesterol fed group (7.6 min) compared to control (2.2 ± 0.4 min) during ischemia (Figure 19B). No VF was observed in hearts from the flax or cholesterol and flax fed animals.

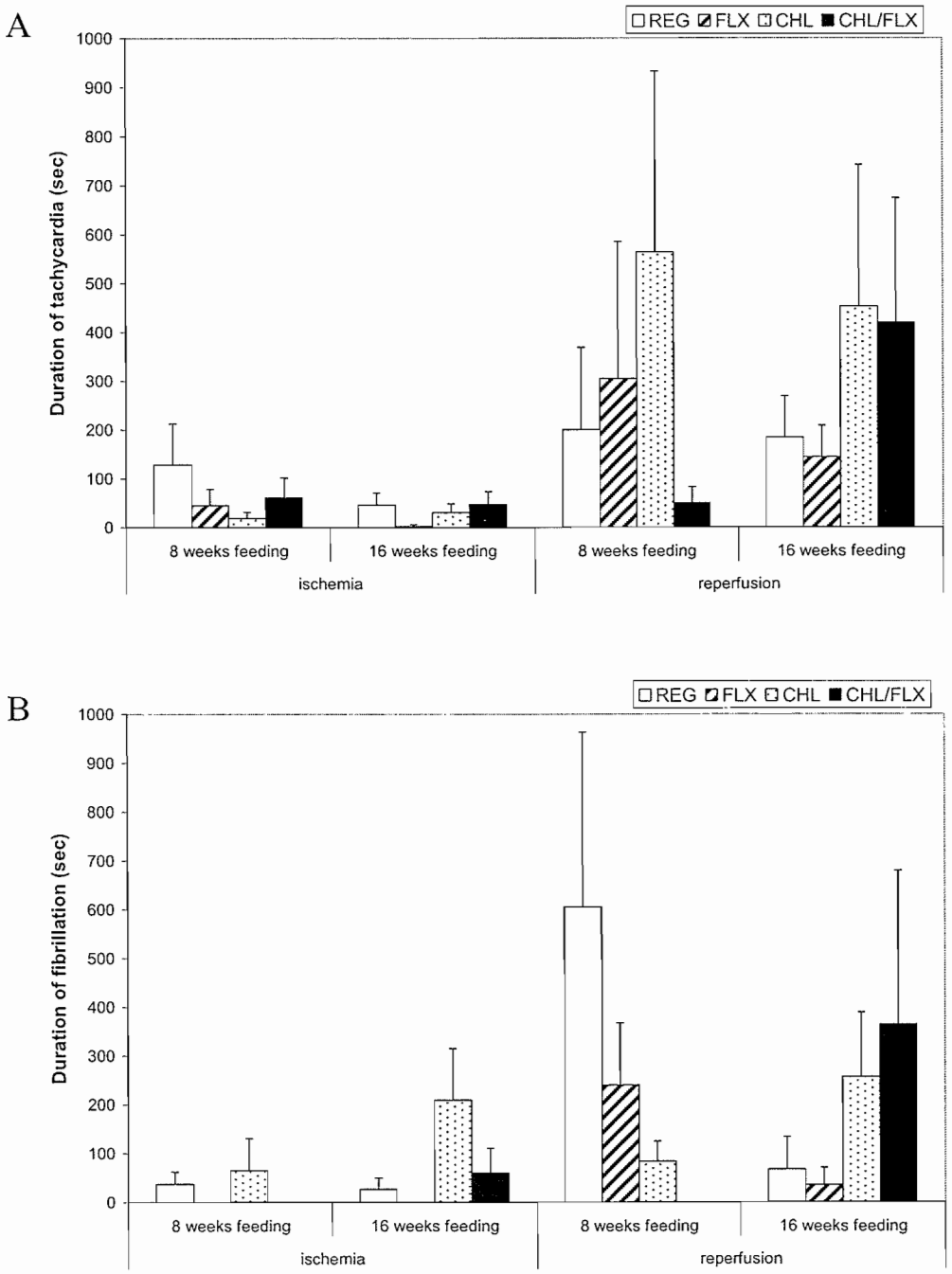


Figure 19. Mean duration of ventricular tachycardia (A) and fibrillation (B) during ischemia and reperfusion in hearts from rabbits consuming regular (REG), 10% flaxseed supplemented (FLX), 0.5% cholesterol supplemented (CHL), or 0.5% cholesterol and 10% flaxseed supplemented diets (CHL/FLX) for 8 or 16 weeks. $P > 0.05$ amongst groups.

The duration of arrhythmia during reperfusion was typically longer than during ischemia. Control hearts fibrillated the longest with an average of 23.5 ± 9.6 min. Hearts from flax fed rabbits followed at 10.6 ± 2.7 min and then by those from cholesterol fed rabbits with 2.5 ± 0.9 min. Hearts from rabbits fed both cholesterol and flax together did not fibrillate.

Duration of arrhythmias following 16 weeks feeding

After 16 weeks feeding, hearts from the rabbits consuming regular diet, cholesterol diet, and cholesterol with flax diet exhibited VT during ischemia an average 2.0 ± 0.6, 1.5 ± 0.8, and 2.0 ± 0.8 min, respectively (Figure 19A). The rabbits consuming the flax alone supplemented diet showed no arrhythmias during ischemia.

Tachycardia durations were longer during reperfusion. Mean durations of VT in hearts from regular and flax consuming rabbits were lowest (4.9 ± 1.8 and 4.7 ± 1.4 min, respectively) followed by the durations from cholesterol fed (10.5 ± 6.4 min) and cholesterol and flax fed rabbits (13.7 ± 7.2 min), both of which had one heart in VT for more than 30 min.

No VF was recorded in hearts from flax fed rabbits while subjected to ischemia (Figure 19B). The duration measured from the fibrillating heart from a regular fed rabbit was about the same as observed at 8 weeks (3.1 min). Hearts from cholesterol consuming rabbits had a mean VF duration of 8.2 ± 1.6 min whereas hearts that came from rabbits that were also consuming cholesterol-flax were about half that at 3.9 ± 2.9 min.

Mean duration of VF during reperfusion was 8.9 min for the heart from the rabbits consuming regular chow, 4.7 min for those from rabbits consuming the flax diet, 6.0 ± 2.8

min for those from cholesterol supplemented animals, and 16.5 ± 13.1 min for hearts from rabbits consuming cholesterol-flax diets, of which one heart fibrillated for more than 40 min.

Effects on cardiac parameters

Status of cardiac hypertrophy

Wet heart weights (g) were obtained following the reperfusion protocol and compared to the total rabbit weight (kg) in order to determine whether any hearts were hypertrophic as a result of the dietary interventions. All groups had a similar heart weight:body weight ratio (Table 7), suggesting that any difference in heart function or susceptibility to arrhythmias was not due to cardiac hypertrophy.

Monophasic action potentials

Heterogeneity across the ventricular wall is a substrate for arrhythmias, so epicardial and endocardial MAP durations were calculated at 90% repolarization ($MAPD_{90}$) for the rabbits following 8 and 16 weeks feeding. At both of these time points, MAP had similar characteristics. At 8 weeks, $MAPD_{90Epi}$ was initially about 25-30 msec longer than $MAPD_{90Endo}$ with the exception of the cholesterol fed group in which they were about the same. Mean $MAPD_{90Epi}$ dropped by 60.8, 73.9, 39.6, and 61.7 msec during the first 10 min of ischemia for hearts from regular, flax, cholesterol and cholesterol flax fed groups respectively (Figure 20A), whereas mean $MAPD_{90Endo}$ dropped only 43.7, 57.7, 35.4, and 44.8 msec (Figure 20B). This narrowed the MAP dispersion between epicardium and endocardium to less than 15 msec. Following 10 min of ischemia, $MAPD_{90}$ in both epicardium and endocardium began to prolong, and this prolongation continued during

Table 7. Heart weight:body weight ratios of rabbits following feeding of regular, or regular feed with flaxseed and/or cholesterol.

Dietary Group	Heart weight : Body Weight*
Regular	1.76 ± 0.06
10% Flaxseed	1.82 ± 0.11
0.5% Cholesterol	1.87 ± 0.09
0.5% Cholesterol/10% Flaxseed	1.88 ± 0.06

* Ratio is obtained by comparing rabbit heart weight in g to body weight in kg. Values represent mean ± standard error for 7-8 samples. $P > 0.05$ amongst groups.

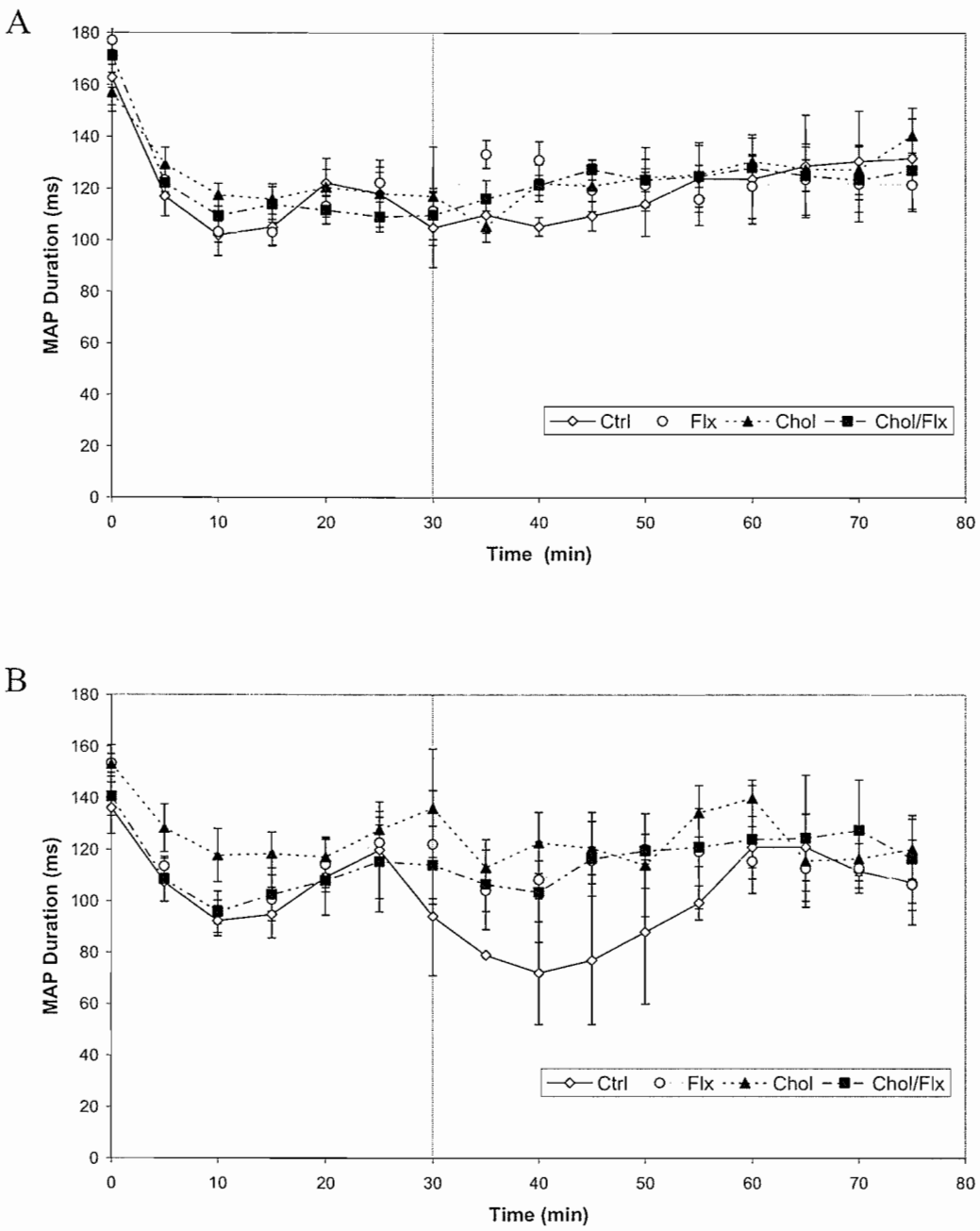


Figure 20. Epicardial (A) and endocardial (B) monophasic action potential recordings (90%) in hearts isolated from rabbits consuming regular (Ctrl), 10% flaxseed supplemented (Flx), 0.5% cholesterol supplemented (Chol), or 0.5% cholesterol and 10% flaxseed supplemented diets (Chol/Flx) for 8 weeks. $P > 0.05$ amongst groups.

reperfusion. There were no significant differences in the degree of MAPD dispersion among the groups of hearts. However, the lack of differences in the epicardial and endocardial $MAPD_{90}$ dispersion reflect the large variability in MAPD detected during ischemia and reperfusion. This variability occurred because MAPD measurements were only made in hearts that did not exhibit VT or VF at the particular time point. Thus, cautious interpretation of the $MAPD_{90}$ data is required. Further experiments would require a much larger group size in order to gather meaningful data to determine if dietary flaxseed affects dispersion of MAPD across the ventricular wall.

MAPD dispersion (an index of heterogeneity) was not significantly different amongst the groups of hearts during ischemia or reperfusion after the longer feeding period. Both epicardial and endocardial MAPD recorded in hearts of rabbits fed for 16 weeks were similar to those following 8 weeks feeding (Figure 21). Although epicardial $MAPD_{90}$ of control and cholesterol fed groups appeared longer versus the two flaxseed fed groups prior to, and at the start of ischemia, the differences were not statistically significant.

Left-ventricular developed pressure

In all hearts, ischemia decreased LVDP to 0-5 mm Hg within 10 min (Figure 22A). Reperfusion triggered a brief rapid recovery of LVDP in the first 5 min, which then evolved into a more gradual recovery until the end of the reperfusion period. At the end of the reperfusion period, developed pressure recovered to 25-35% of the pre-ischemic levels in all the 8 week groups. Rabbits from the 16 week feeding groups also exhibited a similar recovery of LVDP with no differences amongst any of the groups (Figure 22B).

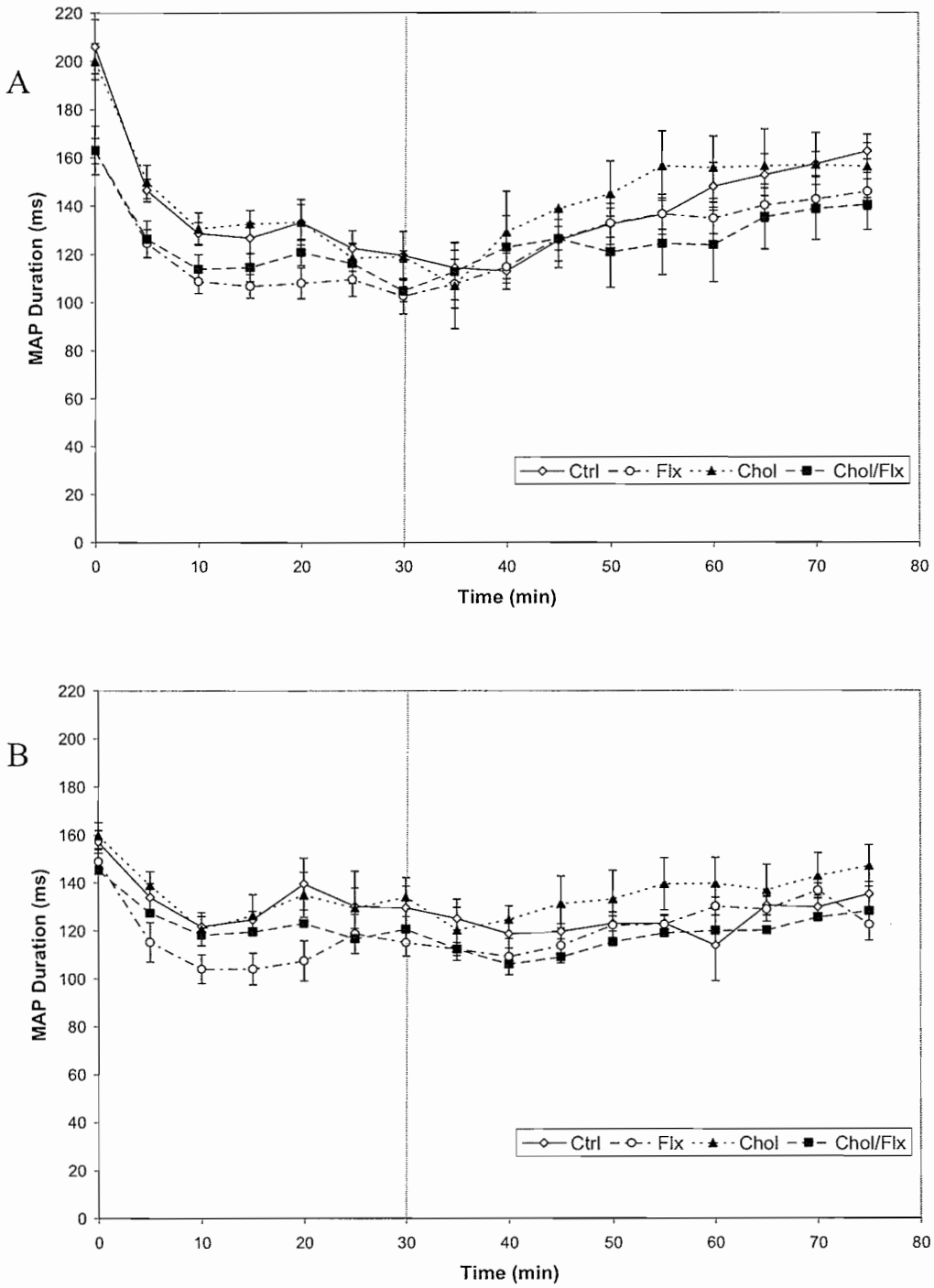


Figure 21. Epicardial (A) and endocardial (B) monophasic action potential recordings (90%) in hearts isolated from rabbits consuming regular (Ctrl), 10% flaxseed supplemented (Flx), 0.5% cholesterol supplemented (Chol), or 0.5% cholesterol and 10% flaxseed supplemented diets (Chol/Flx) for 16 weeks. $P > 0.05$ amongst groups.

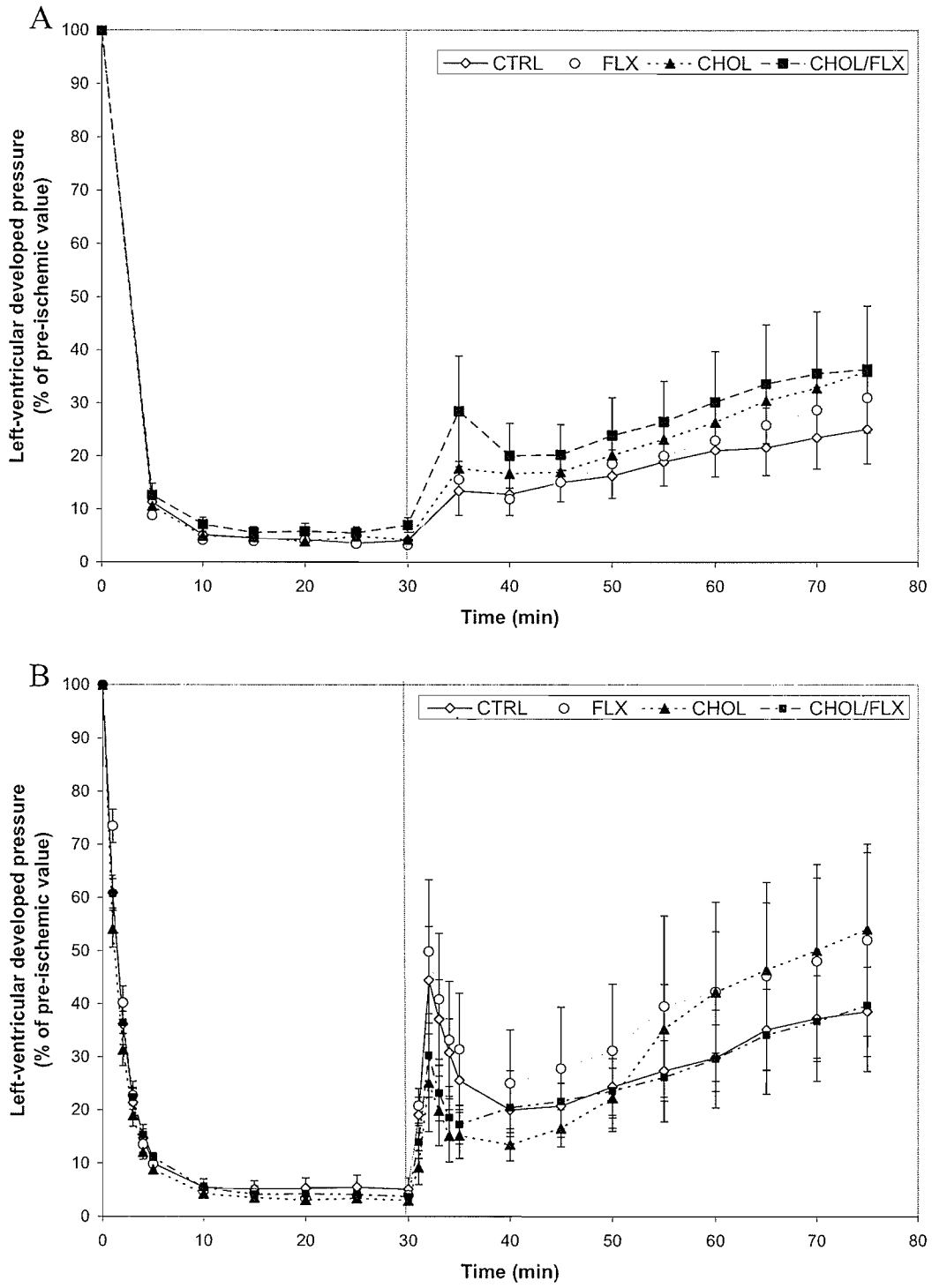


Figure 22. Left-ventricular developed pressure in hearts isolated from rabbits consuming regular (CTRL), 10% flaxseed supplemented (FLX), 0.5% cholesterol supplemented (CHOL), or 0.5% cholesterol and 10% flaxseed supplemented diets (CHOL/FLX) for 8 (A) or 16 (B) weeks. $P > 0.05$ amongst groups.

Left-ventricular end-diastolic pressure

A slow but continuous rise in EDP occurred throughout the ischemic period (Figure 23A). Hearts from the flax fed group exhibited the smallest increase in EDP during ischemia, whereas cholesterol hearts exhibited the largest ($P<0.05$ at T=25). At 8 weeks, final left-ventricular EDP was similar amongst all of the groups at the end of reperfusion. EDP in the hearts from rabbits from the 16 week feeding group followed a similar pattern to that in the 8 week trial. Left-ventricular EDP was similar amongst all of the groups in the 16 week study during ischemia and reperfusion (Figure 23B).

Effects on the QT Interval

The QT intervals of the ECGs were examined to relate the characteristics of electrical activity with arrhythmogenesis. The QT intervals were measured in the paced hearts prior to the ischemia-reperfusion protocol in the Langendorff perfused hearts. Rabbits fed flaxseed exhibited a shorter QT interval than the controls, whereas the longest QT intervals were measured in the cholesterol fed group (Table 8). The addition of flaxseed to the cholesterol supplemented diet significantly shortened the QT interval in the hearts. The flaxseed supplemented groups, FLX and CHL/FLX, exhibited the shortest QT intervals and were also the groups most resistant to arrhythmias during ischemia-reperfusion.

ACUTE EFFECTS OF FATTY ACIDS

FATTY ACIDS AND THE CARDIAC ACTION POTENTIAL CONFIGURATION

The duration of the action potential can influence arrhythmogenesis. To further delineate the potential mechanism for the anti-arrhythmic effects of flaxseed, we examined

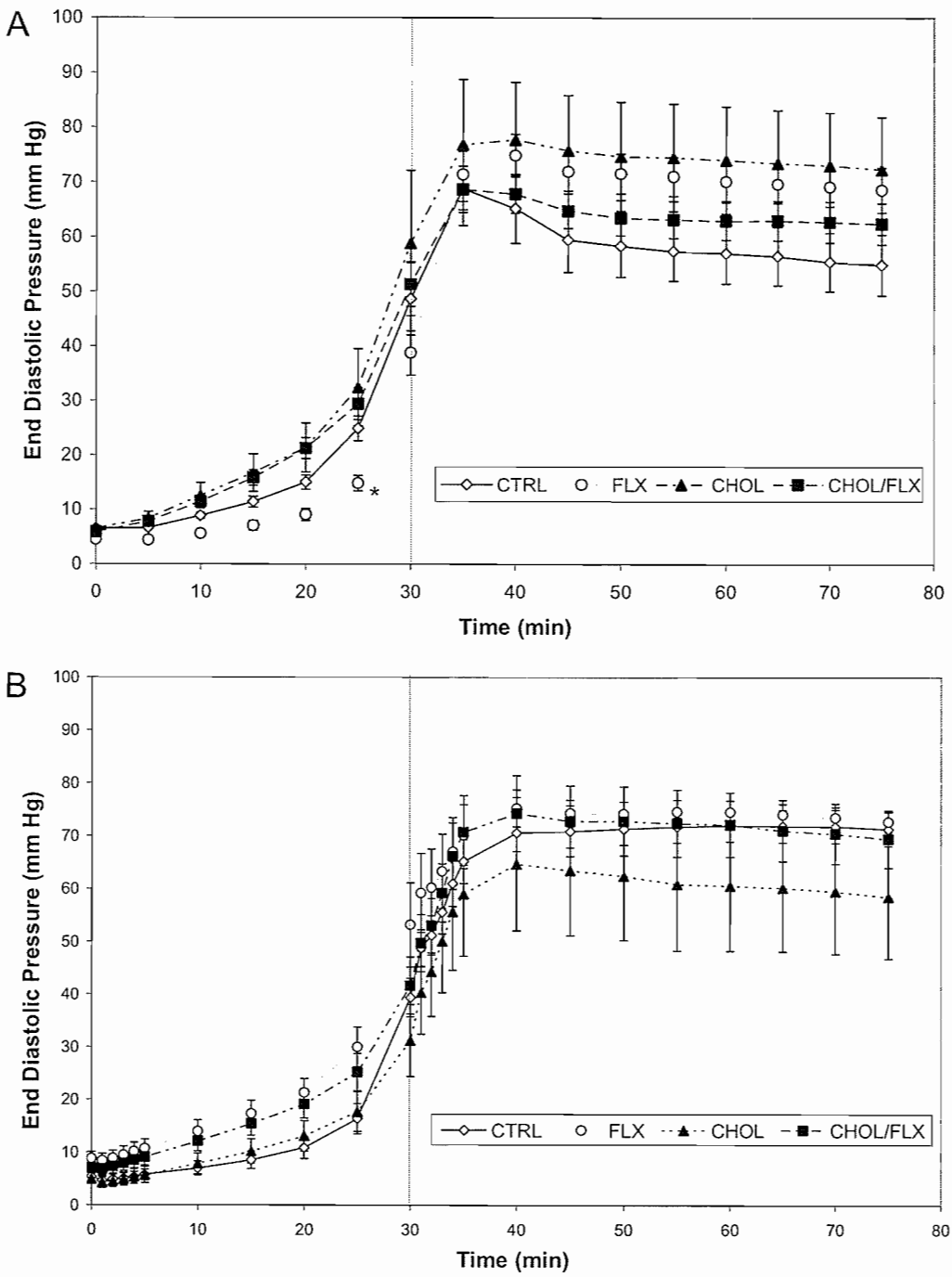


Figure 23. End-diastolic pressure in hearts isolated from rabbits consuming regular (CTRL), 10% flaxseed supplemented (FLX), 0.5% cholesterol supplemented (CHOL), or 0.5% cholesterol and 10% flaxseed supplemented diets (CHOL/FLX) for 8 (A) or 16 (B) weeks. * $P < 0.05$ vs. CHOL.

TABLE 8. QT intervals measured from ECGs recorded in hearts isolated from male New Zealand White rabbits consuming one of the four experimental diets.[1,2]

Dietary Group	QT interval (ms)
REG	234 ± 2^{a}
FLX	226 ± 2^{b}
CHL	241 ± 2^{c}
CHL/FLX	225 ± 2^{b}

[1] All hearts were paced at 2 Hz.

[2] Values are means ± SEM. Values not sharing a common superscript are significantly different, $P < 0.05$. n = 14-16 per group.

the acute effects of ALA, as well as other fatty acids on action potentials in cardiomyocytes isolated from control rabbits. Action potentials were triggered at cycle lengths of 250-5000 msec. Action potential durations were measured at 50 and 90% repolarization (Figure 24).

The monounsaturated fatty acid, oleic acid, had no effect on APD_{50} at any concentration tested (Figure 25A), but shortened APD_{90}. In contrast, the ω-3 PUFA tested, ALA and DHA, significantly shortened APD_{50} and APD_{90} (Figures 25B and C). No fatty acids tested had any effect on the resting potential (-76.9 ± 0.2 mV) or the amplitude of the action potential (113.8 ± 0.5 mV, data not shown).

INFLUENCE OF THE NCX ON THE NEONATAL RAT ACTION POTENTIAL

Together with ion channels that are active throughout the cardiac action potential, the NCX also has influence on the overall configuration [184-186]. The NCX influences the action potential because it is electrogenic and it is responsive to sodium and calcium gradients which are in constant flux within the cell, particularly in proximity of the sarcolemmal membrane.

We were able to measure action potentials in single neonatal rat cardiomyocytes that were controls or had NCX1.1 expression silenced through the use of shRNA [179]. Action potentials from the control neonatal cells had a similar appearance to action potentials recorded in the rabbit *in vitro*, however, the action potentials from cells lacking expression of NCX1.1 were markedly different in appearance (Figure 26). The plateau of the neonatal rat cardiac AP was severely truncated, thus dramatically reducing the APD_{50} in these cells (Figure 27A), but a "tail" was present on the action potentials that resulted in the APD_{90} of the cells being similar in duration (Figure 27B). Resting potential was not different between

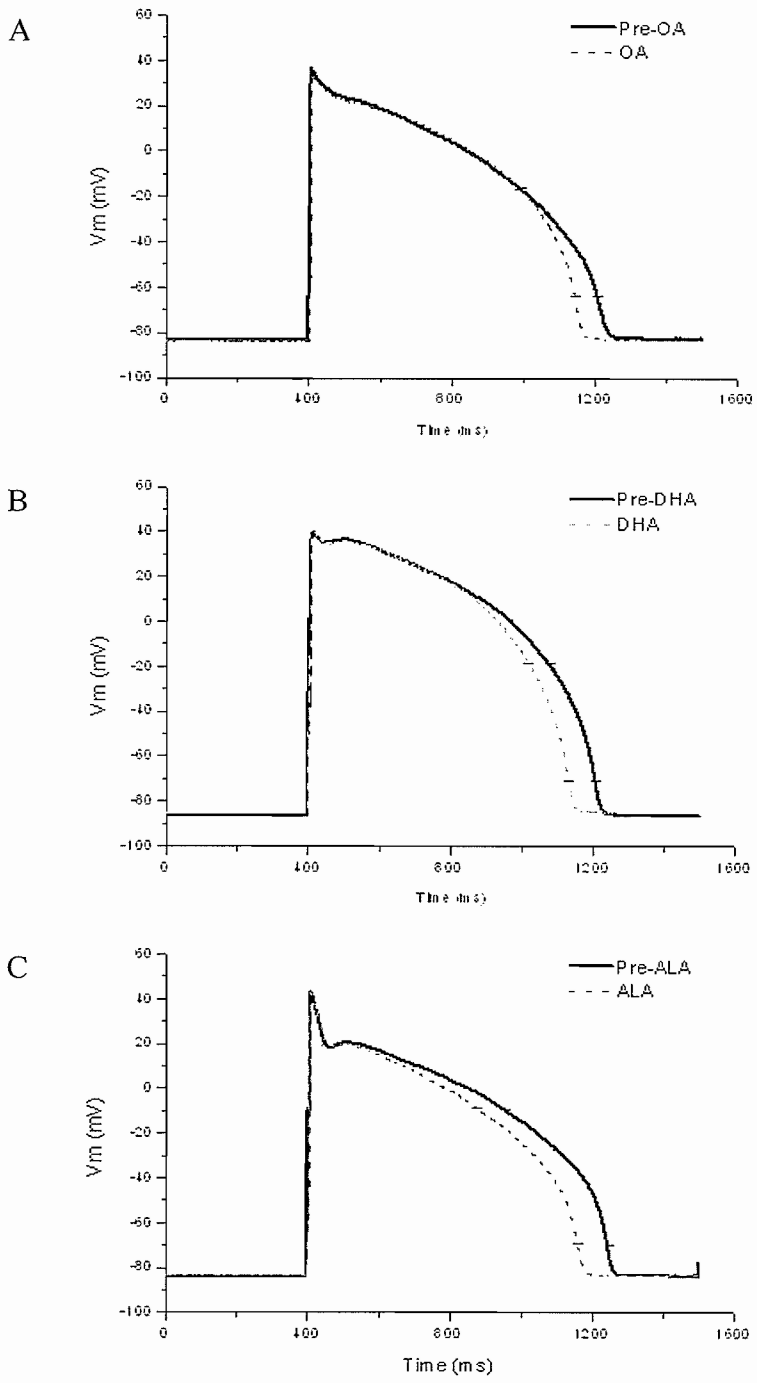


Figure 24. Representative action potentials recorded before and after addition of 25 µM (A) oleic acid, (B) docosahexaenoic acid and (C) α-linolenic acid. Cardiomyocytes were maintained at 23°C and stimulated at 0.5 Hz. Action potential durations at 50% and 90% repolarization are indicated by horizontal bars.

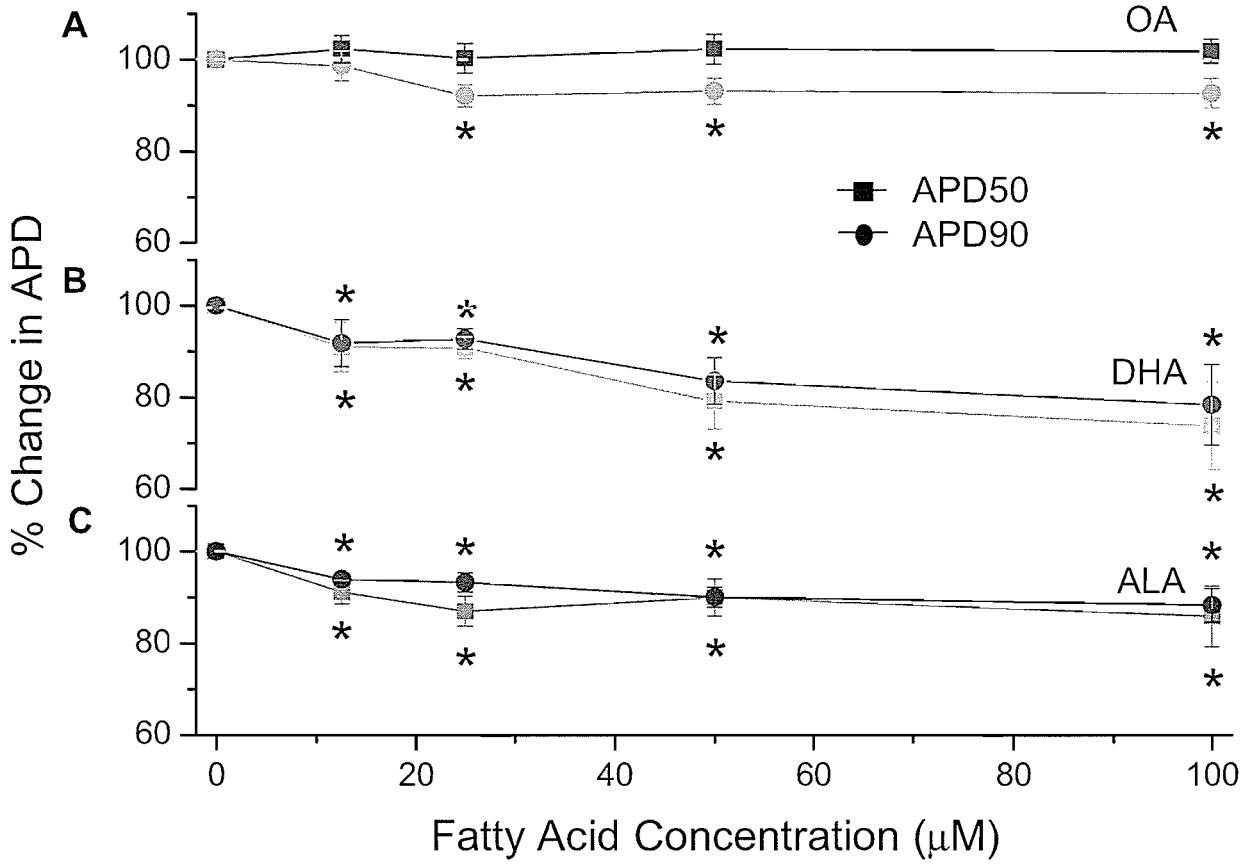


Figure 25. Effects of adding oleic acid (A), docosahexaenoic acid (B), or α-linolenic acid (C) on APD_{50} and APD_{90} in isolated adult rabbit left-ventricular cardiomyocytes. Cells were stimulated at 0.5 Hz in current-clamp mode using the perforated patch clamp technique. Values shown are mean +/- standard error. * $P < 0.05$ vs. pre-fatty acid APD.

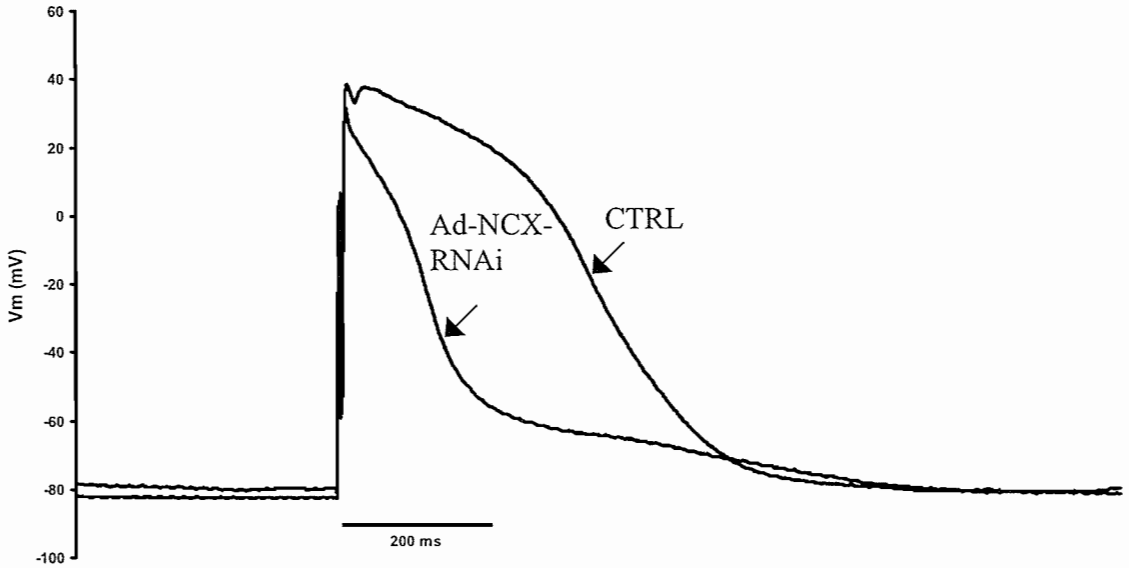


Figure 26. Effect of silencing the NCX on the neonatal rat action potential. Compared to an action potential from a wild type cardiomyocyte, when the NCX is not expressed, the APD_{50} is significantly abbreviated. The APD_{90} are about the same in the two cells, due to the tail present in the neonatal rat action potential.

control (-60.7 ± 3.1 mV) or NCX silenced (-58.5 ± 3.8 mV) cells, and action potential amplitude was only slightly lower in the NCX silenced cells (73.3 ± 2.7 mV) compared to controls (85.9 ± 4.6 mV). Different cycle lengths of stimulation were used to examine the rate-dependent response for the neonatal rat action potentials (Figure 27). As expected, action potentials duration decreased as the stimulation rates increased. This is due to incomplete recovery of L-type Ca^{2+} channels (LTCC) and accumulation of I_{Ks}, which will reduce depolarizing and increase repolarizing current, respectively [187].

Action potentials were also recorded in neonatal rat cardiomyocytes overexpressing NCX1.1. In contrast to the shortened action potentials recorded in the NCX silenced cells, the action potentials recorded from cells with additional NCX expression and activity had an increased plateau duration (APD_{50}) (Figure 28A) and overall duration (APD_{90}) (Figure 28B).

EFFECTS OF ALA ON SPONTANEOUS ACTIVITY OF NEONATAL RAT CARDIOMYOCYTES

Groups of neonatal rat cardiomyocytes exhibited spontaneous contractile activity and trains of action potentials could be recorded using the patch-clamp technique (Figure 29). When ALA was applied to the bathing solution, the rate of contraction decreased in a dose-dependent manner and the cells completely stopped contracting at 100 μM ALA (Figure 30). When the cells were washed with a solution containing fatty-acid free BSA and no ALA, contraction of the cells was restored.

EFFECTS OF ALA ON NEONATAL RAT ACTION POTENTIALS

Neonatal rat cardiomyocytes were stimulated at 0.5 Hz to record action potentials

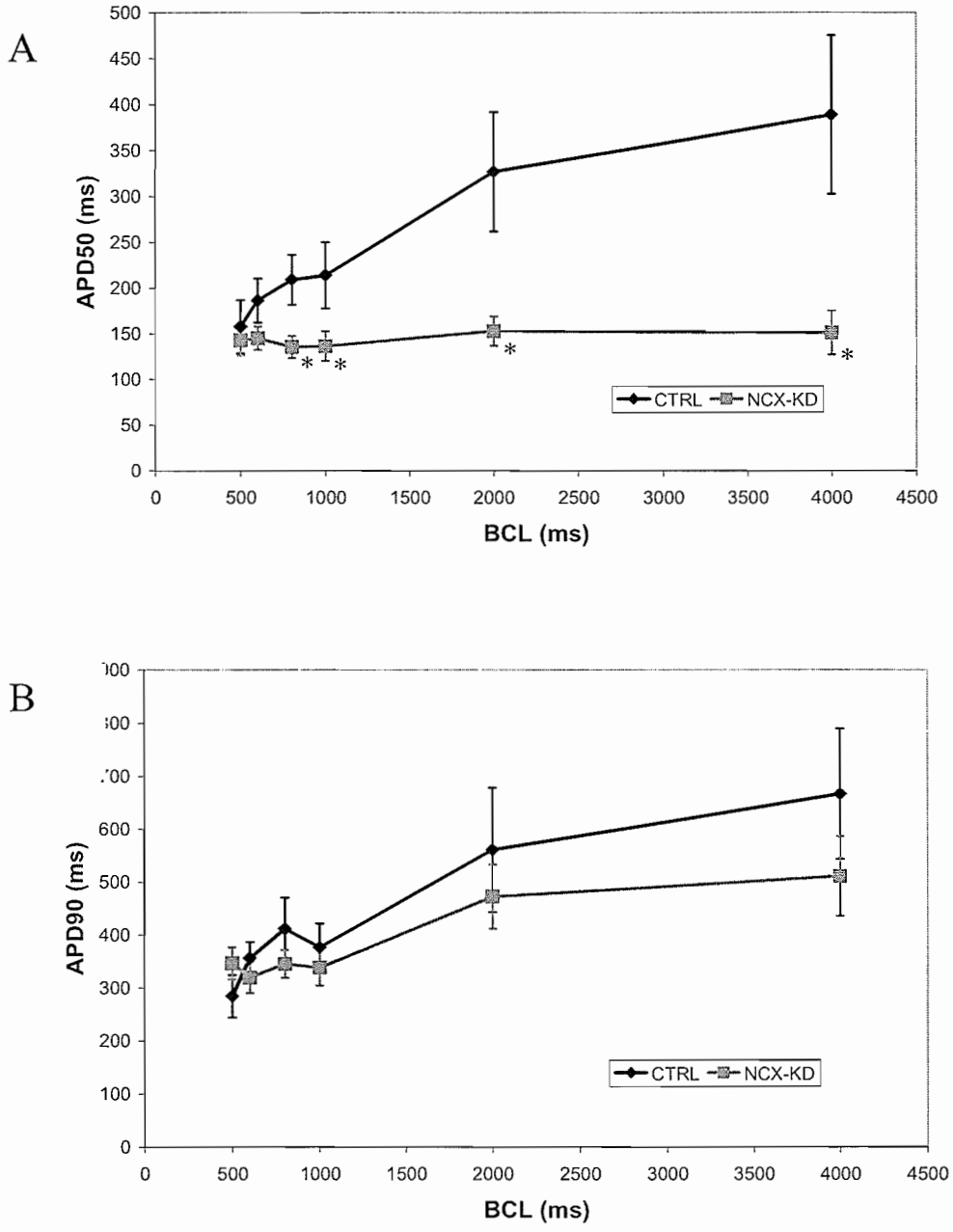


Figure 27. Effect of increasing basic cycle length (BCL) of stimulation on the duration of the action potential at 50% repolarization (APD_{50}) and 90% repolarization (APD_{90}) in neonatal rat cardiomyocytes transduced with either a control (CTRL, n=4-6) or NCX knock-down (NCX-KD, n=8-14) sequence. Values shown are mean +/- standard error. * P<0.05 vs. CTRL.

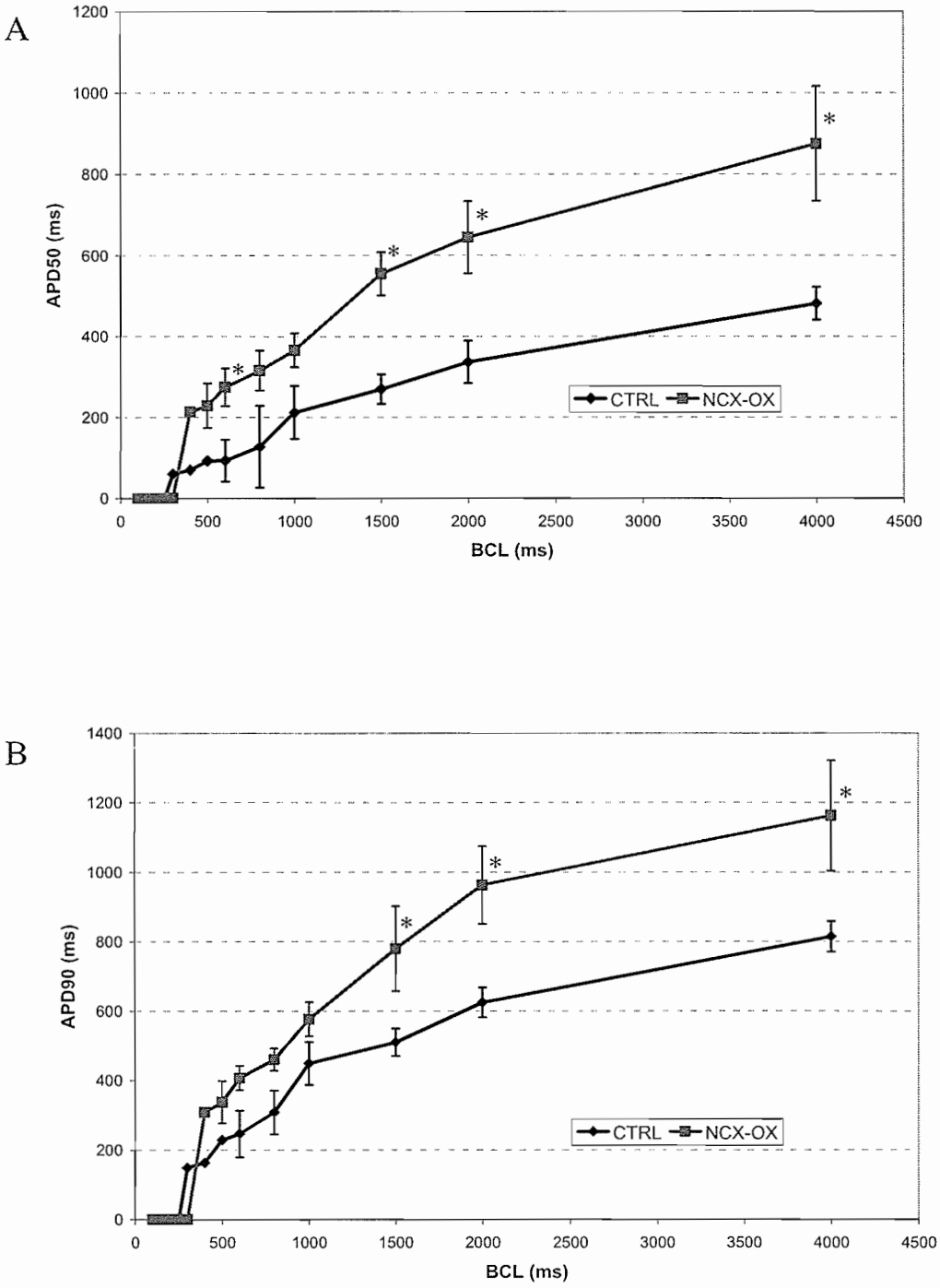


Figure 28. Effect of increasing stimulation cycle length on the duration of the action potential at 50% repolarization (APD_{50}) and 90% repolarization (APD_{90}) in neonatal rat cardiomyocytes transduced with either a control (CTRL, n=4-6) or NCX1.1 (NCX-OX, n=8-14) sequence. Values shown are mean +/- standard error. * $P<0.05$ vs. CTRL.

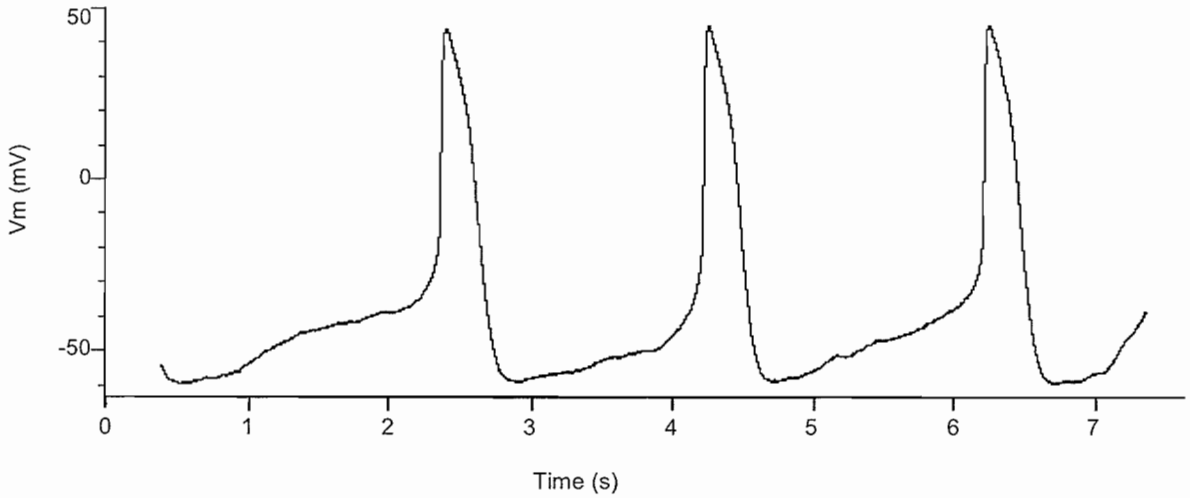


Figure 29. Action potentials recorded in spontaneously beating neonatal rat cardiomyocytes. Note the slow depolarization through phase 4, characteristic of spontaneously depolarizing tissues.

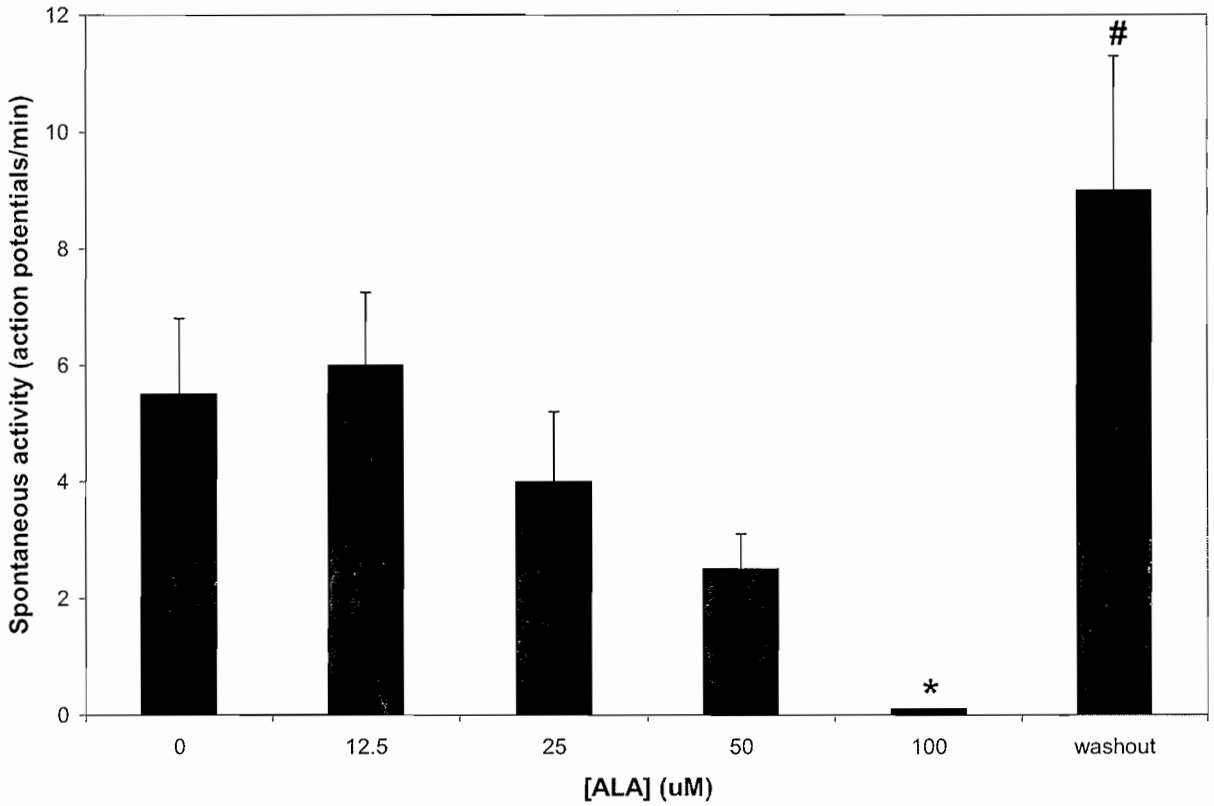


Figure 30. Application of ALA to spontaneously beating neonatal rat cardiomyocytes slowed the contraction rate in a dose-dependent fashion, eventually stopping contraction altogether. When ALA was washed out, cells continued to spontaneously contract. * $P < 0.05$ vs. 0 and 12.5 μM ALA, # $P < 0.05$ vs. 100 μM ALA.

prior to and following the addition of 25 μM ALA to the bathing solution. APD_{50} was reduced to 61.0 ± 15.5% of the control value and APD_{90} was reduced to 68.2 ± 12.5% by ALA (n=3) (Figure 31). Given the small sample sizes, these differences did not reach statistical significance.

POLYUNSATURATED FATTY ACIDS AND THE NCX

NCX Current in control HEK293 cells and cells stably transfected with NCX1.1 or NCX1.3

Given that either overexpressing or silencing the NCX in cardiomyocytes has direct effects on the action potential configuration, we chose to examine the influence of PUFA on NCX activity. The effects of ALA on NCX activity have not been tested to date. HEK293 cells were used to investigate the effects of various fatty acids on NCX activity because these cells do not contain any native NCX1. Western blot analysis of NCX protein confirmed the absence of this protein in these cells (Figure 32). No band was visible in the lane loaded with protein from non-transfected HEK293 cells, indicating a lack of endogenous NCX1 in these cells. However, the expression of NCX in the HEK293 cells was induced using Lipofectamine (Invitrogen). NCX1 protein was detected only in protein extracts from cells transfected with and expressing NCX1.1 or NCX1.3 (Figure 32). The band for NCX1.1 appeared at the reported weight of 120 kDa, while that of NCX1.3 was slightly lower, because it contains 36 fewer amino acids.

NCX current in control HEK293 cells and cells stably transfected with NCX1.1 or NCX1.3Non-transfected HEK293 cells have no native NCX activity and this was confirmed by the absence of an effect of Ni^{2+} application during the voltage ramp protocol (Figure 33A). In contrast, HEK293 cells stably transfected with and expressing the NCX1.1 (Figure

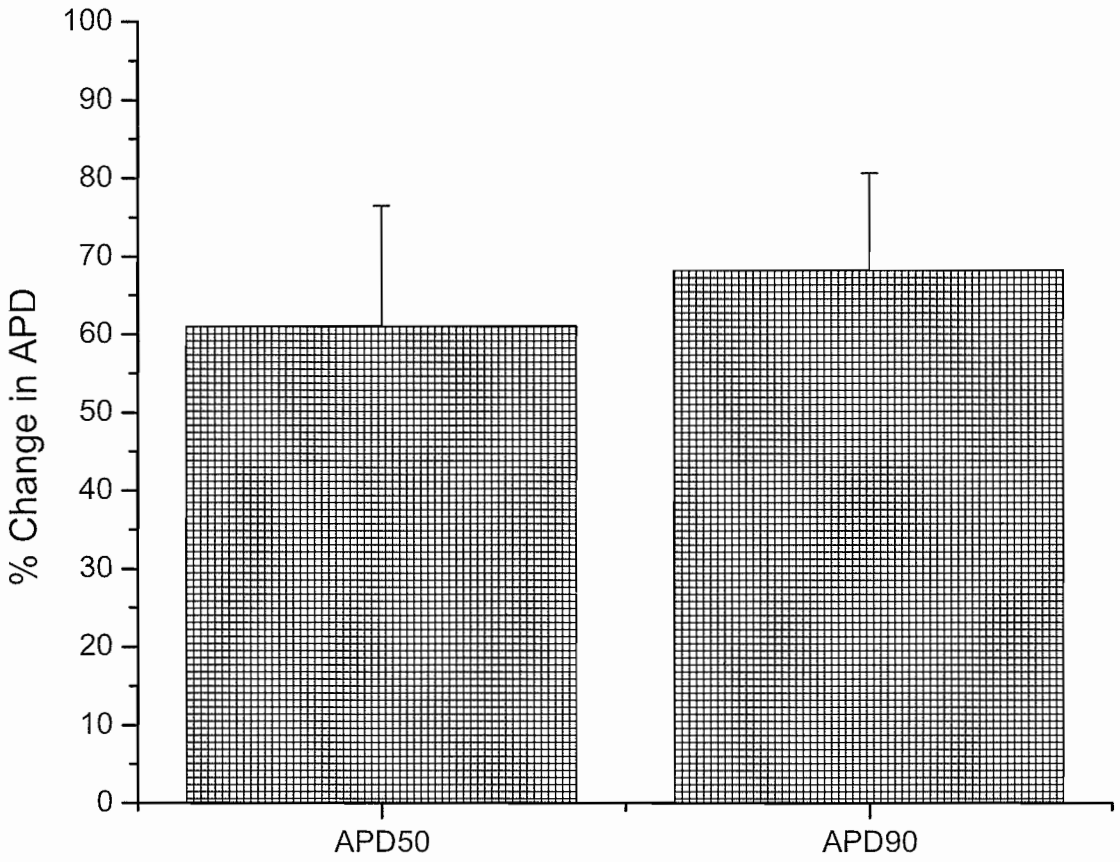


Figure 31. Relative change in neonatal rat cardiac action potential duration (APD_{50} and APD_{90}) following application of 25 µM ALA. Cells were stimulated at 0.5 Hz using the perforated patch-clamp technique. Values shown are mean +/- standard error (n=3).

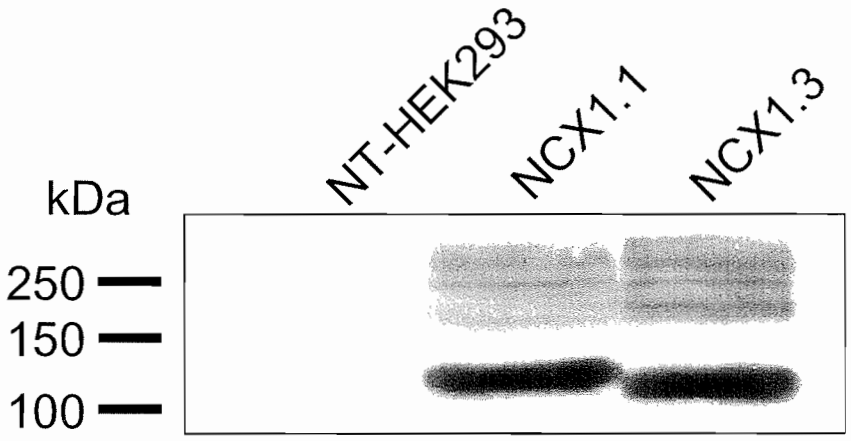


Figure 32. Western blot of total protein extracts from non-transfected HEK293 cells (NT-HEK293), or HEK293 cells expressing NCX1.1 or NCX1.3.

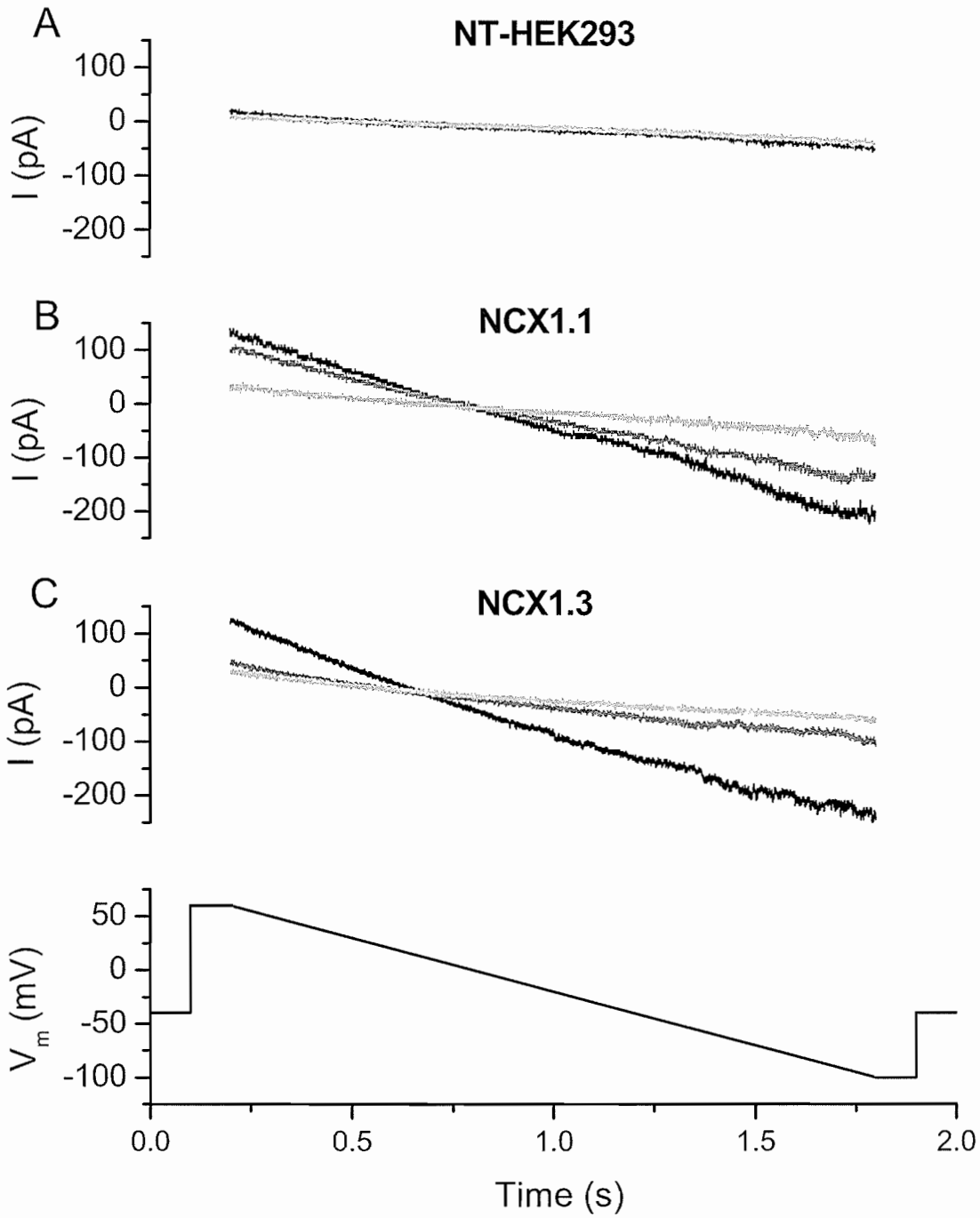


Figure 33. Representative current records in response to a voltage ramp from +60 to -100 mV. Incubation of control HEK293 cells with the NCX blocking ion Ni^{2+} (5 mM) has no effect on the current demonstrating the absence of NCX in these cells (A). In contrast, HEK293 cells expressing NCX1.1 (B) or NCX1.3 (C) show sizeable Ni^{2+} sensitive currents, which can be partially blocked by application of ALA. For each panel, currents are recorded from the same cell, prior to, and following treatments. Control current – black; current following application of ALA – red; current following application of $NiCl_2$ – green.

33B) or NCX1.3 (Figure 33C) genes displayed a sizeable current, which was sensitive to Ni^{2+}. This Ni^{2+}-sensitive current was defined as the total NCX current. The reversal potential for the NCX was not significantly different in the NCX1.1 and NCX1.3 expressing cells (8.09 ± 2.48 mV and 4.57 ± 2.73 mV, respectively) under control conditions. In addition, the maximal inward and outward currents, measured at -100 mV and +60 mV, respectively, were similar in the two cell lines. When normalized for cell capacitance, forward and reverse mode NCX1.1 activity was -11.21 ± 0.87 pA/pF and 7.39 ± 0.52 pA/pF, respectively, and NCX1.3 activity was -12.16 ± 0.80 pA/pF and 9.26 ± 0.84 pA/pF in the respective transport modes. Also shown are representative NCX1.1 and NCX1.3 current recordings following addition of ALA (25 μM) to the bath (Figures 33B and 33C). In both cases, ALA inhibited both forward and reverse mode NCX activity and thus caused a decrease in the recorded current.

Dose-response of ALA on NCX1 isoforms

Dose-response curves for ALA inhibition were constructed in HEK293 cells expressing NCX1.1 or NCX1.3 and subjected to voltage ramp protocols. ALA inhibited the reverse mode NCX1.1 activity (measured at +60 mV) with an IC_{50} of 0.11 ± 0.02 μM (Figure 34A), which was not significantly different from the IC_{50} for forward mode inhibition (0.12 ± 0.02 μM, measured at -100 mV) (Figure 34B). Alpha-linolenic acid also inhibited the reverse (Figure 34C) and forward (Figure 34D) mode activities of NCX1.3, similar to its effects on NCX1.1. However, NCX1.3 was approximately five to seven times more sensitive to ALA inhibition than the NCX1.1 isoform. ALA inhibited the reverse and forward modes

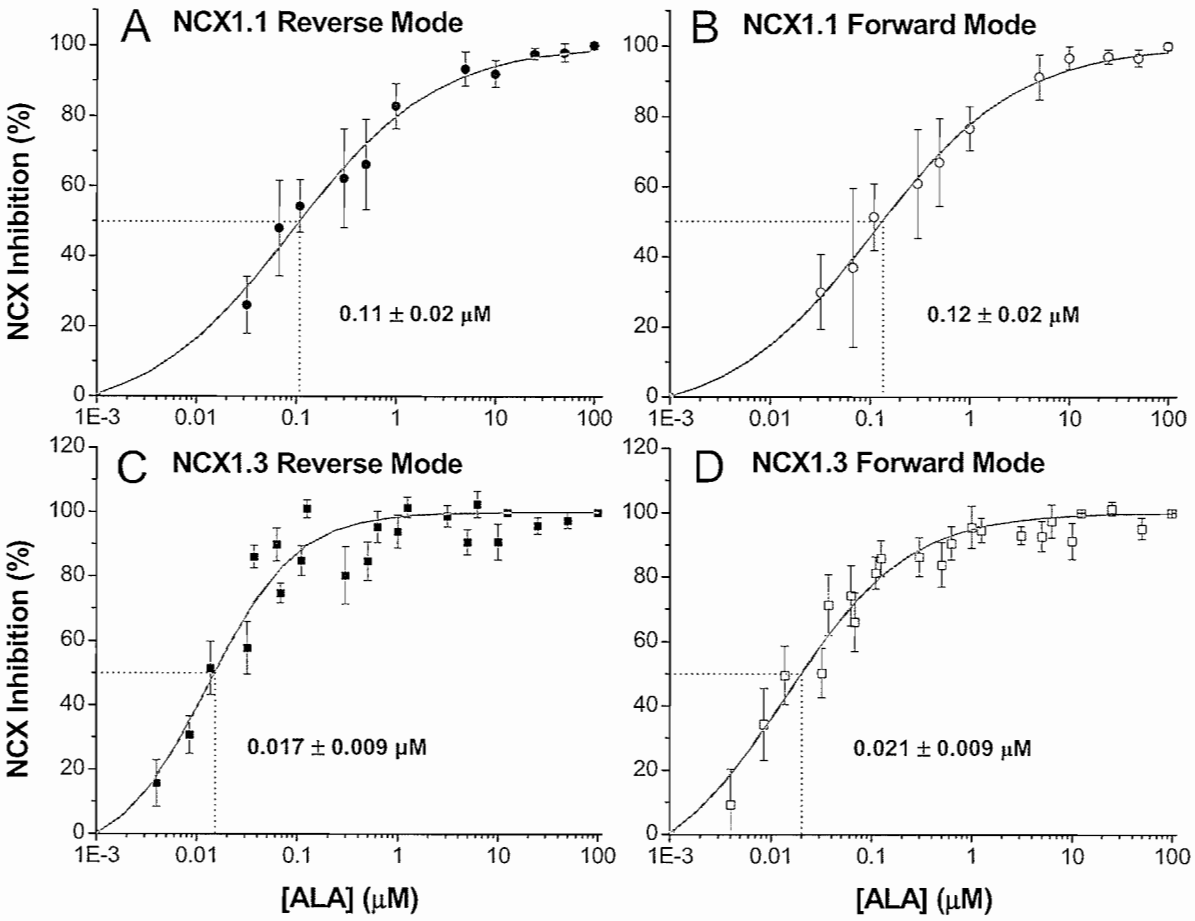


Figure 34. Application of ALA (0-100 μM) to HEK293 cells expressing the cardiac isoform of the NCX produced a dose-dependent inhibition of reverse (A) and forward mode (B) NCX1.1 current (n=10). The dose dependent inhibition is also observed in cells expressing the vascular NCX isoform. ALA inhibited the NCX1.3 reverse (C) and forward mode (D) activity at lower concentrations (n=6-11). Maximal inhibition of NCX1 was determined with 100 μM ALA and all other currents measured in response to each ALA dose were compared to this value. Reverse and forward measurements of NCX1 activity were taken at +60 and -100 mV, respectively.

of NCX1.3 activity at an IC_{50} of 0.017 ± 0.009 μM and 0.021 ± 0.009 μM, respectively. Thus, inhibition of NCX1 by ALA appears to be non-selective for a specific transport mode.

Effects of different classes of fatty acids on NCX1.1 activity

A maximal inhibitory concentration of 25 μM was chosen to assess the effects of different families of fatty acids on the exchanger. As shown in Figure 35A, ALA inhibited NCX1.1, but the effect was only significant at the extreme positive and negative potentials measured. Eicosapentaenoic acid was effective at significantly reducing both modes of exchange activity across most potentials, with the exception of those close to the convergence point of the traces (Figure 35B). The ω-3 PUFA, however, did not completely block NCX1.1 activity, since addition of 5 mM $NiCl_2$ produced additional inhibition of the NCX1.1 exchanger. In contrast, neither the MUFA, OA (Figure 35C), nor the ω-6 PUFA, LA (Figure 35D), had any effect on forward or reverse mode NCX1.1 activity. A closer examination of the potentials to which the NCX1.1 would be exposed to in the heart (-85 to +30 mV), revealed that only EPA significantly altered exchanger activity, although ALA did display a trend towards inhibition. A summary of the inhibitory effects of the different fatty acids on NCX1.1 forward and reverse modes, measured at -100 mV and +60 mV, respectively, is shown in Figure 36. The ω-3 PUFA, ALA and EPA, inhibited forward and reverse modes of NCX activity, while OA and LA had no effect.

Effects of different classes of fatty acids on NCX1.3 activity

We also tested the effects of different classes of fatty acids in HEK293 cells expressing the vascular NCX1.3 exchanger at a maximally inhibitory concentration of 25

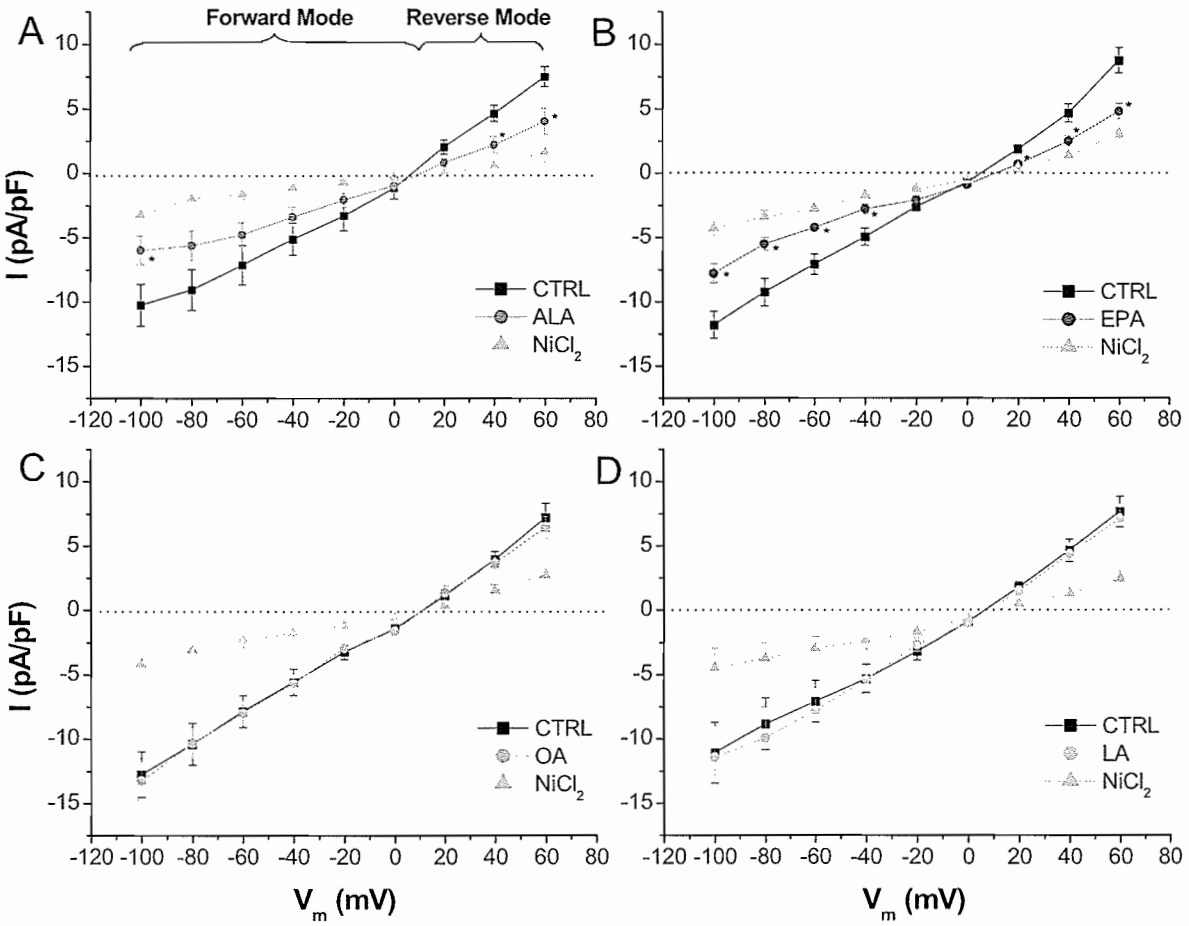


Figure 35. Summary of the inhibitory effects of different classes of fatty acids (25 µM) on NCX1.1. Only ALA (A, n=8) and EPA (B, n=10) inhibited the NCX1.1 current during the voltage ramp protocol. ALA significantly inhibited only at the extreme voltages, whereas EPA inhibited the current throughout the range from +60 to -100 mV. In both cases, NCX activity was further inhibited by 5 mM Ni^{2+} solution, demonstrating that ω-3 PUFA only partially inhibit the NCX. The monounsaturated fatty acid, OA (C, n=14), and ω-6 polyunsaturated fatty acid, LA (D, n=10), had no effect on current. * $P < 0.05$ vs. CTRL.

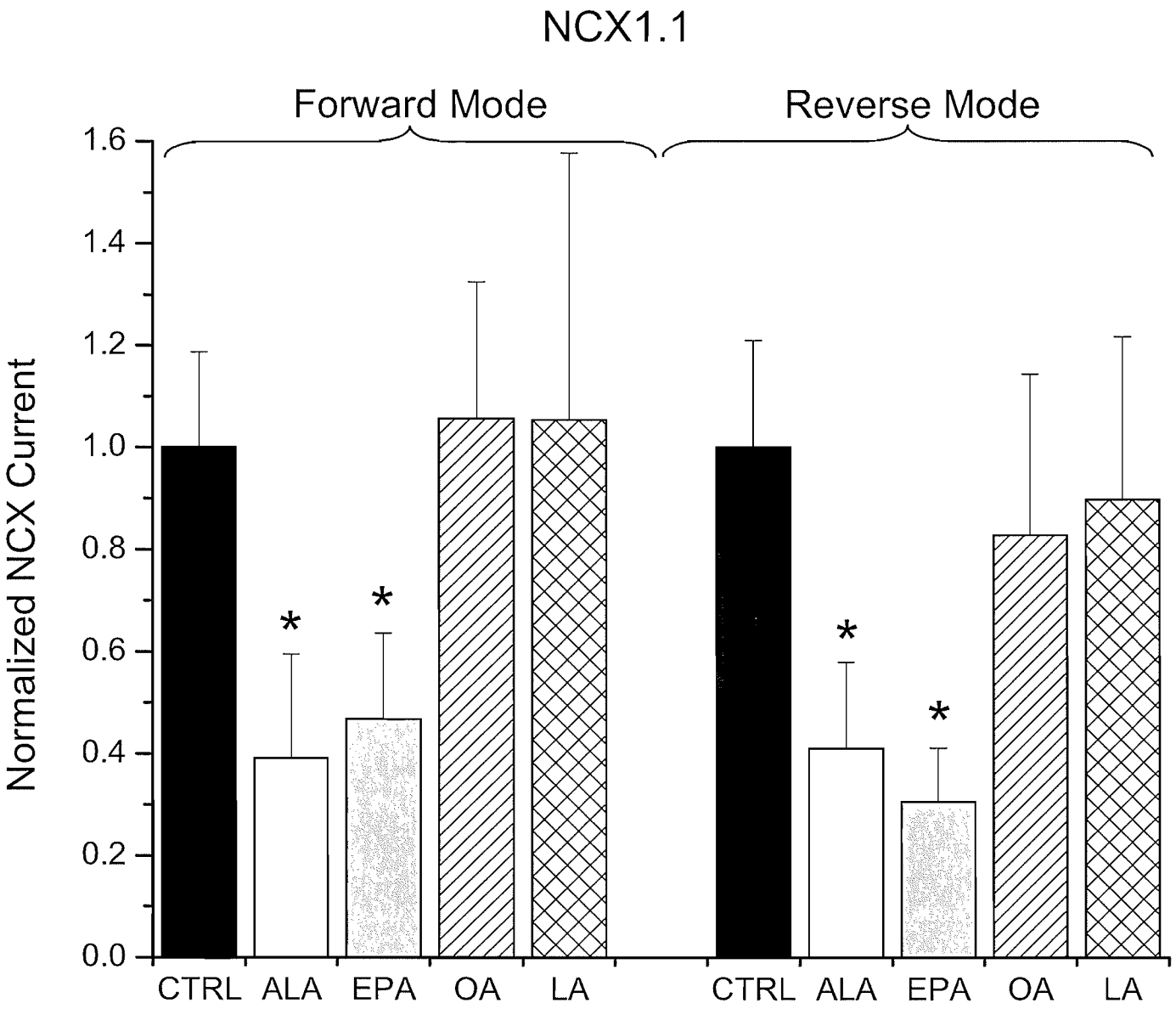


Figure 36. Relative magnitude of NCX current recorded after addition of 25 μM ALA, EPA, LA or OA to HEK293 cells expressing NCX1.1. Forward and reverse modes of NCX activity were measured at -100 and +60 mV, respectively. Total NCX current at these two potentials was calculated by subtracting the current remaining after 5 mM $NiCl_2$ from the pre-$NiCl_2$ current. The current remaining following application of each fatty acid was normalized to controls by dividing it by the pre-treatment, Ni^{2+}-sensitive (NCX) current. Standard error for the control group represents the error associated with the mean of the control and was calculated according to the rules for propagation of error. * $P < 0.05$ vs. CTRL.

μM. Similar to the NCX1.1, ALA and EPA inhibited both modes of NCX1.3 activity, but to an even greater extent (Figures 37A and B). However, the MUFA, OA, also inhibited the forward and reverse modes of NCX1.3 at most potentials (Figure 37C), which is in contrast to its lack of effect on NCX1.1. The ω-6 PUFA, LA, only inhibited NCX1.3 significantly at -100 mV, while some inhibition was observed throughout the range of potentials, albeit not statistically significant (Figure 37D). A closer look at the effects of fatty acids within the physiological range of potentials in the vasculature (-40 to 0 mV) revealed that only significant NCX1.3 inhibition occurred with ω-3 PUFA treatment, but ALA had a greater effect near 0 mV. The lack of blockade at 0 mV may reflect the diminutive NCX1.3 current recorded near the reversal potential. Figure 38 summarizes the inhibitory effects of the various fatty acids on forward and reverse modes of NCX1.3 activity, measured at -100 mV and +60 mV, respectively. All fatty acids tested inhibit forward mode NCX activity by about 50%, whereas ALA and LA have slightly greater inhibitory effects over EPA and OA on reverse mode NCX activity.

Effects of ALA on reverse mode NCX activity in VSMC and cardiomyocytes

After observing significant effects in our heterologous system, we chose to examine the effects of ALA on NCX activity in a native setting of either rat cardiomyocytes (NCX1.1) or VSMC (NCX1.3). Due to the difficulties of recording NCX current directly in these cells, extracellular Li^+ substitution of Na^+ was used to assess reverse mode NCX activity, since the removal of external Na^+ creates an electrochemical gradient that drives the NCX in reverse mode. In VSMC expressing NCX1.3, ALA significantly reduced the rise in

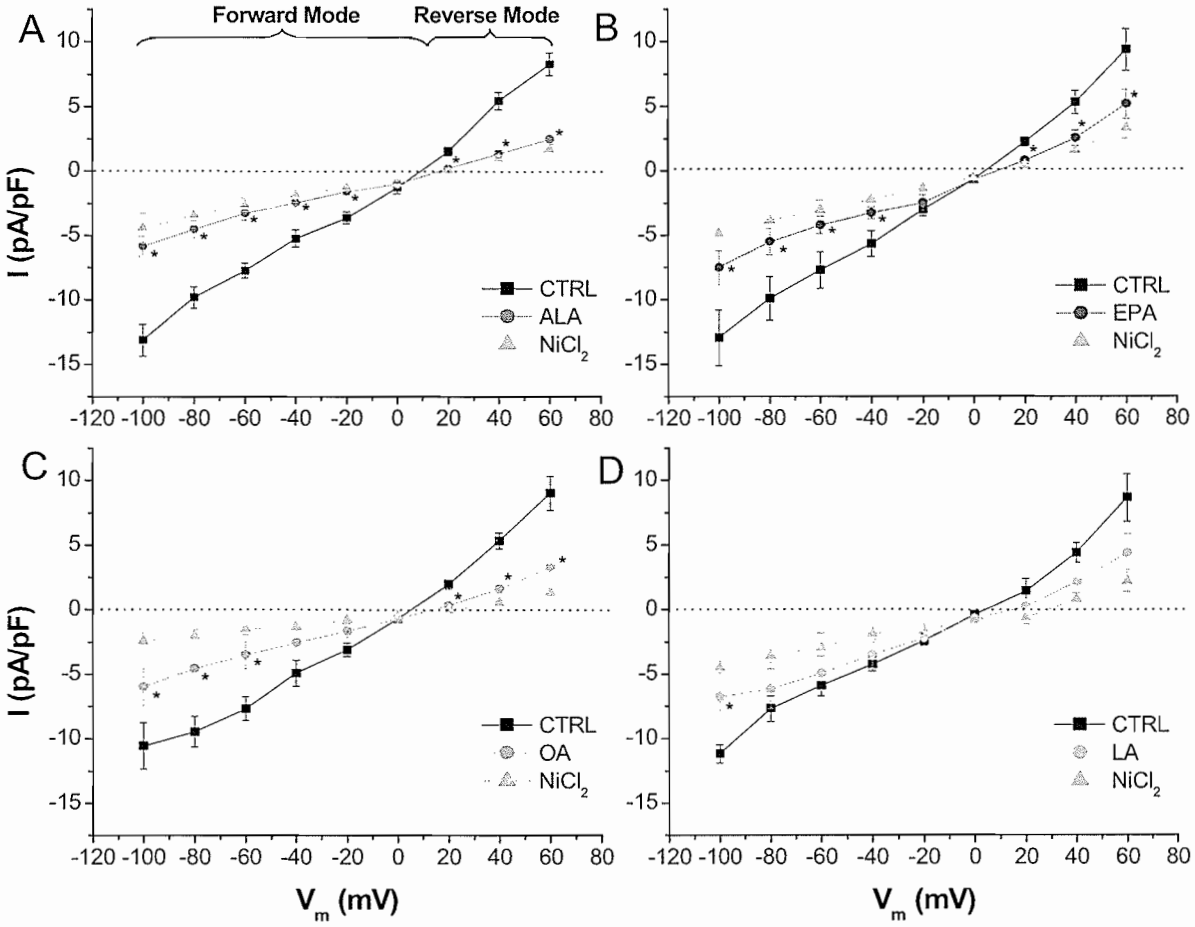


Figure 37. Summary of the inhibitory effects of different classes of fatty acids (25 µM) on NCX1.3. ALA (A, n=11), EPA (B, n=8), OA (C, n=6), or LA (D, n=6), was added to the bath and the voltage ramped from +60 to -100 mV. Both ω-3 PUFA and OA inhibited forward and reverse NCX activity across a range of potentials, but LA only significantly inhibited NCX activity at -100 mV. Application of Ni^{2+} produced further inhibition of the exchanger in all cases, demonstrating an incomplete block by fatty acids. * $P < 0.05$ vs. CTRL.

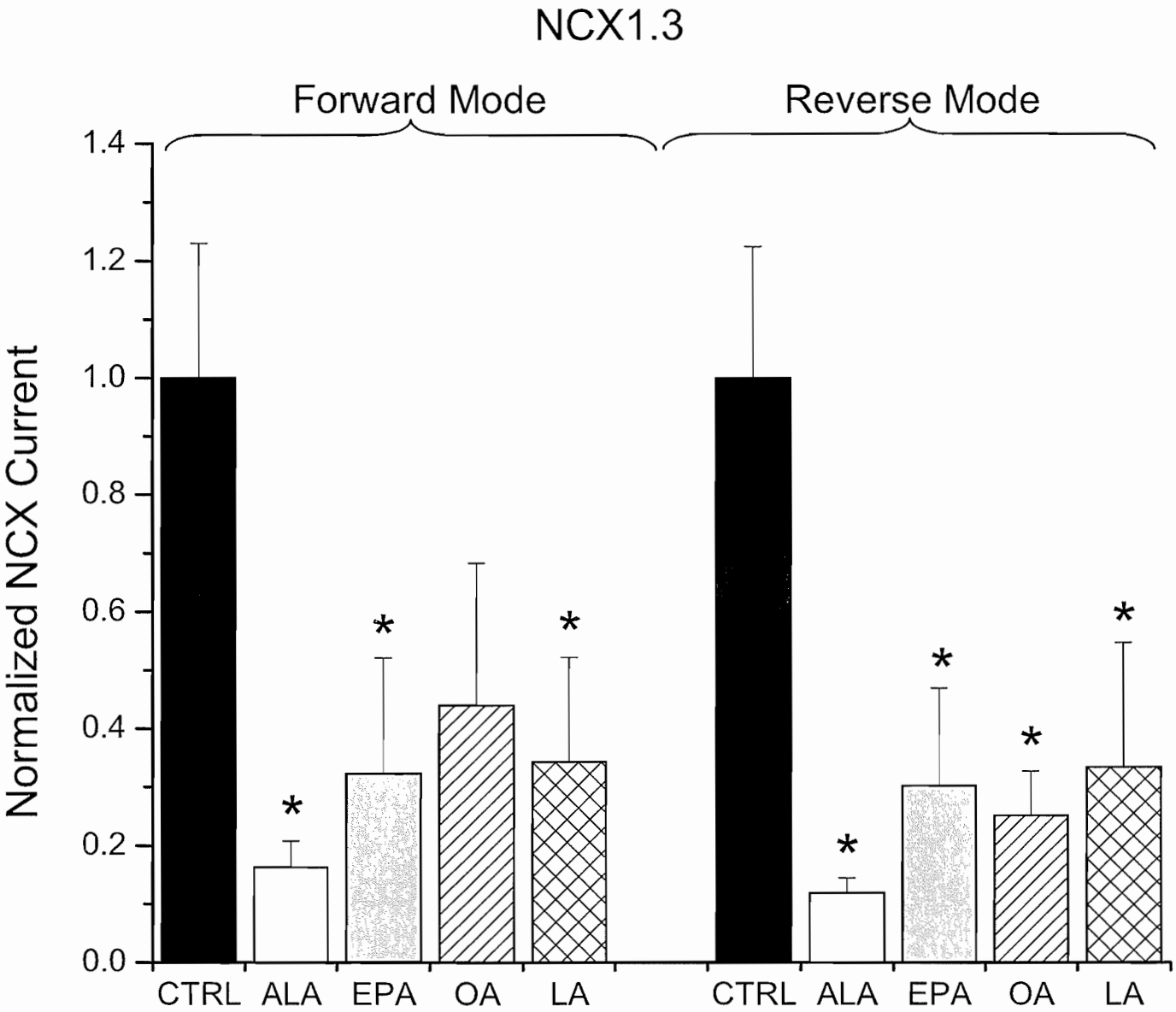


Figure 38. Relative magnitude of NCX current recorded following addition of 25 μM ALA, EPA, LA or OA to HEK293 cells expressing NCX1.3. Forward and reverse modes of NCX activity were measured at -100 and +60 mV as described for Figure 5. * $P < 0.05$ vs. CTRL.

intracellular Ca^{2+} induced by substitution of Li^+ for $[Na^+]_o$ from 108.7 ± 0.8 % to 102.2 ± 0.6 % of baseline values (Figure 39). Therefore, ALA also inhibits reverse mode NCX1.3 activity in native cells. In cardiomyocytes, 25 μM ALA significantly reduced the rise in $[Ca^{2+}]_i$ that accompanies Li^+ substitution from 149.8 ± 5.6 % to 101.6 ± 0.5 % of baseline values (Figure 39). The NCX inhibitor, KB-R7943, blocked the exchanger in a similar manner to ALA. Thus, ALA also inhibits reverse mode Na^+-Ca^{2+} exchange in cardiomyocytes (NCX1.1). These results are consistent with those found in the patch clamp experiments shown in Figures 35 and 37.

Dependence of NCX state on interaction with ALA

Many pharmaceutical agents produce their effects as they bind to a specific site of a protein that may only be accessible during brief periods through a series of conformational changes [188]. We tested the effects of PUFA on various mutants of the cardiac NCX expressed in HEK293 cells. Deletion of the intracellular loop of the NCX, which contains several regulatory sites, abolished any detectable NCX current in transfected HEK293 cells with background currents resembling non-transfected cells. However, deletion of the loop also resulted in removal of the epitope for the NCX antibody, so the only evidence for NCX expression was through evidence of hygromycin resistance, rather than by Western blot.

Currents recorded from the K229Q mutated cells, which do not go into the I_1 inactivated state, were similar in appearance to wild type NCX1.1 (Figure 40). Application of ALA via the bath solution inhibited the NCX, and a subsequent application of Ni^{2+} produced further inhibition. Since these cells do not normally go into the I_1 inactive state, it is possible that interaction of the NCX1.1 and ALA is not dependent on I_1 inactivation.

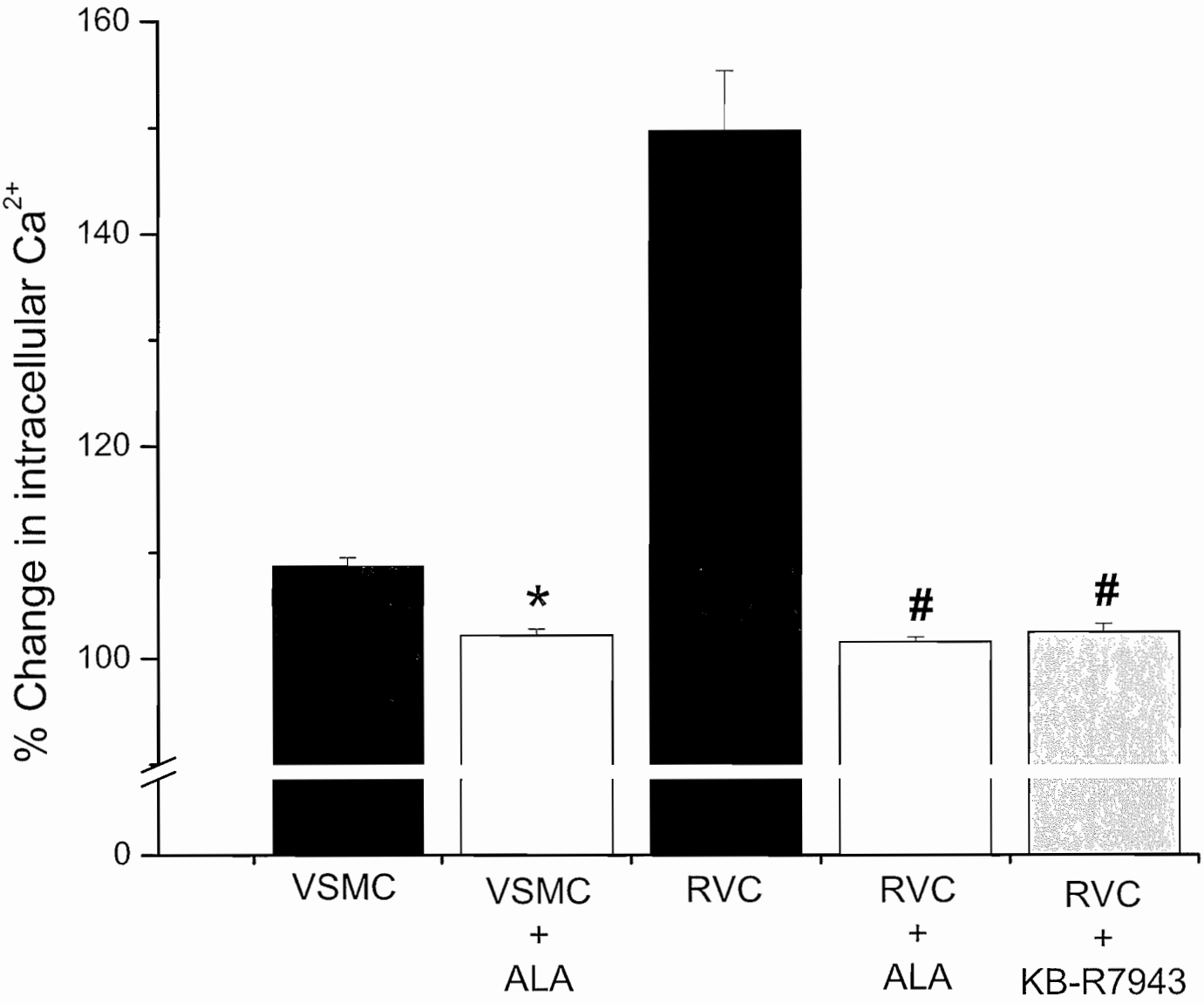


Figure 39. Changes in intracellular Ca^{2+} following substitution of Li^{+} for Na^{+} in the bath solution for rabbit VSMC expressing NCX1.3 or native rat ventricular cardiomyocytes (RVC). Application of 25 μM ALA (white) or KB-R7943 (grey) significantly attenuated the Li^{+}-triggered increase in $[Ca^{2+}]_i$ in both cell types compared to pre-treatment (black). * $P < 0.001$ vs. CTRL VSMC, # $P < 0.001$ vs. CTRL RVC.

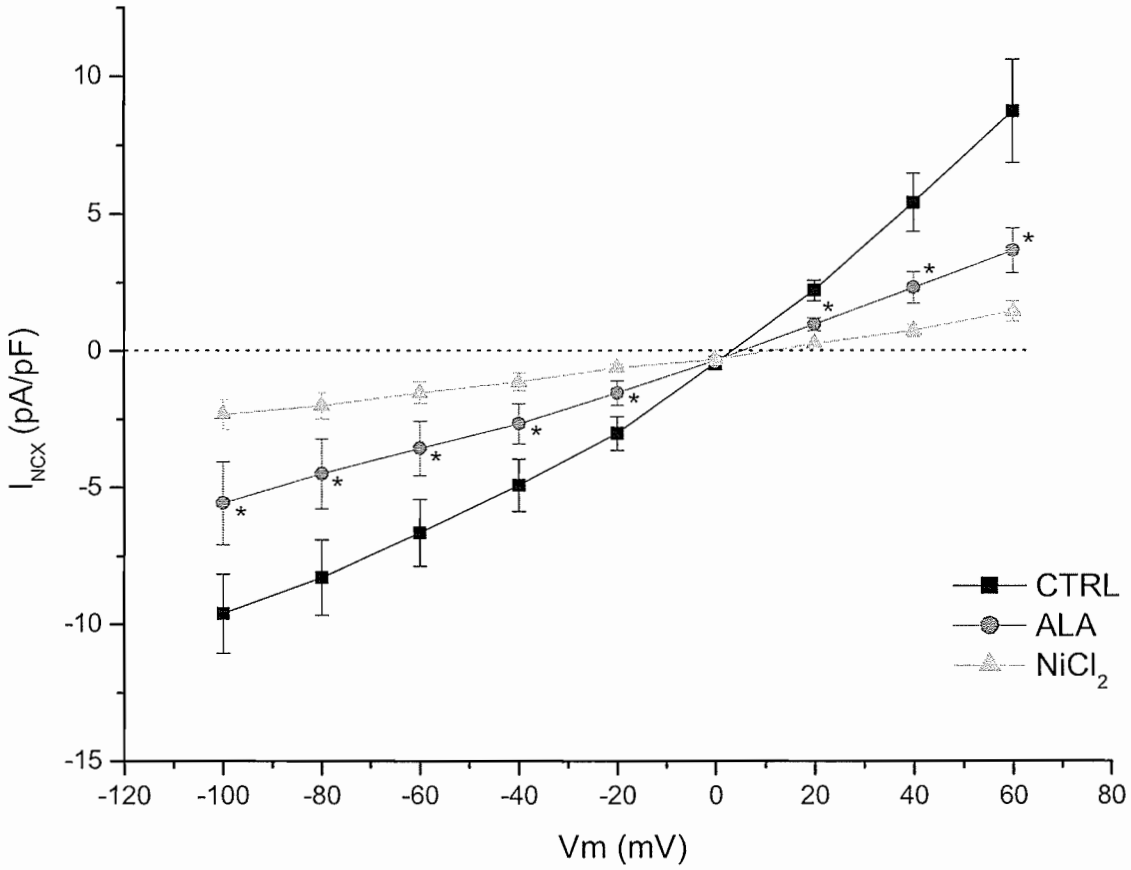


Figure 40. Effect of ALA and $NiCl_2$ on current recorded from HEK293 cells expressing the K229Q NCX1.1 mutant. * $P < 0.05$ vs. CTRL.

ACUTE EFFECTS OF ALA ON I_{KR} IN HEK293 CELLS

Using a stable HEK293 cell line expressing hERG, we measured I_{Kr}. Application of 25 μM ALA had no effect on the voltage of channel activation (-40 mV) or on the voltage of maximal activation (+35 mV), however, ALA did decrease peak I_{Kr} from 73.7 ± 19.7 pA/pF to 44.8 ± 18.6 pA/pF (Figure 41). This represented an overall inhibition of I_{Kr} by ALA of 43.4%. These results were not statistically different due to the relatively small number of cells tested (n=3).

EXPRESSION OF ION CHANNELS

Changes in expression of cardiac ion channel proteins due to diet could affect the size of the currents produced and alter the action potential configuration. Western blots of sarcolemmal cardiac proteins were probed with several antibodies for prominent cardiac ion channels. Table 9 summarizes the channels which were detected in the rabbit cardiac sarcolemmal membrane and membranes of other tissues. Standardization between the groups was not possible so only qualitative comparisons could be made. Notably, we were able to detect a member of the two-pore domain K^+ channel family, TREK-1, which has not previously been detected in the rabbit heart.

ALTERATIONS IN CELLULAR IONIC CURRENTS AND $[CA^{2+}]_I$ FOLLOWING DIETARY FLAXSEED FEEDING

In order to assess changes in key ionic currents responsible for determining the action potential duration following our dietary interventions, Cs^+ current through delayed rectifying K^+ channels and L-type Ca^{2+} current were measured in cardiomyocytes isolated from rabbits

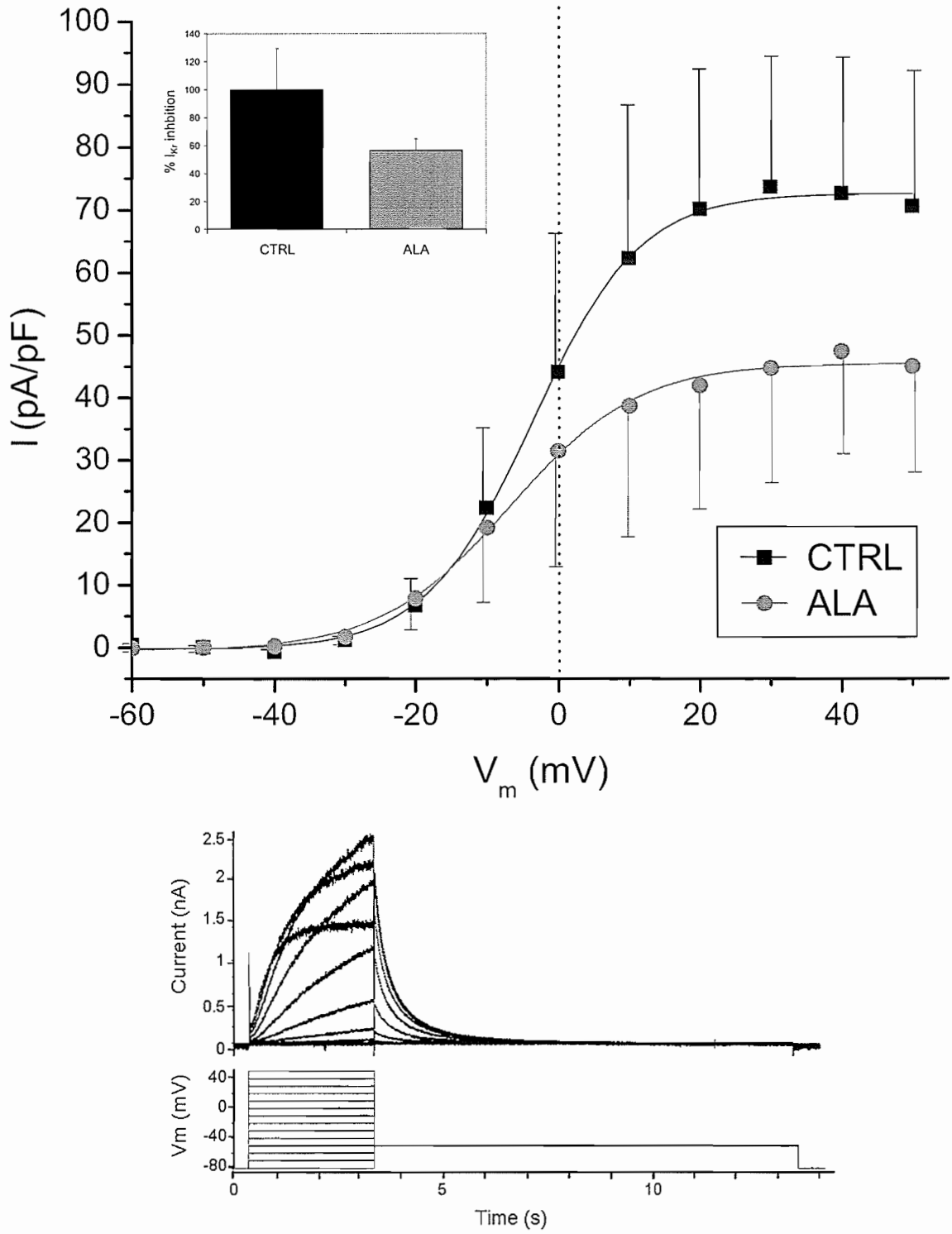


Figure 41. The effect of applying ALA (25 μM) on I_{Kr} in HEK293 cells expressing hERG . ALA was added to the bathing solution which inhibited current up to 43.4% (inset). A representative recording underneath shows the voltage protocol and the tail current, which was measured as the delayed rectifier current.

Table 9: Summary of ion channels detected in rabbit tissues.*

Antibody	Tissue					
	Heart	Brain	Lung	Liver	Kidney	Muscle
Kv1.4	0/4	3/4	0/4	0/4	0/4	0/4
Kv1.5	1/4	1/4	0/4	0/4	0/4	1/4
Kir2.1	3/4	0/4	0/4	0/4	1/4	0/4
Kv4.2	0/4	2/4	0/4	0/4	0/4	0/4
Kv4.3	0/4	1/4	0/4	0/4	0/4	0/4
IsK	1/4	1/4	2/4	1/4	0/4	0/4
HERG	3/4	2/4	1/4	0/4	0/4	0/4
cα1c	0/4	0/4	0/4	3/4	0/4	1/4
TWIK2	3/4	3/4	3/4	3/4	3/4	4/4
TREK1	3/4	4/4	2/4	1/4	1/4	0/4

* For each tissue, cell membrane proteins were isolated from four different control rabbits and run on SDS-PAGE. Western blots were probed with various ion channel antibodies and the number of samples indication interaction with the antibody are shown above.

consuming one of the four prepared diets. Cs^+ currents from all groups were activated ~-60 mV, and by -50 mV the Cs^+ currents from the flaxseed group were significantly larger than controls at all further test potentials (Figure 42). Cells from the cholesterol fed group also had significantly greater currents between -40 and +20 mV and currents from the cholesterol/flax group were larger between -40 and +40 mV.

L-type Ca^{2+} currents showed a similar pattern of change in response to the dietary interventions as the Cs^+ currents. While no significant differences existed between the groups, currents from cardiomyocytes isolated from the regular fed group had the smallest Ca^{2+} currents, followed by the cholesterol, cholesterol/flaxseed, and flaxseed groups (Figure 43A). Recovery of LTCC was not different between the groups, but the LTCC in both cholesterol-containing groups showed a trend of delayed recovery (Figure 43B).

Cells from the flaxseed group had the longest time to peak Ca^{2+} and longest half relaxation time (Figures 44A and B). Systolic and diastolic intracellular Ca^{2+} concentrations were higher in cardiomyocytes from the flaxseed group compared to controls (Figures 44C and D). The cells from the flaxseed group also showed the greatest overall transient rise in Ca^{2+} (Figure 44E).

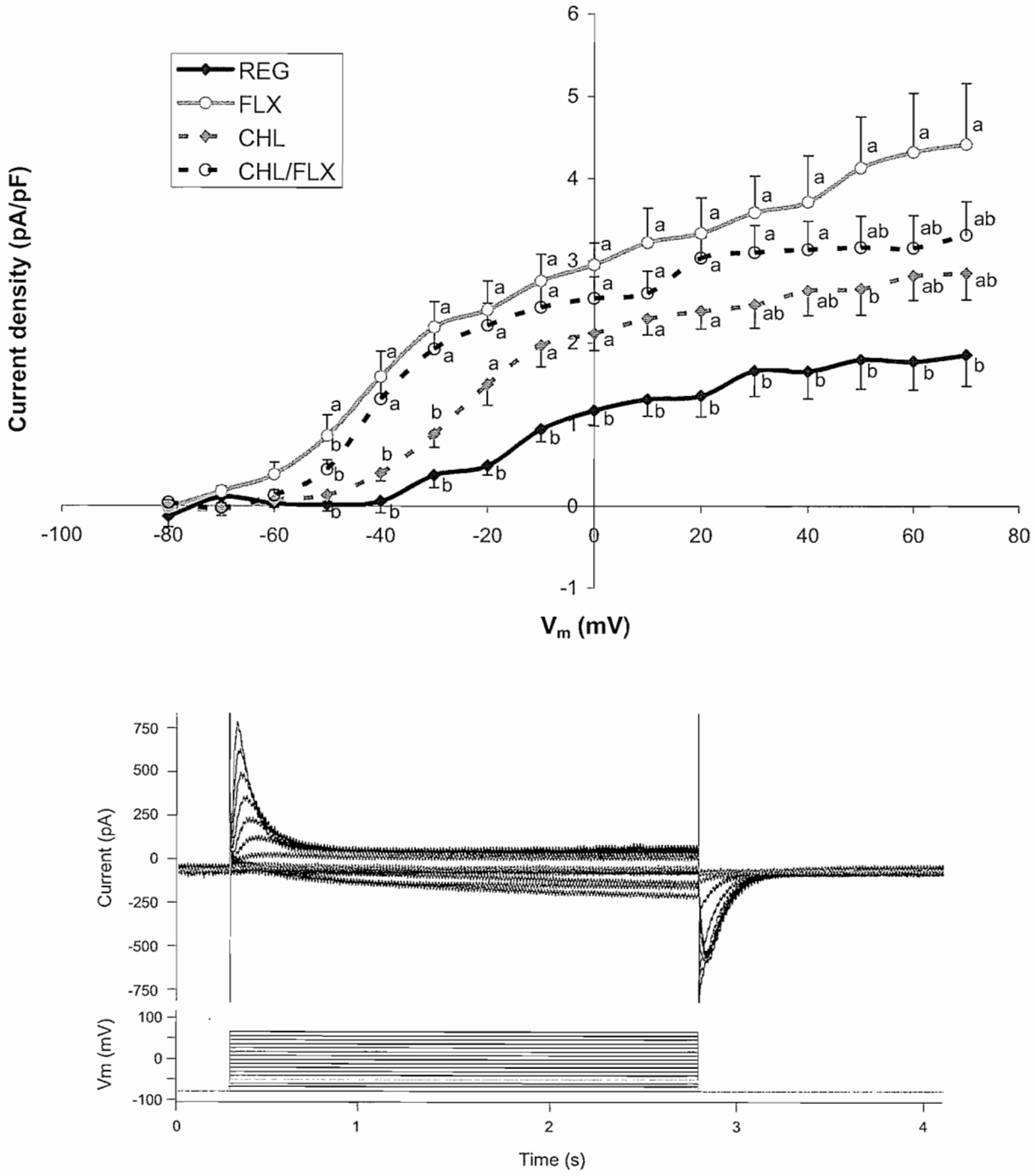


Figure 42. Current density of Cs^+ permeating through delayed rectifier potassium channels in cardiomyocytes isolated from rabbits following dietary treatment with regular or regular diet containing flaxseed with or without cholesterol. Each group represents data from cells of four different rabbits. Points at a membrane potential not sharing a common letter are significantly different ($P < 0.05$). A representative family of currents is shown underneath.

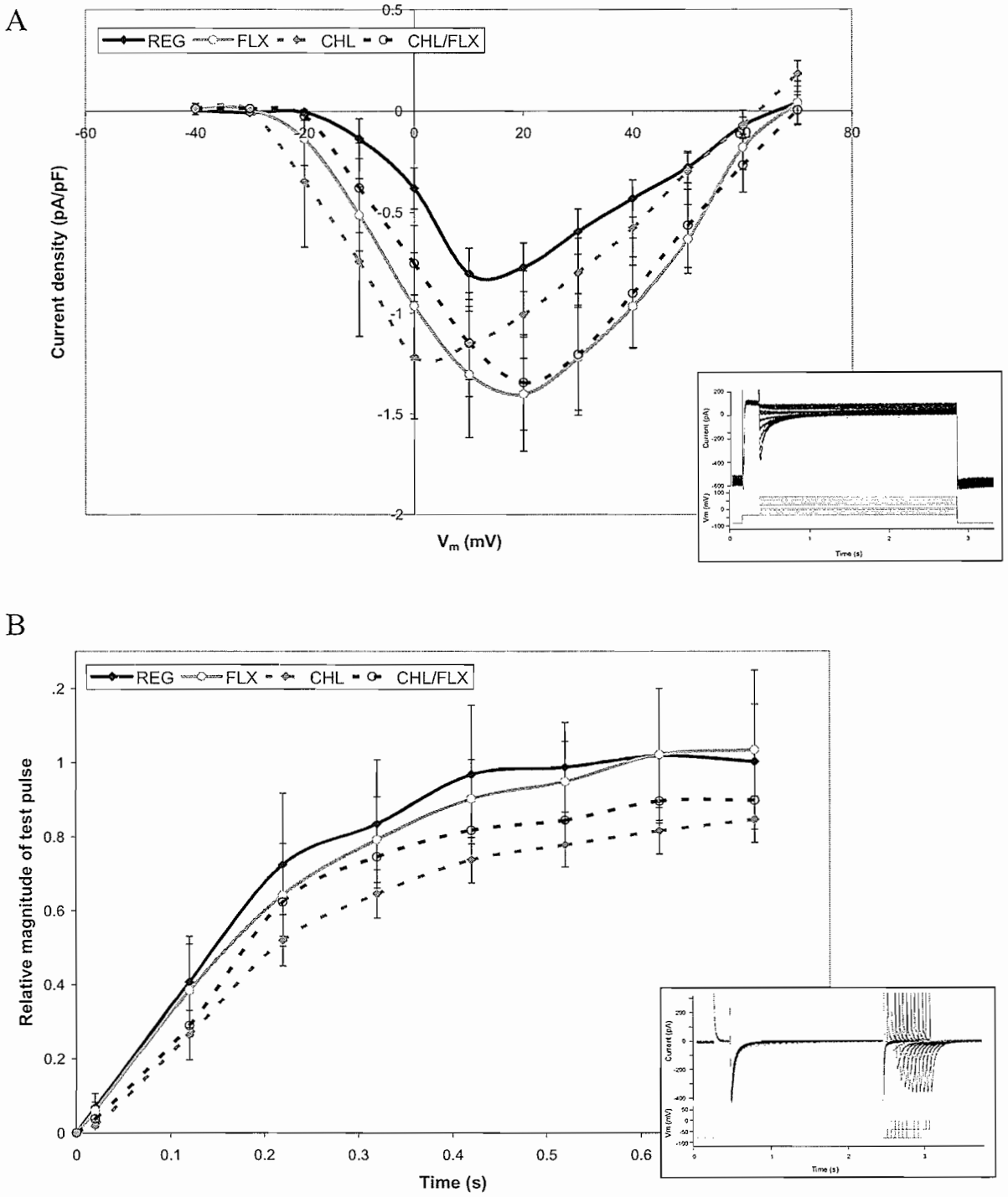


Figure 43. L-type Ca^{2+} currents (A) and channel recovery (B) in cardiomyocytes isolated from rabbits following consuming one of four experimental diets for 8 weeks. Representative recordings are shown on inset figures. Each group represents data from cells from four different rabbits. $P > 0.05$ amongst groups. Representative current recordings are shown on inset figures.

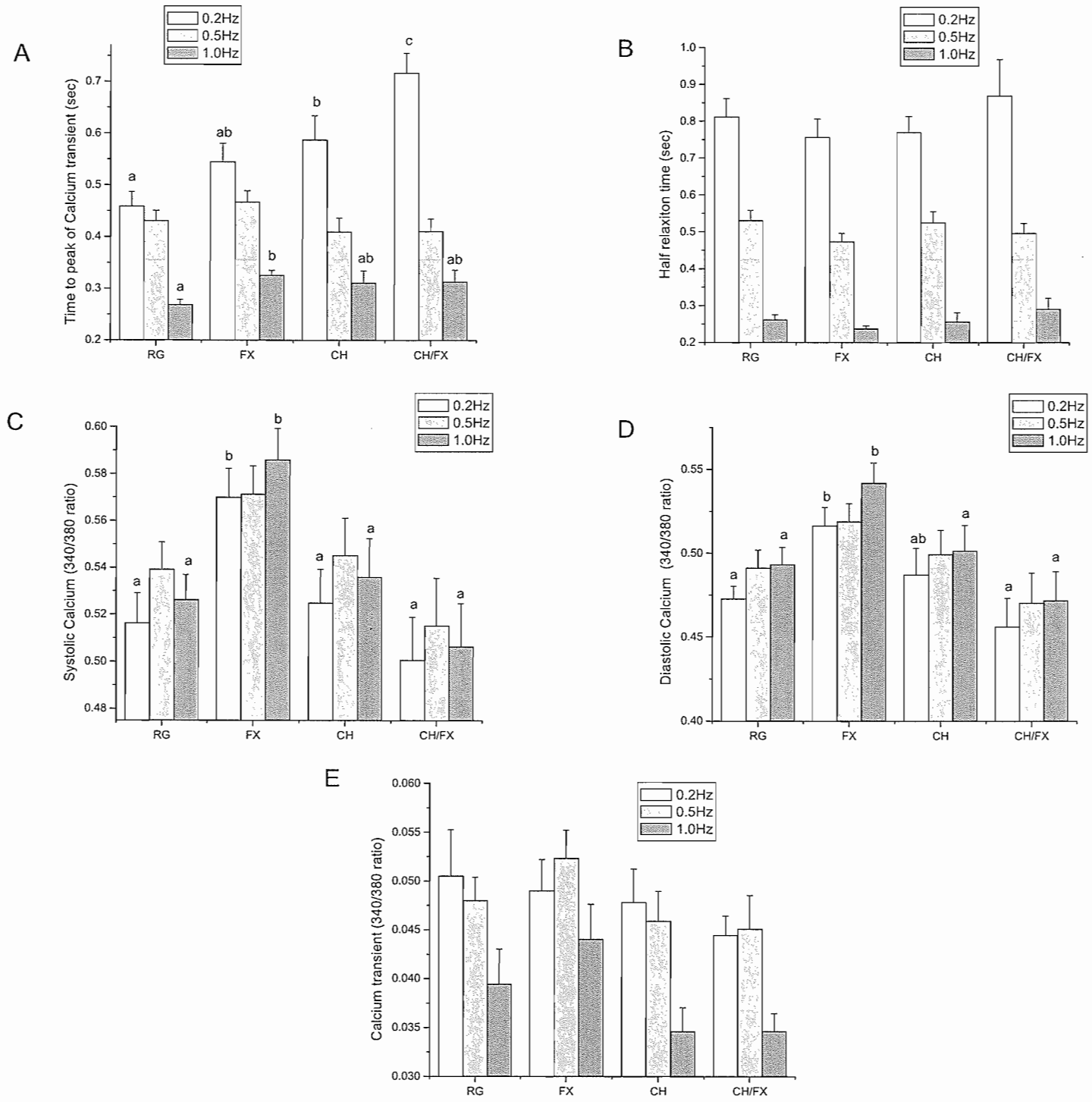


Figure 44. The intracellular Ca^{2+} measurements for time to peak Ca^{2+} (A), cell half relaxation time (B), systolic Ca^{2+} (C), diastolic Ca^{2+} (D) and Ca^{2+} transients (E) in response to stimulation at 0.5, 1, or 2 Hz in rabbit ventricular cardiomyocytes isolated following 8 weeks consumption of regular (RG), 10% flaxseed (FX), 0.5% cholesterol (CH), or 0.5% cholesterol/10% flaxseed (CH/FX) diets. Each group represents data from cells of four rabbits. Groups for each frequency not sharing a common letter are significantly different ($P < 0.05$).

CHAPTER V: DISCUSSION

ALTERATIONS OF TISSUE FATTY ACID PROFILES

It was critical at the outset of our study to determine whether dietary flaxseed would significantly increase tissue ω-3 PUFA levels. The majority of studies have assessed the effects of dietary supplementation with fish oils (EPA and or DHA) on tissue distribution of PUFA. This is likely due to the fact that fish were the source of ω-3 PUFA that provided the beneficial effects against cardiovascular disease in the earliest studies [42, 43, 189]. However, the parent ω-3 PUFA, ALA, especially in the form of flaxseed or flaxseed oil, is much more easily incorporated into other products, simpler to refine, and less expensive than fish oils, but its impact against cardiovascular disease is less studied.

Dietary flaxseed caused a significant increase in plasma ALA in the rabbits. Both the FLX and CHL/FLX groups had significantly higher plasma ALA levels than controls. However, the CHL/FLX group had nearly 20-fold greater plasma ALA than the FLX group, even though they consumed the same amount of flaxseed. This may reflect a cholesterol-facilitated uptake of fatty acids. Thomson *et al.* observed differential effects of fatty acid uptake by the jejunum in rabbits on a high cholesterol diet [190, 191]. This would also be consistent with the 3-fold higher plasma ALA levels in CHL versus FLX rabbits. Our laboratory has also tested the dose dependence of cholesterol on the increase of ALA in the plasma, and found that uptake of fatty acids, including the cardioprotective ω-3 PUFA was linearly related to the cholesterol content of the food. The increased dietary cholesterol clearly provided a more efficient uptake of fatty acids from their feed, even though no exogenous ALA was added to their diets in the form of flaxseed. Of course, since elevated intake of cholesterol can accelerate other aspects of cardiovascular disease, the risk may

offset the potential benefits of higher ω-3 levels.

Dietary flaxseed did not increase the levels of other ω-3 PUFA in the plasma. EPA and DHA were undetectable in the plasma from REG or FLX rabbits, but they were detected in the CHL and CHL/FLX groups. However, their concentrations were negligible (<1% of total fatty acids). Similarly, EPA and DHA levels in cardiac tissue increased only marginally in the CHL and CHL/FLX groups. Thus, it is unlikely that any effects observed in this study were due to any significant changes in EPA or DHA concentrations. Even at the cardiomyocyte level, significant elevations in ALA were observed. Comparison of the levels of the 20-carbon PUFA, AA and EPA, indicates that the lower ratio of ω-6/ω-3 fatty acids favoured a greater metabolic production of ω-3 PUFA via the delta-6 desaturase. This finding highlights the relative importance of a lower ω-6/ω-3 ratio to increase the endogenous production of long chain ω-3 PUFA.

This is the first study to determine the fatty acid profile of cardiomyocytes following their enzymatic digestion and isolation from the whole heart. Importantly, the levels of ALA and even EPA were still elevated even after the isolation procedure. In previous reports that looked at changes in cellular electrophysiological properties following a fish oil intervention [192, 193], no such profile was examined. The fatty acid composition of the cardiomyocytes is central to determining the mechanism thought to be responsible for the electrophysiological effects.

Thus, from plasma, to cardiac tissue, to cardiomyocyte, to the sarcoplasmic reticulum [153], dietary consumption of flaxseed will augment ω-3 levels, specifically for ALA and even its ω-3 metabolite EPA, but not DHA. Our studies, which generate striking antiarrhythmic effects and alterations in the electrophysiologic properties of heart cells, show

that ALA is, on its own, an important modulator of cardiovascular disease, and does not require elongation to longer ω-3 to produce these effects.

ISCHEMIA-REPERFUSION

One of the main goals of our feeding study was to determine if dietary flaxseed exerts antiarrhythmic effects and whether these effects were mediated specifically though ALA. We found that dietary flaxseed exerts a potent antiarrhythmic effect in rabbits. Flaxseed supplementation completely suppressed the VF normally observed during ischemia. Also, when added to a high cholesterol diet, flaxseed moderately decreased VF during ischemia and significantly decreased VF during reperfusion. These effects were specific to VF. Dietary flaxseed did not significantly influence the incidence of VT in this study. No adverse effects were associated with the flaxseed enriched diet in our study. It is also important to recognize that our model used isolated hearts to assess arrhythmogenesis; thus the effects of the high circulating ALA levels observed *in vivo* were removed. Thus, the antiarrhythmic potential of dietary flaxseed may be more pronounced *in vivo* due to the availability of both circulating and endogenous tissue stores of ALA [90].

Although it was clear that the antiarrhythmic action of flaxseed was not due to the generation of significant quantities of EPA or DHA, it is less certain that these effects were due specifically to ALA. However, several arguments would suggest that the dietary flaxseed may have induced the antiarrhythmic action through an elevation of cardiac [ALA]. First, PUFA have known antiarrhythmic capacity [3, 45, 194] and ALA was present in much greater quantities in the heart than the other PUFA. Second, there was a good correlation between the cardiac levels of ALA and the antiarrhythmic effects. Dietary flaxseed increased

the cardiac ALA levels by 7-fold in the FLX group, and 5-fold in the CHL/FLX group compared to the CHL group. The CHL/FLX group had the highest cardiac ALA levels and the lowest incidence of VF in our study. Conversely, the CHL and REG diets exhibited a higher incidence of tachyarrhythmias and had lower tissue stores of ALA. Indeed, this was extended even to the level of the cardiomyocyte itself. Third, ALA has been shown to inhibit arrhythmias when delivered as *in vivo* emulsions [45]. Fourth, we have shown that ALA has the capacity to significantly alter electrical activity in the heart (Figure 24).

MECHANISM OF ANTIARRHYTHMIC EFFECTS

To identify the potential cellular mechanism for the antiarrhythmic effects of flaxseed, we measured QT intervals in the hearts to provide an index of cardiac action potential duration. Dietary flaxseed was associated with a shortening of the QT interval. In contrast, the longest QT interval was observed in the cholesterol-fed group, which also exhibited the highest incidence of VF. Addition of flaxseed to the high cholesterol group resulted in a shortening of the QT interval and an antiarrhythmic effect. This suggests that dietary flaxseed may exert its cardioprotective effect through shortening of the cardiac action potential duration. Indeed, pronounced QT prolongation (either genetic or drug-induced) is often associated with polymorphic tachycardia and Torsades de Pointes [195, 196]. Furthermore, dietary PUFA supplementation with fish oil is also known to shorten the QT interval [46, 121, 197] and this is associated with reduced arrhythmogenesis. In our study, QT intervals were measured under control conditions prior to ischemia. This means that any differences are due to the different fatty acid content of the heart tissue, and the effects of any ionic currents that result. At 16 weeks, a noticeable prolongation of $MAPD_{90}$ in the

epicardium of pre-ischemic hearts from regular and cholesterol fed rabbits relative to the flaxseed groups was also observed.

Our data for QT intervals in the whole heart were further supported by our findings with acute exposure to fatty acids in cardiac myocytes. The ω-3 fatty acids produced a significant shortening of APD_{90} when applied acutely. These results are similar to a recent study in which action potentials recorded in cardiomyocytes from fish oil fed pigs were shortened [198]. However, in our results this effect was not specific to PUFA, since ALA, DHA and the MUFA, OA, produced similar effects. In contrast, only the ω-3 PUFA shortened APD_{50}. These data suggest that ionic currents near the plateau and early repolarization phases may contribute to the antiarrhythmic effect of PUFA. In contrast, class III antiarrhythmic agents suppress arrhythmogenesis by increasing the refractory period, which manifests as a mild to moderate prolongation of the QT interval [199]. We observed a significant increase in reperfusion-induced ventricular fibrillation in our cholesterol fed group. Action potentials in cardiomyocytes from cholesterol fed rabbits are prolonged versus controls [170]. Thus, although it is closely associated with an antiarrhythmic action, it is unclear how QT shortening is antiarrhythmic in our isolated heart model.

There are, however, three possible mechanisms for which a shorter action potential could be antiarrhythmic. First, ischemic tissues have shorter action potentials. It is known that heterogeneity in action potential duration can be a substrate for arrhythmias [200]. In the instance of regional ischemia, if action potentials in the non-ischemic zone shorten as a result of an increase of ω-3 PUFA, then this could reduce the heterogeneity between the regions and make an arrhythmia less likely to occur. Second, shorter action potentials have less calcium entry and require less ATP on a beat to beat basis. Therefore, since energy

requirements may be lower in ω-3 enriched tissue, less stress would exist during times of energy substrate depletion. Third, shorter action potential durations make it is less likely that an early afterdepolarization can occur and initiate an arrhythmia.

CHANGES IN IONIC CURRENTS AND EXPRESSION OF ION CHANNELS

ALA is known to affect various ionic channels and this could underlie its producing of a shorter action potential and antiarrhythmic action. Activation of phospholipase A_2 by increased intracellular Ca^{2+} during ischemia can liberate PUFA incorporated in the cardiac membrane to act on one or more ion channels [158]. A wide range of effects of PUFA on ion channels have been reported but shortening of APD_{50} and APD_{90} could only occur through decreased Ca^{2+} influx, increased K^+ efflux, or a combination of the two. However, with respect to the former possibility, increases in the L-type Ca^{2+} current and $[Ca^{2+}]_i$ were measured in the cardiomyocytes from rabbits administered the flaxseed diet. The increased entry of Ca^{2+} via the L-type Ca^{2+} channels should produce a greater release of Ca^{2+} from the SR store, which can be related to the increase in the Ca^{2+} transient size that we observed. However, previously published results in the literature report an inhibition in L-type Ca^{2+} current when ω-3 PUFA are acutely applied [131-133]. The difference in results may be due to the acute versus chronic settings as we note discrepant results for our I_{Kr} data using our acute or chronic treatments. One study investigating the effects of dietary cholesterol supplementation reported the same increase in L-type Ca^{2+} current we observed, and attributed this to changes in cardiac innervation [170].

Application of increasing concentrations of ALA to spontaneously contracting neonatal rat cardiomyocytes resulted in their quiescence. A similar effect was observed in a

previous report with the application of 5 μM EPA or DHA [201]. Saturated FA, MUFA, and ethyl esters had no effect on the rate of contraction in these cells [201]. Along with the effects ω-3 PUFA have on specific ion channels, they seem to be able to decrease the excitability of the neonatal cardiomyocytes, possibly by altering the threshold of activation. During ischemia, a higher excitation threshold could limit the occurrence of a premature stimulation, especially in depolarized tissue. But, for those with compromised cardiac function, too much stabilization could impair function. This could be a factor in some clinical trials that do not find a beneficial effect of ω-3 PUFA [73, 74]. Thus, careful selection criteria and classification of subjects based on assessment of cardiac function should be practiced to ensure the safety of the patient population as a whole and that outcomes of future studies are valid.

While we and others have shown that *in vitro* application of ω-3 PUFA alters the size of the currents measured in single cells, it is also possible that the actual expression of ion channel proteins is altered with a prolonged dietary intervention. In most instances, fatty acids are involved in the regulation of genes involved in lipid synthesis, transport and metabolism [202, 203], but reports do indicate that ω-3 PUFA can affect the expression of ion channels [204]. Notwithstanding the direct effects of channel activity, changes in current size could result from an increase or decrease in the number of channels expressed in a certain tissue. This could occur, for example, through alteration of peroxisome proliferator activated receptor (PPAR) mediated gene expression. Polyunsaturated fatty acids can alter affinity for PPAR heterodimerization with the retinoid X receptor (RXR), which is required to bind to the PPAR response elements (PPRE) to initiate transcription of certain genes [202, 203, 205, 206]. Fatty acids may also alter expression levels of PPAR itself [206].

Unfortunately, we were unable to quantitatively assess the levels of expression across the experimental groups in our study.

We did, however, identify the TREK-1 ion channel in the rabbit heart. This is the first time that a member of the two-pore domain K^+ channel (K2P) family has been identified in the rabbit heart. The K2P channels, TREK-1, TREK-2 and TRAAK are PUFA-sensitive [207-209]. Activation of these channels by PUFA produces a polarizing effect that could improve conduction in depolarized cells. TREK-1 and TREK-2 are also activated by intracellular acidosis making them ideal candidates to confer protection during ischemia, and their presence has been confirmed in the rat heart [210, 211]. The majority of the research to this point involving K2P channels has been conducted in the brain [208, 209, 212]. Injections of ALA in the brain have been able to attenuate the damage caused by seizures in mice [212]. The cerebral protection was shown to be mediated via opening of K2P channels, and it is possible that activation of K2P channels in the heart could mediate protection via stabilization of the membrane potential in ischemic tissues, which tend to depolarize and become more readily excitable.

The Cs^+ current through K_r channels was augmented in cardiomyocytes from flaxseed fed rabbits relative to controls. This is consistent with the hypothesis that an augmented K^+ efflux may be responsible for the shortened APD_{50} and APD_{90}. However, these results are once again in contrast to experiments where we acutely applied ALA to HEK293 cells expressing. These conflicting results once again emphasize the importance of conducting dietary investigations to clearly identify long lasting effects of physiological relevance. It will be important in the future to determine directly if the number of K_r channels expressed in the heart is altered in response to dietary interventions like flaxseed.

MECHANISM OF EFFECTS OF OMEGA-3 PUFA AND ION CHANNELS

The molecular mechanism whereby ω-3 PUFA and other fatty acids exert their antiarrhythmic effects within the membrane is not certain. However, the mode of action is likely via one or more of three mechanisms. Firstly, the PUFA may be interacting directly with a site on the ion channel, altering its activity. Several studies have provided evidence for the direct binding of fatty acid to ion channels: 1) Application of PUFA to the internal or external side of the cell membrane can determine the degree of effect and whether an effect occurs [127, 128, 148]. This would suggest that the fatty acids are acting directly on a specific site of the channel that is present only on one surface. 2) Administration of ω-3 PUFA can block specific radioligand binding to the sodium channel suggesting a competition for a specific site of interaction [125]. 3) A single point mutation induced in the sodium channel expressed in HEK293 cells significantly diminished the effect of EPA on the inhibition of I_{Na} [126]. This is perhaps the best support for a pharmacological site of interaction for PUFA. 4) PUFA-mediated effects are reversed after addition of delipidated BSA to the membrane [119, 125, 133, 142, 213]. The reversibility of the PUFA-mediated effects suggests the fatty acids are acting directly on the protein since FA that are incorporated into the membrane would not be expected to be so easily scavenged by the addition of delipidated BSA [125]. 5) The concentrations of PUFA that significantly alter ionic currents are normally too low to create a change in the overall fluidity of the membrane by altering the packing of the membrane phospholipids [213].

Despite the lines of evidence identified above, there is still reason to believe that the actions of PUFA are achieved through a second mechanism – an indirect membrane disordering effect. For example, although the experimental [PUFA] are generally considered

too low to induce membrane fluidity changes throughout the whole membrane [213], even small concentrations of ω-3 PUFA in the membrane can affect phospholipid packing and thus channel function provided they are located adjacent to the proteins [124, 214-216]. Studies in which membrane fluidity was altered using benzyl alcohol produced results that are strikingly similar to the effects of ω-3 PUFA on ion channels [123, 124]. A direct relation has been shown between membrane fluidity the degree of unsaturation of the fatty acid [214]. The fact that different classes of PUFA have different effects on ion channels and exchangers means that their simple incorporation into the membrane and the change of membrane fluidity is not the primary mechanism of action. If it were simply a question of membrane fluidity, AA, a highly "bent" ω-6 PUFA, should contribute towards membrane fluidity to a significant extent and result in the same effects as long chain ω-3 PUFA. In many cases it does, but in several instances, AA does not produce the same effects [132, 136]. Possibly, AA is converted to other eicosanoid metabolites, whereas DHA, for example, is not always converted to other products and has more potential to exert its effects as a free PUFA. Use of the non-metabolizable AA analog, eicosatetraynoic acid (ETYA), may help determine the direct effects of AA [136].

A third mechanism could produce a net change in current, without affecting the baseline activity of an individual ion channel. That is, following a long term PUFA dietary treatment, current could be increased or decreased based on changes of expression of ion channels and thus the absolute number of channels present in a membrane. Any net change in ionic current would reflect the overall change produced by any or all of these mechanisms.

ALA AND THE NCX

Our initial measurements of action potentials in NCX deficient neonatal rat cardiomyocytes showed that the NCX is an important player in determining the configuration of the cardiac action potential, affecting the appearance of the plateau phase in particular. While we expected some alterations in the appearance of the action potential since the NCX is electrogenic, we did not expect to see such a dramatic effect. The change may be due to either or both of two factors; the loss of the electrogenic influence of the NCX, or the buildup of intracellular [Ca^{2+}], which could reduce the L-type Ca^{2+} currents. We were also able to record action potentials in neonatal rat cardiomyocytes in which the NCX was adenovirally overexpressed and in these cells action potentials had an increased duration. In pathophysiological settings such as heart failure, elevated expression and activity of NCX is also linked to longer action potentials [184]. In our experiments, elevations of intracellular Ca^{2+} would not be a problem, so augmented I_{Ca} through LTCC would not explain this, so the increase in duration is likely through a direct depolarizing effect of the exchanger.

The direction by which the NCX functions during a normophysiological action potential is controversial. In order to decrease the plateau phase as much as we observed, the cells would have had to lose a depolarizing, inward current, which the NCX produces in forward mode. Some reports suggest that the NCX does function in forward mode nearly exclusively for the duration of the action potential, except at the peak near phase 1, due to the high concentrations of Ca^{2+} in the subsarcolemmal space immediately near the NCX [186, 217]. If the reversal potential of the NCX is calculated using the $[Ca^{2+}]_i$ of the entire cell, it would suggest the NCX operates primarily in reverse mode during the majority of the action potential [186]. Our experiments may support the former case as we see both a direct

inhibition of NCX by PUFA and a shortening in overall APD when FA were applied to cardiomyocytes.

Our study demonstrates that the short-chain ω-3 PUFA, ALA, significantly inhibits both the forward and reverse mode of NCX1.1 activity and that this inhibition is similar to that observed with the long-chain ω-3, EPA. We also report, for the first time, that the vascular NCX isoform, NCX1.3, is inhibited by various classes of fatty acids with a different profile than NCX1.1. Notably, the vascular NCX1.3 was six times more sensitive to ALA than the cardiac NCX1.1 isoform. Also, the MUFA, and to some degree the ω-6 PUFA, inhibited NCX1.3, whereas neither of these classes inhibited the cardiac NCX1.1. Only the ω-3 PUFA inhibited the NCX1.1 isoform and it was unaffected by other classes of fatty acids over the concentration ranges examined.

We found that ALA inhibited the reverse mode of NCX1.1 with an IC_{50} of 0.11 ± 0.02 μM. Although we did not complete a dose response curve for EPA, Figures 35-38 would suggest EPA and ALA inhibited the NCX1.1 to a similar degree. If so, the IC_{50} is eight times lower than reported for EPA on NCX1.1 activity in acutely transfected HEK293 cells [141]. A possible reason for this discrepancy is the different delivery vehicles used in these two studies. We have found that fatty acids suspended in ethanol, as is commonly practised experimentally [120, 124, 131, 139], is not an optimal delivery system. The more physiological mixing with BSA produces a better dissolution of all fatty acids into solution.[172] The identification of such low IC_{50} values further increases the likelihood that these ω-3 PUFA act *in vivo* to alter cardiac function via inhibition of the NCX.

It is noteworthy to consider the circulating ω-3 PUFA levels attainable in the body. Under normal conditions, the free plasma concentrations of ω-3 PUFA are reported to be in

the high nanomolar range, and low micromolar concentrations are certainly possible with dietary supplementation [218, 219]. Our data indicate that such concentrations would produce significant inhibition of NCX activity, especially NCX1.3. Moreover, the IC_{50} for inhibition of both the NCX1.1 and NCX1.3 are lower than those reported for the inhibition of ion channels by ω-3 PUFA (2.1 μM for $I_{Ca\text{-}L}$ [131], 6.0-26.6 μM for I_{Na} [120, 124]). Thus, the protective effect of ω-3 PUFA may reflect a greater contribution of NCX inhibition at low plasma concentrations and inhibition of both the NCX and ion channels at higher plasma levels.

Inhibitors of NCX are currently showing promise as treatments for arrhythmias [112, 117] and heart failure [220]. We previously reported that the dietary inclusion of high ALA content flaxseed exerts a marked antiarrhythmic effect in rabbits [55], and these results are consistent with other studies with ω-3 PUFA in animal models [93, 221]. The beneficial effects of ω-3 PUFA on arrhythmias in humans are becoming increasingly evident. A recent clinical trial [73] tested the effects of 4 g/day of fish oil versus olive oil (control) over a 12 month period in patients that received an implantable cardiac defibrillator (ICD) for secondary prevention (i.e., had a previous symptomatic episode of ventricular tachycardia). The trial found a trend towards a longer time to first ICD event for VT/VF or to death from any cause in the fish oil group. The GISSI-Prevenzione study [222], a large randomized clinical trial that tested the effects of ω-3 PUFA independent of other dietary factors, also found that 0.9 g/day EPA/DHA (1:2 ratio) significantly decreased all-cause mortality (35%), cardiovascular-related mortality (30%), and sudden cardiac death (45%). Results of these clinical trials support the contention that ω-3 PUFA exert their beneficial effects through a reduction in lethal arrhythmias and sudden cardiac death.

The effects of ω-3 PUFA were more potent when studying the NCX1.3. The vascular/renal NCX1.3 isoform was six times more sensitive to ALA than the cardiac NCX1.1, with an IC_{50} of 0.021 ± 0.009 μM and 0.017 ± 0.009 μM for the forward and reverse modes, respectively. Other NCX inhibitors, such as SEA0400, also display preferential inhibition of the vascular NCX over the cardiac isoforms [223, 224]. The greater sensitivity of NCX1.3 to fatty acids suggests that the ω-3 PUFA may offer a potential new treatment for hypertension. Several reports have documented that ω-3 PUFA lower blood pressure in hypertensive animal models [225-227] and humans [228-230]. The ability to lower blood pressure is not specific to a particular class of fatty acids, although fish oils appeared to cause the largest effects [231]. The heightened sensitivity of NCX1.3 to inhibition by fatty acids was also associated with a loss of specificity to different classes of fatty acids. The ω-3 PUFA, ω-9 MUFA, and to some degree ω-6 PUFA, all inhibited the forward and reverse modes of NCX1.3 activity although at higher concentrations of 25 μM.

A comparison of different fatty acids found that the ω-3 PUFA were the most effective in delaying the rise in systolic blood pressure that usually occurs with age in spontaneously hypertensive rats (SHR) [225]. The ω-9 and ω-6 fatty acids also delayed the blood pressure increase, but to a lesser extent. We found a similar rank order of potency for these classes of fatty acids, based on their inhibition of the NCX. In humans, the ability of ω-3 PUFA to lower blood pressure is related to the severity of hypertension present. Relatively high doses (3-4 g/day) of fish oil were reported to decrease systolic blood pressure by 2-3 mm Hg in mild to moderate hypertensive patients [228-230]. However, a 3 mm Hg reduction in systolic blood pressure may be sufficient to reduce mortality from CHD and stroke by 5% and 8% respectively [232]. In contrast, ω-3 PUFA did not have any effect on

blood pressure in normotensive Wistar Kyoto rats [233, 234], or in normotensive patients who received ω-3 PUFA supplements [224, 235]. These findings suggest that the effects of the ω-3 PUFA on the vasculature become manifest only when the systolic blood pressure is elevated. In our study, hearts from the flaxseed group did slightly delay the contracture induced by ischemia following 8 weeks feeding, but this was not apparent in the 16 week group.

In our study, even maximal concentrations of ω-3 PUFA inhibited the total Ni^{2+}-sensitive NCX current by only 50-80%. Thus, a basal level of NCX activity is present that is resistant to ω-3 PUFA inhibition and permits normal cellular function. In fact, heterozygous NCX knockout mice, in which NCX levels are ~50% of normal, fail to show major functional consequences [26]. However, in pathological states such as heart failure, this level of NCX inhibition could affect function due to greatly increased NCX expression and/or activity, or a greater requirement of NCX function in the excitation-contraction coupling process. This could explain why ω-3 PUFA are of benefit in certain disease states, but have no effect in normal healthy patients. Alterations in ionic gradients associated with various pathologies could also influence the characteristics of NCX function. For example, increased $[Na^+]_i$ associated with hypertension would shift the reversal potential for NCX to more negative values and result in greater inhibition of ω-3 PUFA on reverse mode NCX activity, a strategy that seems to reduce vascular tension [224].

Finally, our data indicate that ω-3 PUFA derived from either fish (EPA and DHA) or plant sources (ALA) inhibit the NCX. Both ALA and EPA inhibited the forward and reverse mode of NCX1.1 and NCX1.3 activity in our study. Our results argue against the suggestion that ALA itself is inactive and must be elongated to EPA or DHA to produce a biological

effect. Clearly, ALA directly inhibited NCX1.1 and NCX1.3 yet its elongation to EPA and DHA does not occur in our cell system. It is noteworthy, however, that EPA produced greater inhibition of NCX1.1 activity than ALA over the physiological range of membrane potentials recorded in heart cells (-85 to +30 mV). In contrast, ALA and EPA inhibited NCX1.3 to a similar degree over the full range of membrane potentials studied. Thus, longer chain ω-3 PUFA may affect the cardiac NCX to a greater extent than ALA, whereas the effects of all ω-3 PUFA would be similar on the vascular NCX.

Our data show that ω-3 PUFA, including the short-chain ALA, inhibit NCX1.1 and NCX1.3 activity, but the NCX1.3 isoform is much more sensitive. Conversely, MUFA or ω-6 PUFA of the same hydrocarbon chain length have no effect on the cardiac NCX1.1, but do inhibit the vascular NCX1.3. In pathological conditions, such as heart failure and particularly hypertension, where inhibition of the NCX can restore cardiac function or vascular tone, increased dietary intake of ω-3 PUFA, including ALA derived from plant sources such as flaxseed, may be beneficial.

MECHANISMS OF ALA INTERACTION WITH NCX

Some drugs preferentially bind to certain states of the NCX, so we modified the amino acid lysine to glutamine in the K229Q mutant in order to determine whether the I_1 state is important for the effects of ω-3 PUFA on NCX. This K229Q mutant does not readily go into I_1 inactivation, therefore, this state is not necessary for the mediation of ω-3 PUFA effects. Potential other modes of interaction could include the I_2 site, Ca^{2+} regulatory sites, or perhaps the effect is mediated by a general mechanism as ω-3 PUFA affect nearly all ion

channels examined. The most likely theory is that there is an effect occurring on the lipid membrane that then affects the nearby proteins (ion channels, exchangers, etc) to alter electrophysiological characteristics. Further work should be done to determine the extent to which ALA can alter the membrane fluidity of cardiomyocytes, in particular following feeding with dietary flaxseed.

IMPLICATIONS FOR CANADIANS

Finally, it is important to discuss the relevance of dietary interventions with flaxseed to Canadians. The 10% flaxseed supplementation represents ~13% of the total energetic intake and is comparable to that used in a clinical trial where human subjects consumed 50 g flaxseed/day [236]. Thus, our level of dietary flaxseed supplementation in rabbits has physiological relevance to humans. In view of the compliancy problems inherent in delivering fish and other oils to humans in therapeutic concentrations, the use of a palatable flaxseed product as an alternative dietary supplement may provide a practical, feasible, and important modality to ensure that ω-3 PUFA are delivered to the body in dosages that have clear cardioprotective effects. As the world's leading producer of flaxseed, Canada is in a position to supply flaxseed and ALA to its population and abroad with significant economical benefits, both through increased product commercialization and through savings in the health care costs that would follow a decline in CVD, due to an increased public awareness of the health benefits of locally produced ω-3 PUFA.

SUMMARY

It is difficult to accurately predict the net effect of the action of ω-3 PUFA on the sum

of the ionic currents in the heart. The contrasting results that we have obtained may not be in direct conflict with each other, since we have used two different models for our assessments. Perhaps, the chronic dietary versus acute topical treatments affect ion channel function in different ways. Given that it is the chronic dietary setting that will ultimately play out in individuals, more studies should focus on feeding studies and using tissues from these trials to assess physiological impact of ω-3 PUFA. Unlike pharmacological agents synthesized in the laboratory that go through relatively short, but thorough tests, ω-3 PUFA have been in the food supply for millennia and their effects are based on a tradition of use. At the present time, despite precise knowledge of mechanisms, consumption of any of the ω-3 PUFA seems to be a simple and effective measure for individuals in maintaining good cardiovascular health.

CHAPTER VI: CONCLUSIONS

1. **Dietary flaxseed alters tissue fatty acid composition favouring an increase in ALA. This effect was consistent at the plasma, whole heart, and cardiomyocyte level.**

2. **Dietary flaxseed exerts a potent antiarrhythmic effect during global ischemia-reperfusion of the heart. There is specific reduction in ventricular fibrillation even in a hypercholesterolemic setting.**

3. **The antiarrhythmic effect of flaxseed is associated with a reduction in the QT interval, which is a general index of APD in the whole heart.**

4. **Acute application of ω-3 PUFA (ALA and DHA) to cardiomyocytes reduces action potential duration. A class specific effect was observed on the plateau phase, whereas even the ω-9 MUFA (OA) shortened the overall action potential duration.**

5. **Silencing the NCX dramatically alters the action potential configuration in neonatal rat cardiomyocytes, specifically reducing the plateau. Overexpressing the NCX increases the duration of the action potential.**

6. **Activity of the cardiac NCX1.1 is reduced by ω-3 PUFA (ALA and EPA). The inhibitory effect of ALA is forward and reverse mode independent with IC_{50} values of 0.11 ± 0.02 μM and 0.12 ± 0.02 μM, respectively.**

7. **Activity of the vascular NCX1.3 is reduced by ω-3 PUFA (ALA and EPA), ω-6 PUFA (LA), and ω-9 MUFA (OA). The inhibitory effect of ALA is forward and reverse mode independent with IC_{50} values of 0.017 ± 0.009 μM and 0.021 ± 0.009 μM, respectively.**

8. **The inhibitory effects of ALA on NCX1.1 do not appear to be dependent on the I_1 state of inactivation, since mutation of a key amino acid (229) does not alter the inhibition produced by ALA.**

9. **Delayed rectifying current is augmented in cardiomyocytes isolated from rabbits fed high flaxseed diets relative to controls.**

CHAPTER VII: FUTURE DIRECTIONS

While it is important to determine the pathologies in which dietary supplementation with flaxseed could provide an effective means of protection or treatment, it is also important to further delineate the mechanisms by which flaxseed and ALA produce the significant protective antiarrhythmic effect observed in this study.

Due to the apparently conflicting results of dietary versus acute studies (demonstrated by effects on I_{Kr}), it seems important that further studies use a diet-based model as this is a closer approximation of how dietary supplements are used by humans. Acute studies could be run in parallel and compared to the dietary studies to further compare similarities and differences between the two models and perhaps determine multiple mechanisms of action.

Since ALA and other ω-3 PUFA seem to affect a wide variety of ion channels and exchangers, it seems plausible that at least one way in which they exert their effect is by a general mechanism, such as through altering membrane fluidity and thus the movement of the proteins embedded therein. Measurement of the fluidity of the cardiomyocyte membrane can be determined through techniques such as fluorescent resonance after photobleaching (FRAP) or by measurements of membrane isotropy using fluorescent markers such as trimethylammonium diphenylhexatriene (TMA-DPH). Changes in fluidity could be compared between cells obtained from diet treated animals and cells incubated with specific fatty acids to determine which has the greatest effect on membrane fluidity.

As discussed earlier, changes in the tissue ion channel density due to altered levels of expression could affect the size of currents recorded in experiments. Thus it is important to quantitatively determine the levels of expression of the channels in the heart following dietary interventions with ω-3 PUFA. Our attempts were unsuccessful mainly because our

sarcolemmal preparations lacked a housekeeping protein to use for standardization across the groups. We attempted to use Na^+/K^+ ATPase activity, but this was unsuccessful. Perhaps future attempts should use whole tissue preparations. The results will not provide a picture of channel expression exclusively in the plasma membranes, but at least the presence of several housekeeping proteins should make quantification successful.

We measured the effect of acute application of ω-3 PUFA to rabbit cardiomyocytes on the action potential duration, but do not know the effects on the actual refractory period of the cells. Despite our observation that the action potential was shortened by ω-3 PUFA, it is possible that the refractory period was actually increased, which could contribute to the antiarrhythmic effect of dietary flaxseed and other ω-3 PUFA. Refractory periods could be measured in isolated cardiomyocytes by following a train of action potentials with a test stimulus of decreasing cycle length. The interval at which the test stimulus no longer generates an active response will be the effective refractory period.

The *in vivo* antiarrhythmic effects of dietary flaxseed should also be examined. These studies would be important as regional ischemia-reperfusion is more clinically relevant than the global ischemia protocol we used. By using implanted telemetry devices in rabbits, blood pressure and ECG data could be recorded throughout the feeding, insult and recovery periods. Infarct damage could be assessed through analysis of plasma enzymes creatine phosphokinase and lactate dehydrogenase, as well as measurement of infarct size.

Cell lines expressing single channels are a valuable tool for studying specific channels or exchangers. However, they are most often used to study acute effects of drugs, or in this case, fatty acids. It would be of interest to determine how the characteristics of the different channels change in response to being cultured in lipid defined environments,

specifically with increased ALA, or other fatty acids. Potentially, as the cells divide they would incorporate the available fatty acids into the membrane and this would be a step closer to a physiologically relevant dietary model.

These and other potential studies are of interest for future exploration. While we are quite certain that dietary ALA provides and antiarrhythmic effect, and that this is associated with an overall shortening of QT interval and action potential, the specific mechanisms underlying this effect are unclear. Perhaps it will be the choice of an appropriate model that will provide the most insight as to the electrophysiologic mechanisms, rather than persistent study using models that may not adequately approximate real-life situations. In any case, unlike the development of novel pharmacological therapies, these essential components of our food supply are widespread and readily available. Further research will ensure the population is properly educated to make wise decisions for how they best adapt their diets for optimal health.

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