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# Dietary fish oil prevents asynchronous contractility and alters $Ca^{2+}$ handling in adult rat cardiomyocytes

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**Abstract**

This study examined the effects of dietary incorporation of n-3 polyunsaturated fatty acids (PUFAs) into cardiac membrane phospholipids on $Ca^{2+}$ handling (using Fura-2) and arrhythmic contractility in electrically-stimulated, adult rat ventricular cardiomyocytes. Dietary lipid supplementation with fish oil (FO) for 3 weeks significantly increased the proportion of total n-3 polyunsaturated fatty acids (in particular, docosahexaenoic acid) in ventricular membrane phospholipids compared with a saturated fat (SF) supplemented diet (26.2 ± 0.9% vs 6.9 ± 0.9%, respectively, $P < 0.001$). Cardiomyocytes isolated from the FO group were significantly ($P < 0.001$) less susceptible to isoproterenol-induced arrhythmic contractile activity compared with the SF group over a range of isoproterenol concentrations. Isoproterenol (0.5 $\mu$M) stimulation increased end-diastolic and systolic $[Ca^{2+}]_i$ to a similar extent in both groups. The time constant of $Ca^{2+}$ transient decay was significantly increased in the FO group compared with the SF group (98.4 ± 2.8 ms, $n = 8$ and 86.9 ± 2.1 ms, $n = 8$, $P < 0.01$, respectively). The effect of dietary n-3 PUFA incorporation into membrane phospholipids was not associated with changes in sarcoplasmic reticulum $Ca^{2+}$ content (measured by rapid application of caffeine) or membrane fluidity. The increase in the time constant of decay of $Ca^{2+}$ transients following dietary supplementation with FO may indicate altered functioning of the sarcolemmal $Na^{+}$-$Ca^{2+}$ exchanger by n-3 PUFA incorporation into membrane phospholipids. 

*Keywords:* Ventricular myocyte; $Ca^{2+}$; n-3 fatty acid; Docosahexaenoic acid; Fish oil

## 1. Introduction

Recent evidence suggests that the consumption of polyunsaturated fatty acids (PUFAs) of the omega 3 (or n-3) class (of marine origin) reduce both the incidence of and mortality from coronary heart disease and this may be related to the antiarrhythmic activity of these PUFAs [1–3]. Experimental animal studies have also reported that dietary n-3 PUFAs change myocardial phospholipid fatty acid composition and protect against the development of both ischaemic and reperfusion induced arrhythmias in rats and marmosets [4,5]. In recent studies we have reported that n-3 PUFAs (in particular, eicosapentaenoic acid and docosahexaenoic acid) also display antiarrhythmic activity when added acutely to cardiomyocytes isolated from the hearts of young adult rats, with antiarrhythmic activity being associated with an increase in cardiomyocyte sarcolemmal lipid fluidity [6–9]. Acute n-3 PUFAs (and to a lesser extent, dietary n-3 PUFAs,) have also been reported to block and alter the voltage-dependence of whole cell $Na^{+}$ and $K^{+}$ currents in adult and neonatal rat cardiomyocytes [10–14]. Collectively, these results suggest a general reduction of cell excitability by acutely added n-3 PUFAs. Furthermore, acute addition of n-3 PUFAs to cardiomyocytes can both reduce the availability of $Ca^{2+}$ for sarcoplasmic reticulum (SR) uptake and inhibit the SR release mechanism [15]. These mechanisms could potentially prevent spontaneous release of SR $Ca^{2+}$ and therefore, the development of arrhythmias. Indeed, it has been suggested that this may be a mechanism underlying the antiarrhythmic effects of acute n-3 PUFAs at the level of the SR [15–17].

Previous studies of this nature have provided a useful insight into the possible mechanism of the antiarrhythmic action of these fatty acids when acutely applied (which do not incorporate into the membrane phospholipids). However, the effects of dietary n-3 PUFA modification of the membrane phospholipid composition on cardiomyocyte $Ca^{2+}$ handling has not been reported. Therefore, the pur-

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pose of this study was to determine whether dietary effects of incorporation of n-3 PUFAs into cardiac membrane phospholipids alters $Ca^{2+}$ transients using a cellular model of arrhythmia, which we have recently developed [14], using adult rat cardiomyocytes. This study therefore aimed firstly, to establish whether an antiarrhythmic effect of a fish oil dietary supplement (compared with saturated fat), could be observed in isolated rat cardiomyocytes and, secondly, to determine the $Ca^{2+}$ handling properties of the cardiomyocytes since $Ca^{2+}$ overload plays a central role in arrhythmogenesis [18]. This study was also designed to establish whether dietary lipid supplementation influenced SR function as has been shown to occur with respect to the acute addition of n-3 PUFAs [15,16].

## 2. Methods

### 2.1. Animals and dietary oils

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

Male Sprague Dawley rats consumed a modified laboratory rat chow based on the AIN 96 G mix with 7% fat supplied by the addition of high oleic sunflower oil (kindly provided by Meadow Lea Foods Ltd., Ryde, NSW, Australia) and water provided *ad libitum*. Room temperature was maintained at 23°C with constant (55%) humidity, and lights were maintained on a 12 hour light (8 am–8 pm)/dark cycle. Animals were maintained on the above diet to 9 weeks of age then assigned to one of two groups containing the above diet supplemented with either saturated fat (SF) or fish oil (FO) (both 10% added lipid) for a period of 3 weeks prior to the preparation of isolated cardiomyocytes (saturated fat was from Metro Quality Foods, Greenacres, NSW, Australia; fish oil was kindly provided by RoPUFA, Hoffmann La Roche, Basel, Switzerland). The percentage of total fat, carbohydrates and protein in the experimental diet was 17%, 57% and 18%, respectively. The final fatty acid compositions of the diets are shown in Table 1.

Table 1
Fatty acid composition (wt%) of the lipid supplemented diets

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

Data shown are mean for two samples per dietary group. The shorthand notation for fatty acid structure is "a:b (n-c)". "a" represents the total number of carbon atoms in the fatty acyl chain; "b", the number of double bonds separated by single methylene groups; and "n-c", also written as "omega-c" (or ω-c), denotes the number of carbon atoms between the first double bond and the methyl end of the chain. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set.

[a] FAME, fatty acid methyl esters.

[b] 18:1 contains n-9 and n-7 isomers.

[c] n/d, not detected.

### 2.2. Perfusion and culture media

Calcium-free Tyrode perfusion media, contained (in mM) 137.7 NaCl, 4.8 KCl, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 11 glucose, 10.0 (N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]) (HEPES), pH 7.40. $CaCl_2$ was added prior to use to give the appropriate concentrations indicated. Tyrode solution was prepared using ultra-pure (Milli-Q) water and was filtered through a 0.22 μm Millipore (Bedford, MA, USA) filter prior to use and gassed with 100% $O_2$. DMEM culture medium was supplemented with 1 mM $Ca^{2+}$, 10 mM HEPES, 25 mM $NaHCO_3$, 100 U/mL Penicillin G, 100 μg/mL Streptomycin, 0.05 μg/mL Amphotericin B, 2 mM carnitine, 5 mM creatine, 5 mM taurine and 1 mg/mL delipidated bovine serum albumin (BSA).

### 2.3. Isolation of cardiac myocytes

Hearts were excised and perfused in a retrograde manner on a Langendorff apparatus (non-recirculating) with Tyrode solution containing 1 mM $Ca^{2+}$ for 4 min as described previously [6]. The heart was then perfused with nominally $Ca^{2+}$-free Tyrode solution (non-recirculating) for 2 min. Buffers were maintained at 37°C and gassed with 100% $O_2$ during the Langendorff perfusion. The heart was further perfused in a recirculating manner for 20 min with Tyrode solution supplemented with 20 μM $Ca^{2+}$, 45 U/mL collagenase, 0.2 U/mL protease and 0.1% (w/v) delipidated BSA at 37°C. After perfusion with collagenase, the ventricles were removed and agitated in Tyrode solution containing 40 μM $Ca^{2+}$, 1.5% (w/v) BSA and 30 mM 2,3-butane-dione monoxime at 25°C. The suspension was filtered through 250 μm nylon-mesh gauze and made to a final volume of 50 mL. The concentration of $Ca^{2+}$ was increased stepwise in increments to 1 mM over 45 min. Aliquots of the cardiomyocyte suspension (approximately 2 mL) were added to petri dishes containing glass coverslips (#0, 6 mm × 17 mm) coated with laminin (25–50 μg/mL) or taken for via-

bility assessment. Rod-shaped cardiomyocytes adhered to coverslips within 60 min at 24°C. Coverslips were washed twice with DMEM culture medium (1 mM $Ca^{2+}$) pre-equilibrated with 5% $CO_2$: 95% $O_2$ to remove non-adhering cardiomyocytes. The final preparation contained over 95% rod-shaped, quiescent cardiomyocytes. Cardiomyocytes were maintained in DMEM in a humidified incubator at 37°C, gassed with 5% $CO_2$ in air, and were used within 4 hours.

### *2.4. Measurement of cardiomyocyte contraction and response to isoproterenol*

Cardiomyocytes on coverslips were placed in a custom-designed superfusion chamber and superfused with Tyrode buffer containing 1 mM $Ca^{2+}$ at 37°C and allowed to equilibrate for 2 min. A video camera mounted on an inverted Olympus microscope housed in a perspex chamber, transferred images to a computer connected between the camera and a monitor. An on-line, real-time computer program was used (LabVIEW, National Instruments, Victoria, Australia) to measure cell length as shown in Figs. 1A and 1B. Contractility was induced by electrical-field stimulation using a Grass S4 stimulator. The cells were routinely stimulated with a pulse duration of 5 ms at a frequency of 1 Hz using two platinum wire electrodes located at either side of the superfusion chamber. Asynchronous contractile activity was induced by superfusion with progressively increasing isoproterenol concentrations (0.01 $\mu$M to 3 $\mu$M) in the presence of electrical-field stimulation. Asynchronously contracting cells (in the presence of electrical-field stimulation) were visually scored and defined as those cells that exhibited a contraction rate exceeding the rate of applied electrical stimulation (i.e., cells not contracting in synchrony with the applied electrical stimulus). Four preparations per heart, consisting of at least 80 cardiomyocytes were counted for each rat heart.

### *2.5. Measurement of $[Ca^{2+}]_i$ using Fura-2*

Isolated cardiomyocytes on laminin coated coverslips (#0 glass) were transferred to a petri dish containing Tyrode solution supplemented with 1 mM $Ca^{2+}$, 2% (w/v) BSA and 30 mM 2,3-butane-dione monoxime and 5 $\mu$M Fura-2 (acetomethoxy (AM) form) at 24°C for 15 min. The coverslips were then transferred to a custom-designed superfusion chamber (#0 glass base) located on the stage of an inverted epifluorescence microscope (Nikon, Tokyo, Japan) and superfused at 2.5 mL/min with Tyrode buffer containing 2 mM $Ca^{2+}$. For fluorescence measurements cells were illuminated sequentially at 340 ± 10 nm and 380 ± 10 nm with a UV light source (175 W Xenon lamp) and Lambda DG-4 high-speed filter changer (Sutter Instrument Company, Novato, CA, USA). The emitted fluorescence was detected with a photomultiplier at 510 ± 40 nm. An adjustable rectangular diaphragm in the light-path preceding the photomultiplier restricted measurement of fluorescence to a single cell. The signals from the photomultiplier were fed through an A-D converter (BIOPAC Systems, Santa Barbara, CA, USA) and digitized at 200 Hz and passed through a low pass band filter and output to a computer for real-time analysis. After subtraction of background, the ratio of fluorescence signals at 340 nm/380 nm (R) were calculated and converted to $[Ca^{2+}]_i$ using the following formula [19],

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

where $R_{max}$ represents the maximum fluorescence ratio (calcium saturation) which was determined at the end of an

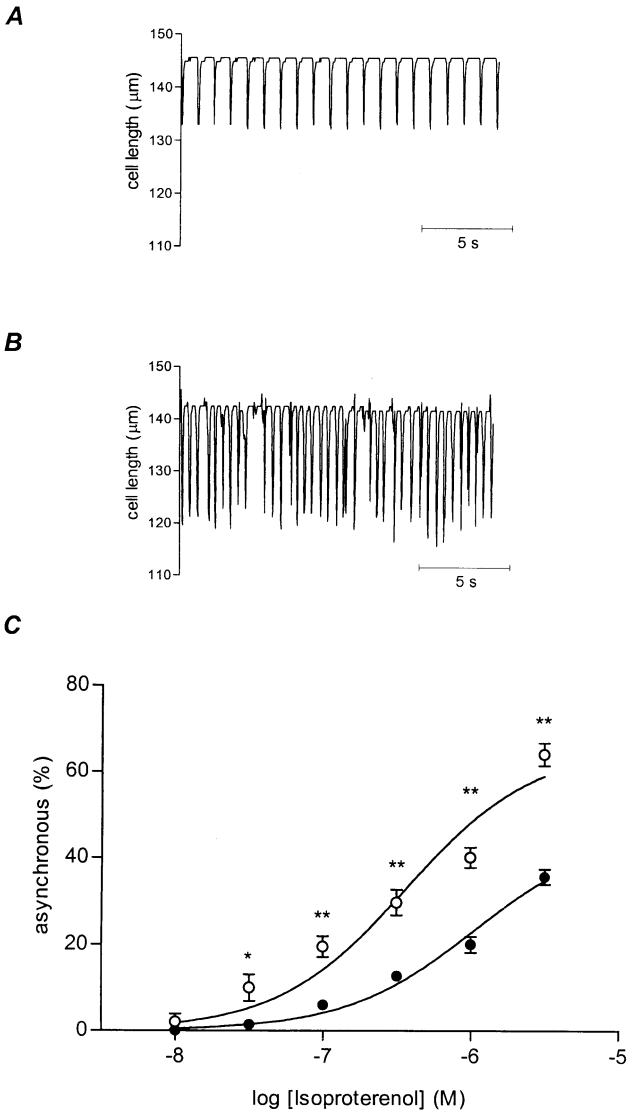


Fig. 1. Asynchronous contractility in rat cardiomyocytes. (A) original record demonstrating the change in cell length during steady-state (synchronous) contractility induced by electrical stimulation at 1 Hz. (B) The same cell showing the development of asynchronous contractility following treatment with 1 $\mu$M isoproterenol with electrical stimulation at 1 Hz. (C) The percentage of asynchronous contracting cardiomyocytes in response to increasing concentrations of isoproterenol. Diets were supplemented with either 10% saturated fat, SF (○) or 10% fish oil, FO (●). Data are means ± SEM for $n = 6$ animals per dietary group. $^*P < 0.05$, $^{**}P < 0.001$ for FO vs SF by two-way ANOVA.

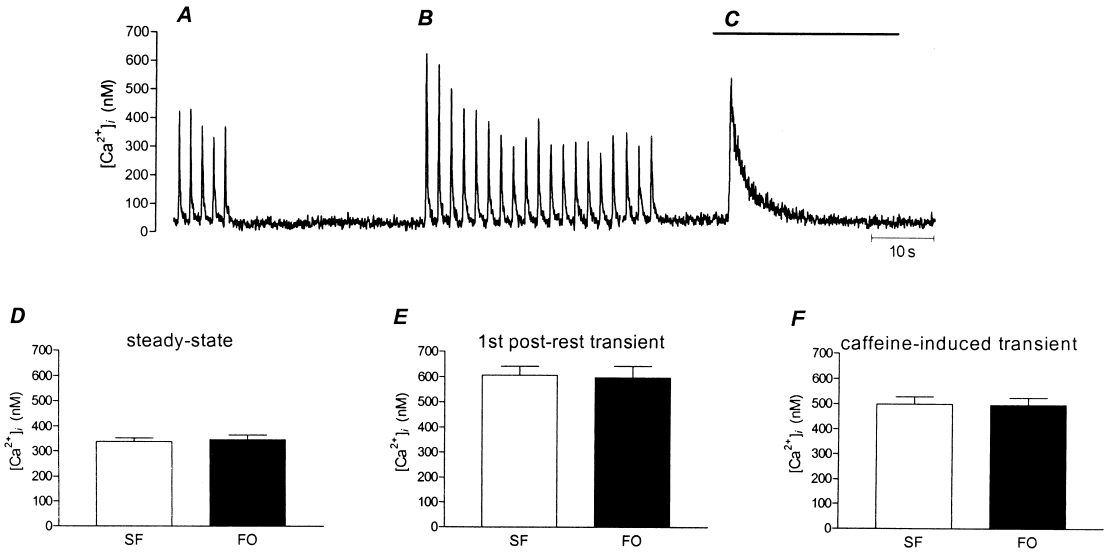


Fig. 2. Indirect measurement of SR $Ca^{2+}$ content in rat cardiomyocytes. (A–C) Representative $Ca^{2+}$ transients in an isolated rat cardiomyocyte measured using Fura-2. (A) Steady-state $Ca^{2+}$ transients elicited at a stimulation frequency of 0.5 Hz. (B) 30 s post rest potentiation and the negative staircase effect of $Ca^{2+}$ transients characteristic of rat cardiomyocytes prior to the new steady-state. (C) After rapid application of 20 mM caffeine in the absence of electrical stimulation, indicated by the horizontal bar. (D) Developed $[Ca^{2+}]_i$ during steady state. (E) developed $[Ca^{2+}]_i$ of the first $Ca^{2+}$ transient following 30 s rest. (F) Developed $[Ca^{2+}]_i$ of the caffeine-evoked $Ca^{2+}$ transient. Data are means ± SEM for $n = 32$ cardiomyocytes from 8 animals per dietary group. Abbreviations, saturated fat (SF) and fish oil (FO).

experimental protocol by treating cells with the membrane permeating agent, digitonin (20 $\mu$M). The minimum fluorescence ratio $R_{min}$ was determined by treatment with 5 mM EGTA and 20 mM caffeine, pH 7.3. $K_d$ is the dissociation constant for Fura-2 in simple ionic solution (225 nM), and $\beta$ represents the ratio of emission intensities at 380 nm excitation at saturating and calcium-free conditions.

After 5 min equilibration, cells were stimulated at 0.5 Hz for 2 min, using two platinum wire electrodes placed within the superfusion chamber and connected to a DS9A Digitimer stimulator (Digitimer Ltd., Herts, England). This procedure allowed for measurement of baseline calcium transients which included the end-diastolic $[Ca^{2+}]_i$ (the $[Ca^{2+}]_i$ between successive contractions), and the systolic $[Ca^{2+}]_i$ (the maximum $[Ca^{2+}]_i$ during contractions). In all experiments involving electrical stimulation, at least 5 consecutive $Ca^{2+}$ transients were ensemble averaged to obtain the $Ca^{2+}$ transient parameters (using AcqKnowledge software; SDR Clinical Technology, Sydney, NSW, Australia).

### 2.6. SR $Ca^{2+}$ content and $Ca^{2+}$ transients in cardiomyocytes

Sarcoplasmic reticulum (SR) $Ca^{2+}$ content was determined using two procedures. Firstly, after steady-state $Ca^{2+}$ transients were established at 0.5 Hz, a 30 s rest period was applied to allow the SR to load with $Ca^{2+}$ and then electrical stimulation was resumed at 0.5 Hz. The peak height of the first $Ca^{2+}$ transient post rest was analyzed and taken as an indirect measure of relative SR $Ca^{2+}$ content (post-rest potentiation) [20]. Alternatively, SR $Ca^{2+}$ content was determined by rapid application of caffeine [21]. Steady-state $Ca^{2+}$ transients were initially recorded for 1 min and then 20 mM caffeine was rapidly applied to the cell of interest via a rapid-application system using a reservoir placed 30 cm above the level of the chamber (in the absence of electrical stimulation). The outlet tube for caffeine application was placed approximately 500 $\mu$m upstream from the cell and delivered at a flow rate of 5 ml $min^{-1}$. A representative example is shown in Fig. 2. To test the effect of the SR $Ca^{2+}$-ATPase inhibitor, 2,5-Di-tert-butylhydroquinone (DBHQ) on SR $Ca^{2+}$ pump function [22], cardiomyocytes were stimulated at 0.2 Hz and $Ca^{2+}$ transients measured at steady-state before and after addition of 10 $\mu$M DBHQ for 4 min. The effect of DBHQ was reversible upon washout (data not shown). $\beta$-adrenergic agonist stimulation was carried out using 0.5 $\mu$M isoproterenol (4 min) at stimulation rates of 0.2–2 Hz. To determine the time constant ($\tau$) of the decay of $Ca^{2+}$ transients, exponential rates of decay were fitted to a single exponential function:

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

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

### 2.7. Fatty acid analysis

Total lipids were extracted from the ventricular tissue, the standard colony diet and dietary oils using a slight modification of the method of Bligh and Dyer [23]. For

analysis of ventricular lipid composition tissue was homogenised in 1 mL water using a Tenbroeck hand-held homogeniser. 4 mL of 2-propanol was added and the mixture boiled for 30 s. After cooling, 8 mL of chloroform was added, the mixture shaken, and the organic phase collected. The organic phase was evaporated to dryness under $N_2$. The phospholipids were separated from the other lipid classes by thin layer chromatography (TLC) on silica gel 150A-LK5D plates (Whatman, Clifton, NJ, USA), and developed in a solvent system of petroleum ether:acetone (3:1 [v/v]). The phospholipids remaining at the origin were scraped from the plate. Phospholipid fatty acid methyl esters (FAMEs) were prepared by heating the samples at 50°C overnight in 1% (v/v) $H_2SO_4$ in methanol. FAMEs were extracted using hexane and contaminants removed using a Biosil (silicic acid) column. All solvents used for lipid extraction, TLC and preparation of FAMEs contained the antioxidant butylated hydroxytoluene (0.05% w/v). FAMEs from lipid extracts of the lipid supplemented diets, and the myocardial phospholipid extracts, were analysed by GLC. GLC was performed using a Hewlett Packard HP 5710 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) fitted with a 50 metre BPX70 capillary column (Scientific Glass Engineering, Melbourne, Victoria, Australia). The FAMEs were separated using a carrier gas (hydrogen) flow of 35 cm/s with a temperature gradient of 130°C to 230°C at 4°C/min. A cold on-column injector was used with the flame ionisation detector temperature set at 250°C. FAMEs were identified using authentic lipid standards (Nu-Chek-Prep Inc., Elysian, MN, USA) by GLC. The proportions of the total fatty acids were normalised to a value of 100%.

### 2.8. Membrane fluidity measurements

Membrane fluidity was determined by measuring the steady-state fluorescence anisotropy ($r_{ss}$) of the probe TMAP-DPH according to a modification of a method described previously [10,24]. This probe readily partitions into the polar headgroup region of the cell membrane bilayer [25]. Isolated ventricular cardiomyocytes attached to laminin-coated glass coverslips were washed in Tyrode buffer. Cardiomyocytes were then loaded with 1 $\mu$M TMAP-DPH for 35 min at 37°C. Cells on coverslips were then placed in a glass cuvette and $r_{ss}$ values were measured according to the following formula as described previously [25],

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

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

### 2.9. Chemicals

DMEM culture medium, bovine serum albumin (BSA, fraction V), carnitine, creatine, taurine, 2,3-butane-dione monoxime, protease (type XIV), laminin and isoproterenol, were from Sigma Chemical Co. (Castle Hill, NSW, Australia). Digitonin was from Calbiochem-Novabiochem GMBH (Bad Soden, Germany). Collagenase was from Yakult Honsha Co., Ltd (Tokyo, Japan). Solutions of isoproterenol (5 mM stock) were prepared daily in 10 mM ascorbic acid and kept on ice. Penicillin/Streptomycin was from GIBCO-BRL (Melbourne, Victoria, Australia). 2,5-Di-tert-butylhydroquinone (DBHQ) was from Biomol (Plymouth Meeting, PA, USA). N-((4-(6-phenyl-1,3,5-hexatrienyl)phenyl)propyl) trimethyl-ammonium p-toluenesulfonate (TMAP-DPH) and Fura-2 AM was from Molecular Probes (Eugene, OR, USA). All other chemicals were of the highest grade available.

### 2.10. Statistics

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

## 3. Results

Prior to lipid supplementation, rat body weights were not significantly different. The body weights at 9 weeks of age were 360.7 ± 9.2 g ($n = 8$) and 374.7 ± 9.5 g ($n = 8$), for the SF and FO groups, respectively, and following 3 weeks dietary fat supplementation with either SF or FO, body weights increased to 474.2 ± 10.3 g ($n = 8$) and 474.6 ± 2.4 g ($n = 8$), respectively (not significantly different). Immediately following isolation of cardiomyocytes, viability was assessed as the percentage of rod-shaped cells without membrane blebs, and was 73.1 ± 3.9% in the SF group and 76.7 ± 2.2% in the FO group (not significantly different).

### 3.1. Fatty acid composition of rat ventricular phospholipids

The fatty acid composition of the lipid supplemented diets are shown in Table 1. The FO diet contained approx-

Table 2
Fatty acid composition (wt%) of the ventricular phospholipids after dietary lipid supplementation

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

Data shown are mean ± SEM for 6 animals per dietary group. The shorthand notation for fatty acid structure is given in Table 1 legend. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set.

[a] FAME, fatty acid methyl esters.

[b] 18:1 contains n-9 and n-7 isomers.

[c] n/d, not detected.

Superscripts indicate significant differences at

[d] $P < 0.05$,

[e] $P < 0.01$,

[f] $P < 0.001$ vs SF (by Students unpaired *t*-test).

SF; saturated fat group, FO; fish oil group.

imately 29% total n-3 polyunsaturated fatty acids (PUFAs) whilst the SF diet supplement contained approximately 1% n-3 PUFAs. The FO diet also contained comparatively less saturated fat and monounsaturated fat.

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

### *3.2. Effect of dietary lipid supplementation on isoproterenol induced asynchronous contractile activity of cardiomyocytes*

Fig. 1A shows the change in cell length of a synchronously contracting cardiomyocyte and after treatment with the β-adrenergic receptor agonist, isoproterenol, asynchronous (arrhythmic) contractile activity develops (Fig. 1B) in cardiomyocytes as shown previously [7,9,26,27]. Fig. 1C shows that the onset of asynchronous contractile activity was evident at lower isoproterenol concentrations in cardiomyocytes isolated from the SF group in comparison with the FO group. This effect was statistically significant between the SF and FO groups at the concentrations of isoproterenol indicated in Fig. 1. It was not possible to accurately determine the $ED_{50}$ dose of isoproterenol needed to elicit asynchronous contractile activity due to the fact that maximum levels of asynchronous contractility were not achieved because of the nature of the experimental protocol. However, it would appear that cardiomyocytes isolated from the SF rats are significantly more sensitive to isoproterenol (at concentrations ≥30 nM) in terms of the development of asynchronous contractile behaviour than cells isolated from rats fed the FO supplemented diet.

### *3.3. Effect of dietary lipid supplementation on $[Ca^{2+}]_i$ transients in cardiomyocytes*

To determine the level of Fura-2 loading, the value of the fluorescence ($F_{380}$) was compared in all cells studied. The $F_{380}$ values were 3.68 ± 0.12 units ($n = 96$) from the SF group and 4.03 ± 0.14 units ($n = 96$) from the FO group (not significantly different). To determine SR $Ca^{2+}$ content, two alternate experimental protocols were used on the same cell. A representative recording of $Ca^{2+}$ transients is shown in Fig. 2A–C. Fig. 2A shows $Ca^{2+}$ transients under steady-state conditions achieved at 0.5 Hz. This was then followed by a 30 s rest period with no electrical stimulation. The first $Ca^{2+}$ transient peak (as shown in Fig. 2B) immediately after this rest period was used as an indirect measure of SR $Ca^{2+}$ content by measuring the developed $[Ca^{2+}]_i$ (calculated by subtracting the minimum (end diastolic) $[Ca^{2+}]_i$ concentration from the maximum (systolic) $[Ca^{2+}]_i$ concentration). In addition, an alternative procedure for determining SR $Ca^{2+}$ content using caffeine (20 mM) was carried out as described in "methods" (Fig. 2C). The mean of the developed $[Ca^{2+}]_i$ in all hearts (total of 32 cardiomyocytes analysed for each group) was similar (approximately 340 nM) between the SF ($n = 8$) and FO ($n = 8$) groups at steady-state, as shown in Fig. 2D. The 30 s post rest $Ca^{2+}$ transient was increased to approximately 600 nM for both SF and FO dietary groups (not significantly different) as shown in Fig. 2E. When caffeine was rapidly applied, a large release of $Ca^{2+}$ from the SR was evident. Under these conditions, the $Ca^{2+}$ released is removed from the cytoplasm by non-SR pathways and the caffeine-induced $Ca^{2+}$ transient declined more

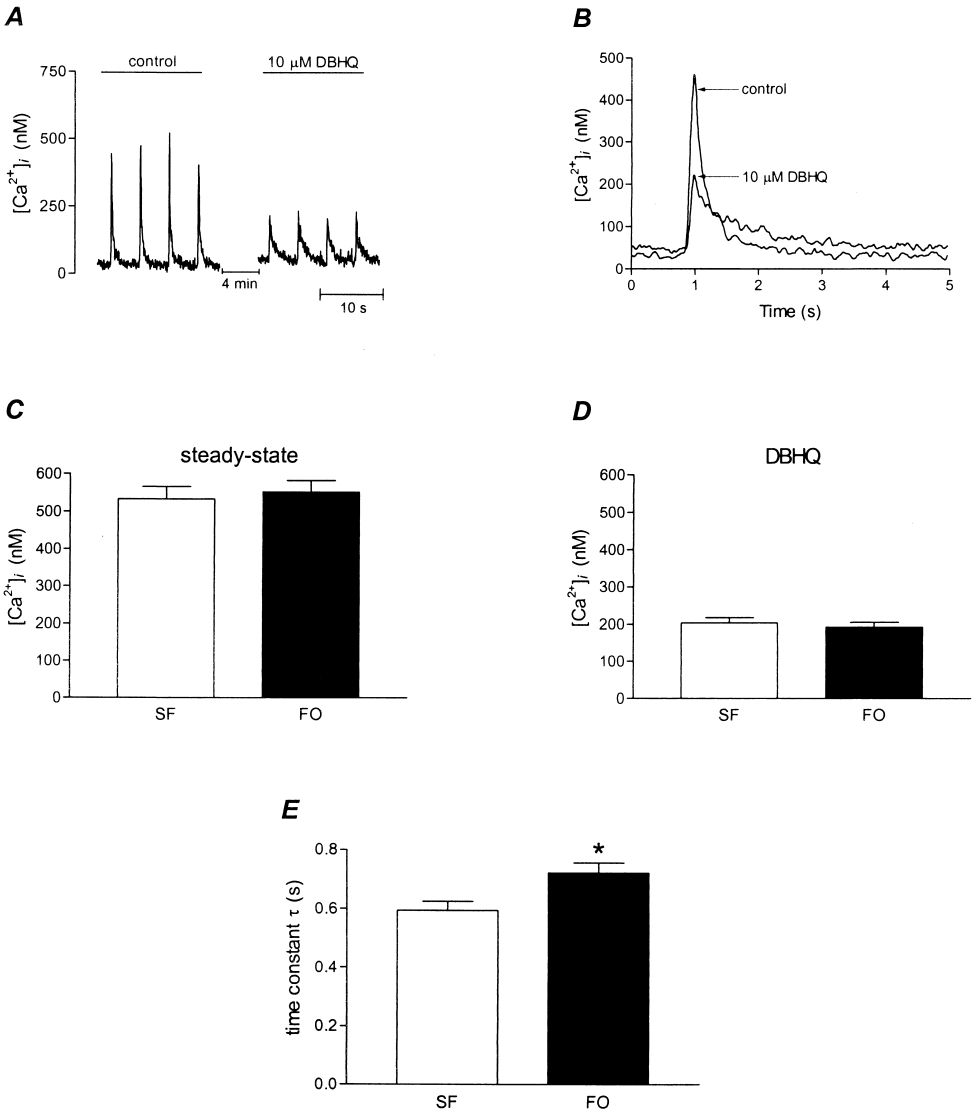


Fig. 3. Effects of the SR $Ca^{2+}$ pump inhibitor, DBHQ, on $Ca^{2+}$ transients in rat cardiomyocytes. (A) Representative $Ca^{2+}$ transients in an isolated rat cardiomyocyte measured using Fura-2 in the absence (control) or following 4 min treatment with 10 $\mu$M DBHQ at a stimulation frequency of 0.2 Hz. (B) Ensemble average of $Ca^{2+}$ transients from panel A. (C) Comparison of SF and FO dietary groups for developed $[Ca^{2+}]_i$ during steady state at 0.2 Hz. (D) Developed $[Ca^{2+}]_i$ following 4 min treatment with 10 $\mu$M DBHQ. (E) Time constants were determined by single exponential fit of the declining phase of the electrically stimulated $Ca^{2+}$ transients following DBHQ treatment. Data are means ± SEM for $n = 32$ cardiomyocytes from 8 animals per dietary group. Abbreviations, saturated fat (SF) and fish oil (FO). *$P < 0.05$.

slowly than electrically stimulated $Ca^{2+}$ transients, probably due to the inhibition of SR $Ca^{2+}$ uptake by caffeine. The maximal concentration of $[Ca^{2+}]_i$ as measured by this protocol, was less than the 30 s post rest potentiation protocol described above (approximately 500 nM as shown in Fig. 2F), and was similar in value for both the SF and FO groups. This is probably because the post rest transient includes $Ca^{2+}$ contributed from both the SR and sarcolemma. However, the results obtained by this latter protocol similarly demonstrate that there was no significant difference between cells from the SF and FO dietary supplemented groups in relation to cardiac SR $Ca^{2+}$ content.

DBHQ was used to inhibit uptake of $Ca^{2+}$ by the SR as shown previously [28]. Fig. 3A shows a representative recording of electrically stimulated $Ca^{2+}$ transients at a frequency of 0.2 Hz before and after application of 10 $\mu$M DBHQ for 4 min. The ensemble average of the $Ca^{2+}$ transients from Fig. 3A is shown in Fig. 3B. DBHQ treatment resulted in a significant reduction in the value of the peak $Ca^{2+}$ transient and an increase in the time constant of decay ($\tau$) of the $Ca^{2+}$ transient. Figs. 3C and 3D show the developed $[Ca^{2+}]_i$ under control conditions and following DBHQ treatment, respectively, with there being no significant difference between the dietary groups. However, the time constant of $[Ca^{2+}]_i$ decay ($\tau$) in the FO group ($n = 8$) was significantly greater ($P < 0.05$) compared with the SF group ($n = 8$) as shown in Fig. 3E, indicating a more rapid $Ca^{2+}$ efflux via sarcolemmal $Ca^{2+}$ exchangers in the SF

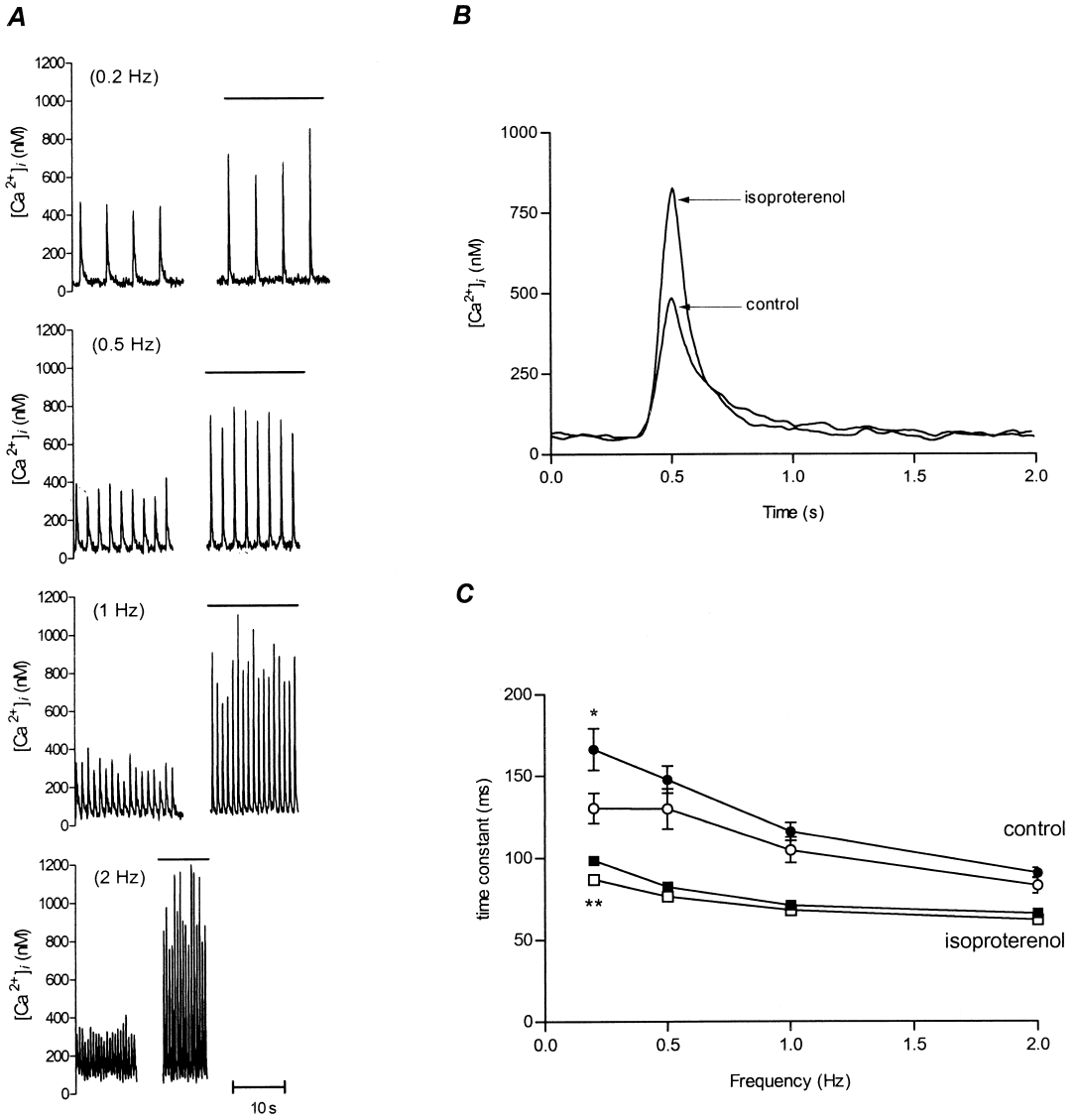


Fig. 4. Effects of isoproterenol on $Ca^{2+}$ transient decay at 0.2–2 Hz in rat cardiomyocytes. (A) Representative $Ca^{2+}$ transients in an isolated rat cardiomyocyte measured using Fura-2, in the absence or presence (indicated by the horizontal bar) of 0.5 $\mu$M isoproterenol at stimulation frequencies of 0.2, 0.5, 1 and 2 Hz. (B) Representative ensemble average of five $Ca^{2+}$ transients following electrical stimulation at 0.5 Hz under control conditions or in the presence of 0.5 $\mu$M isoproterenol as indicated. (C) Time constants were determined by single exponential fit of the decay phase of the electrically stimulated $Ca^{2+}$ transients following electrical stimulation at frequencies of 0.2–2 Hz during control conditions, comparing saturated fat, SF (○) or fish oil, FO (●) supplementation, and following 0.5 $\mu$M isoproterenol treatment, SF (□) or FO (■). Data are means ± SEM for $n = 29$–31 (FO) or $n = 32$ (SF) cardiomyocytes from 8 animals per dietary group. *$P < 0.05$ SF vs FO under control conditions, **$P < 0.01$ SF vs FO in the presence of isoproterenol, by two-way ANOVA.

group. The contribution of the SR to the $[Ca^{2+}]_i$ transient provides approximately 62% of the $Ca^{2+}$ available for transients and therefore, for contraction. This result compares to 42% in toad pacemaker cells [28] and 32% in guinea-pig ventricular cells [20].

To investigate whether $Ca^{2+}$ handling following $\beta$-adrenergic receptor stimulation was different between cardiomyocytes isolated from the two dietary groups, isoproterenol (0.5 $\mu$M) was used to increase the cellular $Ca^{2+}$ load. To determine whether $Ca^{2+}$ handling was frequency-dependent, various electrical stimulation frequencies were used. The isoproterenol treatment increased the amplitude of $Ca^{2+}$ transients at all stimulation frequencies tested. A representative recording of $Ca^{2+}$ transients over the stimulation range 0.2–2 Hz is shown in Fig. 4A. The ensemble average of five $Ca^{2+}$ transients from a single cardiomyocyte stimulated at 0.5 Hz in the absence and presence of isoproterenol is shown in Fig. 4B. Isoproterenol increased the amplitude of $Ca^{2+}$ transients and decreased the time constant for $Ca^{2+}$ decay. The time constant for mean $Ca^{2+}$ decay in cardiomyocytes from the FO group ($n = 8$) was significantly greater ($P < 0.05$) than that of the SF group ($n = 8$) in the absence of isoproterenol as shown in Fig. 4C. Furthermore, treatment with isoproterenol significantly decreased the time constant of the decay phase of $Ca^{2+}$ transients (in both groups) indicating a more rapid $Ca^{2+}$ seques-

tration by the SR and/or $Ca^{2+}$ efflux via sarcolemmal $Ca^{2+}$ exchangers. In the presence of isoproterenol, the time constant of the decay phase of $Ca^{2+}$ transients was significantly higher in the FO group compared with the SF group (98.4 ± 2.8 ms and 86.9 ± 2.1 ms, respectively, $P < 0.01$).

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

### *3.4. Steady-state fluorescence anisotropy (membrane fluidity) of rat cardiomyocytes*

We have previously demonstrated that acute addition of micromolar concentrations of n-3 PUFAs significantly decreased the anisotropy of the probe TMAP-DPH in cardiomyocytes (i.e., increased sarcolemmal membrane fluidity) [10]. Therefore, this study investigated whether incorporation of n-3 PUFAs into membrane phospholipids by dietary fish oil supplementation (compared with saturated fat) increased membrane fluidity in a similar manner. The fluorescence anisotropy ($r_{ss}$) of the FO cardiomyocytes (0.241 ± 0.003, $n = 8$) was not significantly different from SF cardiomyocytes (0.241 ± 0.005, $n = 6$), indicating the membrane fluidity (as detected by TMAP-DPH) was unchanged by the nature of dietary fat supplementation. It is noteworthy from the data presented in Table 2 that the proportion of total saturated fatty acids in the ventricular phospholipid fatty acids was not altered by the dietary lipid treatments although there were significant changes in the n-6/n-3 PUFA ratio.

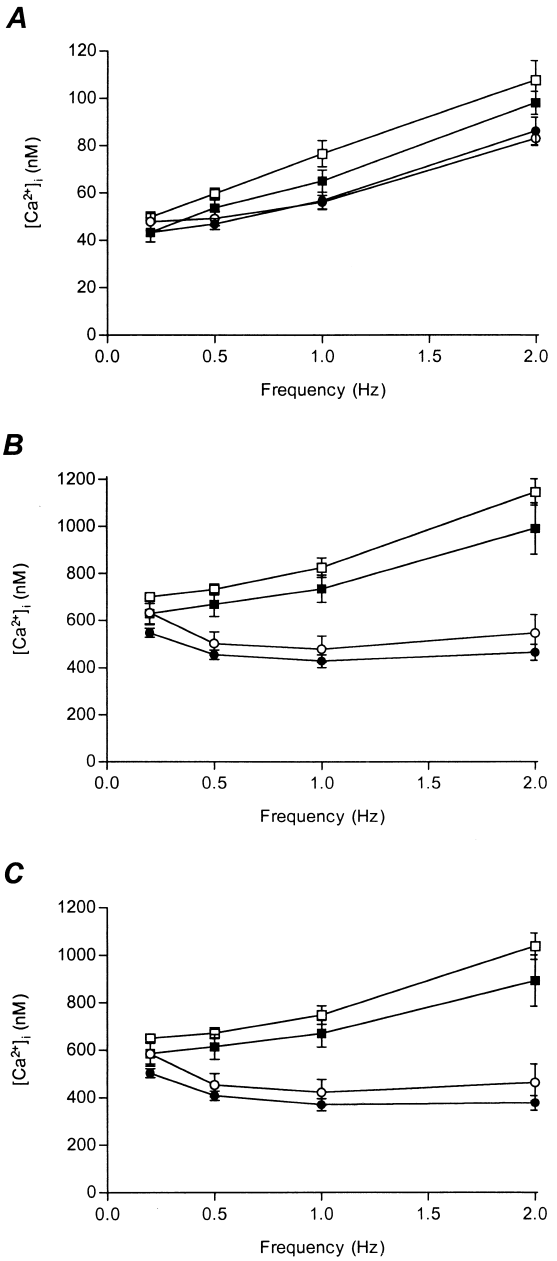


Fig. 5. Effects of electrical stimulation frequency and isoproterenol on $[Ca^{2+}]_i$. $Ca^{2+}$ transients in isolated rat cardiomyocytes were measured using Fura-2 at stimulation frequencies of 0.2, 0.5, 1 and 2 Hz during control conditions comparing saturated fat, SF (○) or fish oil, FO (●) supplementation, and following 0.5 μM isoproterenol treatment, SF (□) or FO (■). (A) End-diastolic $[Ca^{2+}]_i$, (B) systolic (peak) $[Ca^{2+}]_i$, (C) developed $[Ca^{2+}]_i$ (systolic − end diastolic). Data are means ± SEM for $n$ = 29–31 (FO) or $n$ = 31–32 (SF) cardiomyocytes from 8 animals per dietary group.

## 4. Discussion

Clinical, epidemiological and other laboratory based studies have clearly demonstrated that dietary n-3 polyunsaturated fatty acids (PUFAs) are associated with a significant reduction in the incidence of fatal cardiac arrhythmias [1–5]. Laboratory-based studies on potential antiarrhythmic mechanisms have focussed on the action of free fatty acids and their acute effects on various transsarcolemmal ion currents with the predominant effects of the n-3 PUFAs appearing one of dampening the electrical excitability of cardiac myocytes and preventing unwanted aberrant electrical disturbances and $Ca^{2+}$ sparks leading to arrhythmic

contractility [12,14,29–32]. In contrast, this study investigated the antiarrhythmic effects following dietary supplementation of the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in an adult rat cardiomyocyte model of arrhythmia which we have recently developed [6,9,14]. We demonstrate that dietary fish oil (FO) supplementation for 3 weeks significantly increased the proportions of EPA and DHA in the cardiac membrane phospholipids, in comparison with that following dietary supplementation with saturated fat (SF). This was associated with a significant decrease in the proportion of cardiomyocytes contracting in an asynchronous manner in response to the $\beta$-adrenergic receptor agonist, isoproterenol, and a likely decrease in sensitivity to isoproterenol in the FO group relative to the SF dietary group. Dietary induced changes in cardiomyocyte arrhythmia susceptibility to isoproterenol were not attributable to differences in the proportion of the total ventricular saturated fatty acids since these remained similar in both groups. However, the higher proportions of total n-3 PUFAs incorporated into membrane phospholipids, in particular DHA, exerted an anti-arrhythmic effect. This protective effect of FO supplementation was associated with an increase in the time constant for the decay of $Ca^{2+}$ transients in the FO group but was not associated with a change in the membrane lipid fluidity as determined by the fluidity probe TMAP-DPH.

Increasing concentrations of isoproterenol induced a steady increase in the percentage of cells undergoing asynchronous contractile activity and may be related to $Ca^{2+}$ overload [33]. Therefore, during measurement of $Ca^{2+}$ transients we chose a concentration of isoproterenol that would not induce all cells into $Ca^{2+}$ overload and asynchronous contractility. At the cellular level this form of aberrant contractile behaviour in part reflects the behaviour of the whole myocardium to arrhythmogenic stressors associated with ischaemic arrhythmias, excessive $\beta$-adrenergic receptor activation and/or calcium overload. We have previously reported that isoproterenol-induced asynchronous contractile activity in adult rat cardiomyocytes could be rapidly prevented or terminated by the acute addition of low micromolar concentrations of n-3 PUFAs, but not n-6 PUFAs or saturated fatty acids [6,9]. One possible antiarrhythmic mechanism by which the incorporated n-3 PUFAs could manifest their effects, could relate to an altered membrane lipid environment. The n-3 PUFA induced changes in membrane-composition are likely to alter membrane physical properties, which, in turn could affect many membrane-bound enzyme activities [24,34–36]. Collectively, this could reduce sensitivity and response of the $\beta$-receptor signalling pathway [37,38].

While we have also demonstrated that membrane fluidity as measured using the probe TMAP-DPH, was not significantly changed by dietary incorporation of n-3 PUFAs into cardiac membrane phospholipids (as opposed to acute addition of n-3 PUFAs), others have found a decrease in membrane fluidity with a concomitant rise in membrane cholesterol levels in neonatal cardiomyocytes cultured with an EPA-enriched culture medium [24]. The results of our present study contrast with our previous studies in which we have reported that the addition of free, n-3 PUFAs to cardiomyocytes increased membrane fluidity [7,14]. This suggests that the free form of n-3 fatty acids, as opposed to esterified n-3 PUFAs associated with membrane phospholipids, are able to perturbate the lipid membrane bilayer resulting in membrane fluidity increases and ion channel blockade. Interestingly, benzyl alcohol, a membrane fluidising agent [39], also displays ion channel blocking properties in a manner similar to that of the free n-3 PUFAs [7,14,40].

It has been reported that in guinea-pig cardiomyocytes an increase in SR $Ca^{2+}$ content results in arrhythmias (after-contractions) [41]. Therefore, it is plausible that animals on a diet that is "cardioprotective" (antiarrhythmic) may have a decreased level of $Ca^{2+}$ in their SR stores. However, using Fura-2, we have shown in this study that the SR $Ca^{2+}$ content was similar in cardiomyocytes from both the FO and SF dietary groups indicating that there must be an alternative explanation to reconcile the antiarrhythmic properties attributable to the FO diet. Our previous study showed that when dietary n-3 PUFAs were incorporated into cardiac membrane phospholipids, the steady-state cell contraction amplitude (and post-rest potentiation) were similar when comparisons were made between cardiomyocytes isolated from rats from control (SF) and FO dietary groups [14]. This result contrasts to that obtained when n-3 PUFAs are added acutely to isolated cardiomyocytes in which a rapid, negative inotropic effect and a decrease in amplitude of $Ca^{2+}$ transients are induced [11,15]. This acute effect of n-3 PUFAs is believed to be related to inhibition of the sarcolemmal L-type $Ca^{2+}$ current [29,31] as well as direct effects on the $Ca^{2+}$ content of the SR via SR uptake and SR release processes [15–17]. Interestingly, we demonstrated that there was no significant difference in $Ca^{2+}$ transients between the SF and FO groups when the L-type $Ca^{2+}$ channel opener, Bay K8644 was used (unpublished observations).

Although this study demonstrated that the resting cell $[Ca^{2+}]_i$ was slightly higher in the SF group compared with the FO dietary group, it is possible that the higher $[Ca^{2+}]_i$ may result from an increased propensity for $Ca^{2+}$ sparks in cells isolated from the SF group (Dr David Saint, University of Adelaide, unpublished observations). This could predispose cells from the SF dietary group to increased arrhythmic contractile activity in the presence of isoproterenol. The slower rate of $Ca^{2+}$ extrusion from the cytoplasm in cells from the FO group as evident from the time constant of $Ca^{2+}$ decay, appeared independent of the SR $Ca^{2+}$ ATPase pump activity since, in the presence of DBHQ, a SR $Ca^{2+}$ ATPase inhibitor, higher values were also obtained for the DBHQ-sensitive $Ca^{2+}$ transient decay rates in the FO group (data not shown). This result suggests that the differences we observed in the extrusion of $Ca^{2+}$ from the cytoplasm

may involve sarcolemmal $Ca^{2+}$ transporters, in particular the $Na^{+}$-$Ca^{2+}$ exchanger.

In conclusion, a possible action of n-3 PUFAs when incorporated into the membrane phospholipids following short-term dietary administration may involve moderate alteration of $Ca^{2+}$ transients and decay rates. The n-3 PUFAs incorporated into membrane phospholipids may also act as a pool for the generation of free n-3 PUFAs which are released by the action of phospholipases activated during ischaemia or in other circumstances [42]. These free n-3 PUFAs would then be able to undergo metabolic alterations to produce antiarrhythmic eicosanoids and prostaglandins [43] and/or to act directly on various ion channels and membrane-associated enzyme systems at the cell surface as well as at the SR. It is therefore likely that membrane incorporated n-3 PUFAs exert multiple effects on the cardiac myocardium and act synergystically to elicit antiarrhythmic and cardioprotective effects at a number of functionally important sites.

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