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Polyunsaturated dietary fats change the properties of calcium sparks in adult rat atrial myocytes

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Abstract

This study investigated the effects of dietary omega-3 polyunsaturated fatty acids on calcium handling mechanisms in cardiac myocytes, with the hypothesis that this effect underlies some of the antiarrhythmic properties of these compounds. Adult male Sprague Dawley rats had their standard chow supplemented with either lard (57% saturated and 40% monounsaturated fat), canola oil (60% monounsaturated, 33% polyunsaturated) or fish oil (78% polyunsaturated). Isolated cardiac atrial myocytes from these animals were loaded with fluo-3AM and examined with laser scanning confocal microscopy. The dietary interventions resulted in considerable changes in the membrane phospholipid composition of cardiac cell membranes, particularly the ratio of n-6 to n-3 (2.17 with lard supplement and 1.28 with fish oil supplement). Calcium sparks in myocytes from rats which received saturated fat were significantly more prolonged than those from rats which received fish oil. (Lard = 105.4 ± 18.9 ms; Fish oil = 43.5 ± 4.7 ms: mean ± s.e.m). The results for canola oil were intermediate (56.4 ± 9.0 ms). The prolongation of the sparks in rats fed lard was primarily due to a higher proportion of sparks with long plateaus and/or slowed kinetics in this group. The frequency of sparks was not significantly different in cells from any group. We conclude that calcium handling mechanisms in rat atrial myocytes are affected by inclusion of different fats in the diet, correlated with changes in the cell membrane phospholipid composition, and speculate that this may underlie some of the antiarrhythmic properties of these dietary compounds.

Keywords: Dietary fats; Polyunsaturated fatty acids; Atrial fibrillation; Cardiac calcium handling; Cardiocytes; Calcium sparks

1. Introduction

A number of epidemiological studies have shown that consumption of a diet high in n-3 polyunsaturated fatty acids (PUFAs) can confer protection from coronary heart disease [1,2,3]. Dietary PUFAs have been shown to have a similar protective action in animal studies, particularly in relation to cardiac arrhythmias [4,5,6,7]. More recently, it has been reported that some PUFAs can exert powerful antiarrhythmic actions when applied acutely, for example as an intravenous infusion in surgically manipulated animals [8,9].

The mechanisms underlying the antiarrhythmic actions of fatty acids are not fully understood, but it seems likely that it is due in large part to block of ionic currents. It has been shown that free fatty acids of the n-3 class are potent blockers of the cardiac sodium current [10,11]), potassium currents [12,13] and L-type calcium current [14]. Although the effects of free fatty acids on membrane currents are well documented, the effects of modification of the membrane phospholipid composition by *dietary* PUFAs on these same currents has not yet been widely reported. However, our own data [15] indicate that Na^+ and K^+ currents are not substantially changed in animals in which cell membrane composition has been dramatically altered by dietary administration of fats.

A paradox arises, then, as to how *dietary* PUFAs protect against arrhythmias. An explanation may lie in the fact that arrhythmias can have a diversity of origins. Indeed, a large contribution to arrhythmogenesis can arise from changes in calcium handling at the cellular level, which leads to a class of arrhythmias called "calcium overload" arrhythmias. These arrhythmias arise when abnormal calcium release from the sarcoplasmic reticulum (SR) results in large transient increases in intracellular calcium, leading to propagating waves of calcium release and the generation of large inward currents as a consequence of greatly increased electrogenic Na-Ca exchange [16].

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There are very little data in the literature on changes in calcium handling induced by fatty acids, either as free acids or incorporated into the membrane phospholipid. As noted above, L-type calcium currents are blocked by free fatty acids [14], but the effects on other calcium handling mechanisms, such as ryanodine receptor channels or Ca-ATPases are not well documented. There is currently very little data in the literature on the effects of *dietary* fatty acids on calcium handling, although *in vitro* studies suggest that the sarcoplasmic Ca-ATPase may be affected [17]. With this in mind, we have performed experiments to examine calcium handling in cells isolated from animals whose diets were supplemented with either saturated fats (lard), n-6 PUFAs (canola oil) or n-3 PUFAs (fish oil). We used calcium sparks, recorded with fluorescence laser microscopy, as an indication of changes in calcium handling.

2. Methods and materials

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

2.1. Dietary supplementation

Three groups of 6 male Sprague-Dawley rats (350–450 g) were gavaged with 3 ml of either lard (saturated fat), canola oil (predominantly monounsaturated and n-6 polyunsaturated fats) or fish oil (predominantly n-3 polyunsaturated fats) daily for a period of three weeks. During this time, rats were fed standard rat chow *ad libitum* in addition to the fat supplement. The composition of the supplements is given in Table 1.

Table 1
Composition of dietary supplements

Lipid (%)	Lard	Canola oil	Fish oil
14:0	8.36	—	—
16:0	32	4.55	0.16
18:0	16.9	1.5	0.4
18:1 (n-9)	35.7	58.8	4.63
18:2 (n-6)	1.34	21.7	—
18:3 (n-3)	0.7	12.1	0.5
20:1		1.1	10.2
20:5 (n-3)			48.0
22:5 (n-3)			1.2
22:6 (n-3)			26.2
Σ	95	99.75	91.29
Lipids by class: (%)	**Lard**	**Canola oil**	**Fish oil**
Saturated	56.89	6.23	1.72
Monounsaturated	40.08	60.04	15.81
Polyunsaturated	2.02	33.82	77.7
n-6	1.34	21.76	1.77
n-3	0.68	12.06	75.93

2.2. Cell isolation

Thirty minutes after an injection of heparin (2000 units i.p.), animals were anesthetized with pentobarbitone sodium (60 mg/kg IP). The heart was removed, washed in an ice-cold, oxygenated, calcium-free Tyrode's solution for 1 min, and then perfused, via an aortic cannula, with the Tyrode's solution containing 1 mM calcium warmed to 37°C for 4 min. This was followed by 6 min of perfusion with calcium free Tyrode's solution at a perfusion rate of between 9 and 10 ml min^{-1} to remove blood from both the coronary vasculature and ventricular chambers. The Tyrode's solution contained (mM): NaCl, 134; HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid), 10; KCl, 4; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; glucose, 11, and was adjusted to pH 7.4 with 1.0 M NaOH. After perfusion with calcium free Tyrode's solution, the heart was perfused with 15 μM calcium Tyrode's solution containing collagenase (0.1 mg ml^{-1}, Yakult), protease (0.04 mg ml^{-1}, Sigma), defatted bovine serum albumin (0.8 mg ml^{-1}) and BDM (2.5 mg ml^{-1}). After approximately 20 min the atria were removed and gently teased apart in calcium free Tyrode's solution containing BDM (3 mg ml^{-1}) and defatted bovine serum albumin (13 mg ml^{-1}) to separate the cells. The calcium concentration of the cell suspension was gradually increased to 60 μM, then 210 μM, then 460 μM, then finally 1 mM over 40 min. Except for the perfusion of the heart, all the preparation and maintenance of the cells was at room temperature (25°C).

2.3. Recording and analysis of calcium sparks

Cells were loaded with 30 μM of the AM ester of Fluo-3 (Molecular Probes) in conjunction with 2.5 ml ml^{-1} of 10% pluronic acid for 30 min at room temperature (22–25°C). After this 30 min period, the loading solution was removed and replaced with Tyrode's solution containing 1 mM calcium. The cells were then plated on to a laminin (50 μg ml^{-1}, Sigma) coated coverslip and placed on the stage of a Nikon Diaphot inverted microscope. Calcium sparks were viewed using the fast line scan mode of a Bio-Rad MRC-1000 Krypton-Argon laser scanning confocal microscope in fluorescence mode with excitation at 488/10 nm and emission detection at 522/32 nm. The objective lens was a 40× Nikon water immersion lens with a numerical aperture of 1.15. All experiments were performed at room temperature (22–25°C).

Spark area, duration and width were measured using the computer program Scion Image. Sparks were analyzed by setting the threshold level so that the background was completely removed just before the spark (in many cases this was performed separately for each spark). Spark width, duration and area were measured at the level of threshold.

Spark width was measured at the widest point across the spark and duration as the longest length along the length of the spark. Events were identified as sparks if the width of the event was not more than 5 μm and no less than 0.5 μm. Spark brightness was calculated from the profile of the spark. Peak intensity was determined as a percentage of the background immediately prior to the spark. For an event to be considered as a spark, the peak of the event had to be a minimum of 50% greater than the background value.

2.4. Membrane phospholipid measurements

Membrane phospholipid composition was determined according to the method outlined by Bligh and Dyer [18]. Note: The numeric description accompanying the fatty acid names follows the convention (x:y n-i) where x = length of carbon chain, y = number of double bonds and i = position of first double bond from the end of carbon chain having the methyl group. The position of the first double bond is often referred to as "omega".

2.5. Statistical analyses

At least ten sparks were measured from each cell, and at least 6 cells from each animal. Data from these sparks was averaged for each cell then for each animal. In calculating statistical parameters (mean, s.e.m, etc) the "n" value used was the number of animals. The averaged data from the rats receiving the lard, canola oil and fish oil supplements were compared using a one way ANOVA assuming unequal variance and a P value of 0.05 was taken as significant.

3. Results

The experiments in this study were done at room temperature. This is partly to improve the viability of the cells, but also for practical reasons (it is difficult to arrange a temperature controlled stage on the confocal microscope). It would be expected that calcium sparks would be more brief at physiological temperatures, but that the differences noted between spark types would persist. Part of the difficulty in interpretation of the results stems from the large variability in some of the variables (particularly the frequency of sparks). Since we cannot know the membrane composition of the individual cells that we use for confocal microscopy, it is possible that this variability in frequency is due to variations in membrane phospholipid composition, particularly if the relation between spark frequency and some component of the membrane is particularly steep. The study was limited to some extent by the higher than expected n-3 fatty acids in the cell membranes of all groups, not just the fish oil supplemented group. This is probably due to the the dietary manipulation that we used, ie. supplementation of standard rat chow by gavage with different fats or oils. The standard rat chow may itself have comparitively high (and variable) PUFA content, leading to a less obvious difference in cell membrane composition than we anticipated. In an ideal study a completely fabricated diet would be used for all groups, rather than using supplementation of standard diet to manipulate fatty acid intake, thus ensuring a low PUFA intake in the "lard" group.

Table 2
Cardiac membrane phospholipid composition of atrial cells

Lipid (%)	Lard	Canola oil	Fish oil
16:0	21.5	17.1	17.9
18:0	25.5	26.0	26.9
18:1 (n-9 + n-7)	10.1	11.1	9.7
18:2 (n-6)	7.6	8.8	6.1
20:4 (n-6)	19.3	20.5	17.5
20:5 (n-3_	—	—	1.5
22:5 (n-3)	3.2	3.1	3.5
22:6 (n-3)	8.9	9.7	13.9
Σ	96.1	96.3	97
Lipids by class (%)	**Lard**	**Canola oil**	**Fish oil**
Saturated	48.91	44.9	47.16
Monounsaturated	10.84	11.69	9.74
Polyunsaturated	40.45	43.41	43.1
n-6	27.42	29.72	24.04
n-3	12.64	13.39	18.87

Table 2 shows the membrane phospholipid composition of atrial cell membranes from the three dietary groups. The membrane phospholipid composition of atrial myocytes was significantly altered by the dietary manipulations: the main difference was a change in the total n-3 fatty acid component in cells from animals supplemented with lard (12.64%), compared to those supplemented with fish oil (18.87%). In particular, the ratio of n-6 to n-3 PUFAS was 2.17 in the lard supplemented rats and 1.28 in the fish oil supplemented rats.

Fig. 1 shows typical x-t plots of calcium sparks. To generate these plots, a single horizontal line is scanned repeatedly and sequential scans stacked vertically, resulting in a plot which contains both spatial temporal information. It was noticeable by visual inspection of the line scans that sparks in cells isolated from animals supplemented with lard were larger and longer lasting than those in cells from animals supplemented with fish oil. The summary data for all sparks is shown in Fig. 2. The mean area of sparks was 31.6 ± 6.72 μm^2 in cells from rats receiving lard as a supplement and 10.7 ± 1.06 μm^2 in cells from rats receiving fish oil ($P < 0.05$, n = 6 for canola and lard, n = 5 for fish oil) The mean area for canola supplemented rats was intermediate between these two (17.6 ± 2.83 μm^2: not significantly different from either lard or fish oil). The increase in spark area was primarily due to an increase in duration: 105.4 ± 18.9 ms in lard supplemented vs 43.5 ± 4.7 ms in fish oil supplemented rats (Fig. 2B). The mean frequency of sparks in the different groups were not significantly different (10.6 ± 4.3, 6.0 ± 3.2 and 6.1 ± 2.2 min^{-1} in lard, canola and fish oil respectively), although there was

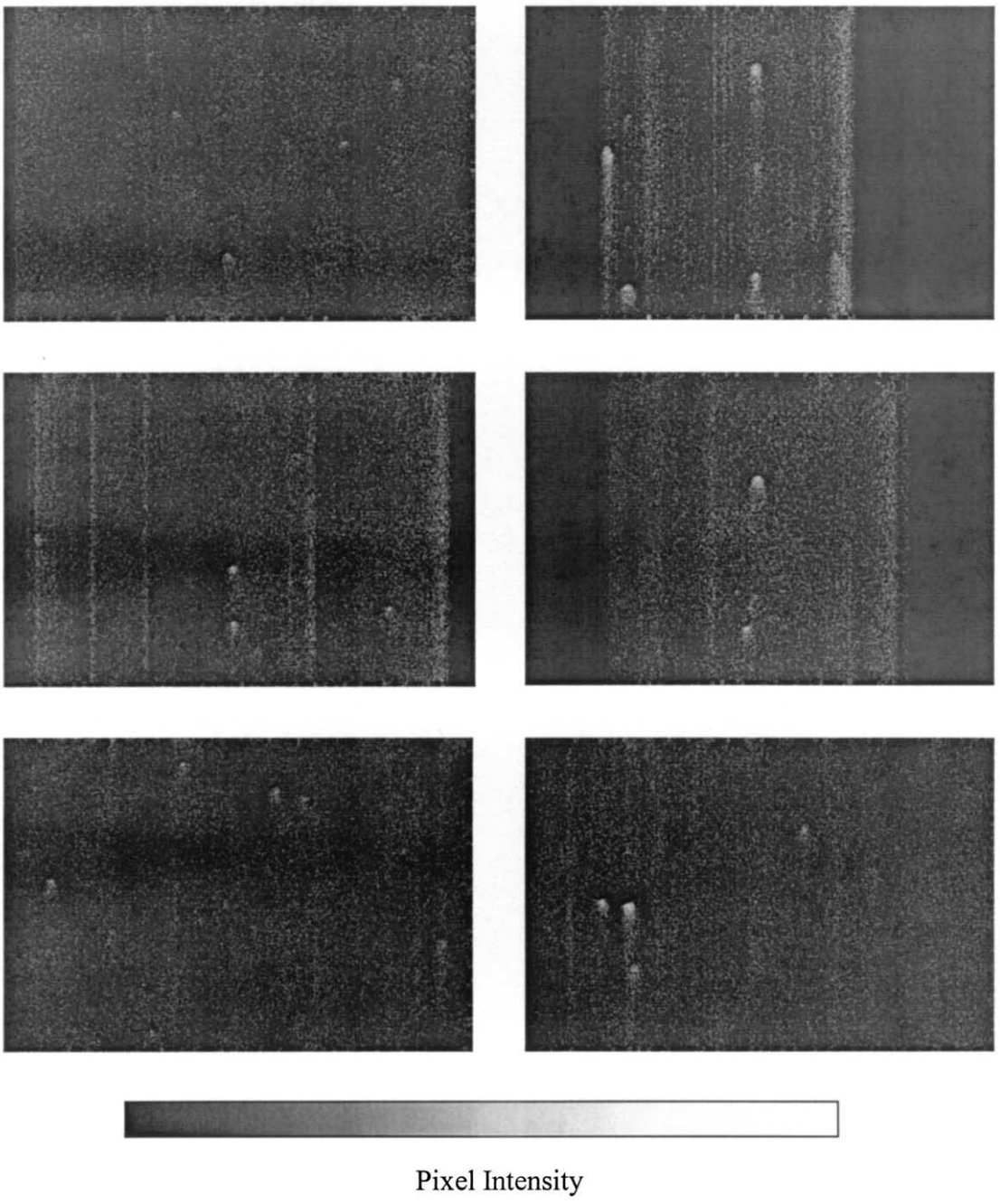

Fig. 1. Typical line scan images of calcium sparks in cells isolated from rats whose diet was supplemented with fish oil (left hand side) or saturated fat (right hand column). Total image width is 96 mm and the height is 1.05 s for all images.

a large cell to cell variability, which led to large error bars (Fig. 3).

As well as measuring duration and area with the threshold crossing algorithm noted in the methods, we also attempted to measure the rate of decay of sparks by fitting a single exponential function to the declining phase. While this approach worked well for many sparks, a large proportion of sparks in all groups could not be fit with an exponential decline, and instead exhibited a range of characteristic decay profiles. In other cases, although the decay phase of the spark could be reasonably well fitted by a single exponential, the rise time was very slow, leading to a long time to peak. The net effect of this great variability in spark profile meant that exponential fits could not be made to many sparks, and that the duration of the sparks (measured by the threshold crossing algorithm) was not well correlated with the rate of decay. In order to gain a better measure of the changes in spark characteristics in the groups, we therefore sub-classified all sparks measured into 3 main types. These are illustrated in Fig. 3. Type 1 is the “idealised”

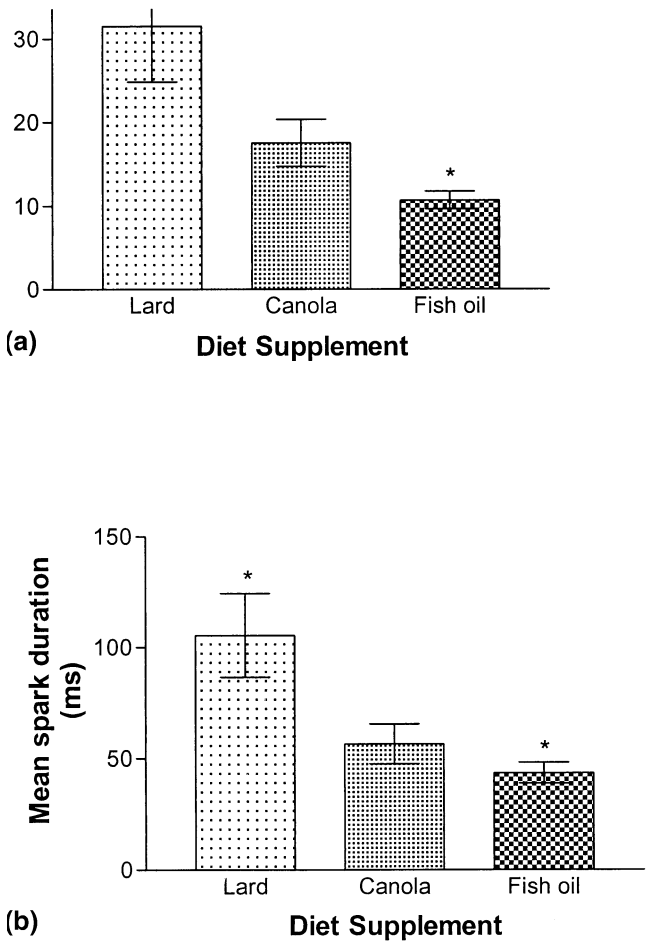

Fig. 2. Panel A: Mean spark area (μm^2 ± SEM) of cells from rats supplemented with lard (n = 6), canola oil (n = 5) and fish oil (n = 5). * Significant at $P < 0.05$. Panel B: Mean duration (ms ± SEM) of cells from rats supplemented with lard (n = 6), canola oil (n = 5) and fish oil (n = 5). * Significant at $P < 0.05$.

spark, with a rapid rise and essentially single exponential decay. Between 63% and 77% of sparks in the various groups were of this type. Other spark types noted were those with a rapid rise but with a marked step in the decay phase (type 2), or a very slow rise, rounded peak and very slow (but apparently exponential) decay. (type 3- note time scale). The proportion of sparks falling into each category was markedly different in the three dietary groups. The bar graph in Fig. 4 summarizes the data for 208 sparks (in cells from 6 rats) in lard supplemented rats, 126 sparks (5 rats) in cells from canola supplemented rats and 174 sparks (5 rats) in fish oil supplemented rats. The proportion of "ideal" sparks (ie rapid rise and exponential fall) was highest in the fish oil group (77% vs 70.6% with canola and 63.4% with lard). The proportion of type 3 (very slow rise and fall) was essentially the same in the three groups. There was a dramatic difference in the proportion of sparks with marked steps/plateaus in the decay phase (type 2 in our classification: 19.7% with lard, 4.8% with canola and 1.8% with fish oil).

4. Discussion

Polyunsaturated fatty acids (PUFAs) have been the subject of intense investigation since it became clear that, when present in the diet, they confer protective effects against cardiovascular disease (for review see Leaf et al. [19]). As noted above, this protective effect extends to an antiarrhythmic action [4,6,20]. Free PUFAs have been shown to exert antiarrhythmic effects, either in single cell models [21,22,23], isolated hearts [20] or in whole-animal models [8,9], and part of this action is undoubtedly due to a decreased electrical excitability as a consequence of block of sarcolemal ionic currents involved in the action potential; I_{Na} [10,11], I_K [12,13] and L-type calcium current [14]. However, many arrhythmias are not due to changes in electrical excitability, but rather are associated with abnormal calcium handling by the sarcoplasmic reticulum. This raises the possibility that part of the antiarrhythmic effects of fatty acids may be mediated through an action on calcium handling. Consistent with this notion, it has recently been shown that free PUFAs can indeed alter calcium handling at the level of the SR in cardiac myocytes [24]. This latter study showed that EPA reduced the rate and amplitude of spontaneous calcium waves in isolated myocytes, an effect which the authors concluded was due to inhibition of the calcium release process. The SR calcium store load was also increased by free EPA, although the underlying mechanism was not clear (ie. increased SERCa2 pump activity or reduced leak).

In principle, calcium sparks should yield complementary information on the calcium release process. In a study of the effects of free fatty acids on calcium handling, Xiao et al [14] reported that free EPA did not affect sparks, which would seem to indicate that the release process itself is not affected.

Whether alteration of membrane phospholipid composition by dietary interventions can affect calcium handling in a way similar to free fatty acids is, at present, not known. There have been reports that the activity of the SR Ca-ATPase in cardiac membranes *in vitro* is either reduced by dietary n-3 PUFAs [17,25] or unchanged [26,27], but effects on other processes, such as calcium release, have not been reported.

The data presented here shows that dietary intervention, and the concomitant change in the n-3 PUFA content of membrane phospholipids, can indeed affect calcium handling in atrial myocytes. We find that sparks in cells with a higher ratio of n-3 to n-6 fatty acids in their membrane phospholipids (ie fish oil supplemented animals), have a smaller area and duration. The mechanism underlying these effects is not clear. The decreased average area (and duration) with fish oil supplementation is likely to be due to a

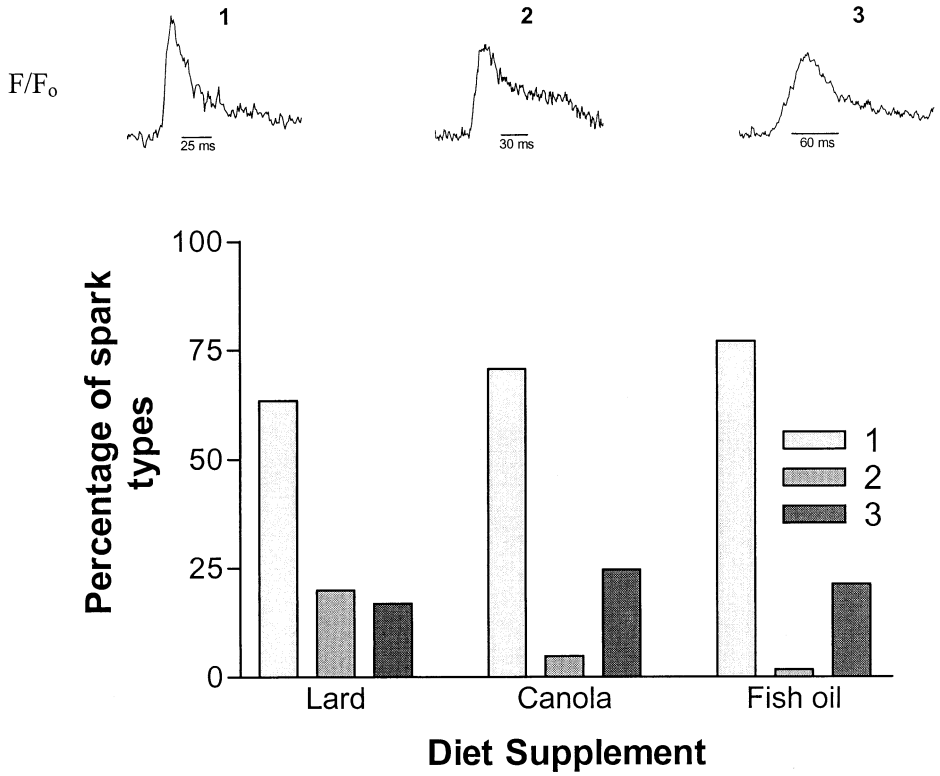

Fig. 3. Upper panels: Plots of sparks (fluorescence increase vs. time) showing examples of sparks fitting the definition of each type; type 1; steep increase followed by a steep, exponential decline (bar = 25 ms), type 2: steep increase and sharp peak, then an initial exponential decrease to a plateau phase lasting tens of ms, then a final decrease to background level (bar = 30 ms), type 3; a gradual increase followed by a prolonged peak then a slow decrease (note time scale, bar = 60 ms). Lower panel: Proportion of spark of types 1–3 from rats supplemented with lard (n = 208 sparks, 6 rats), canola oil (n = 126, 5 rats) and fish oil (n = 174, 5 rats).

reduced calcium flux out of the SR during the spark, although it could also be due to changes in calcium buffering in the cytoplasm (which seems unlikely), or reduced calcium sequestration by the phospholipid membranes themselves (which is possible, but hypothetical). A reduction in calcium flux out of the SR could itself be a consequence of reduced store load, and a consequently lower gradient for calcium. One can deduce differences in store load from the frequency of sparks, since spark frequency has been reported to be related to store load (a greater store load leading to a higher frequency) [28,29,30]. Our data showed no significant difference in spark frequency between the different groups, suggesting that the store load is not likely to be substantially changed in the different groups. The finding that SR store load is not affected by the same sort of dietary treatment as used here has recently been confirmed [31].

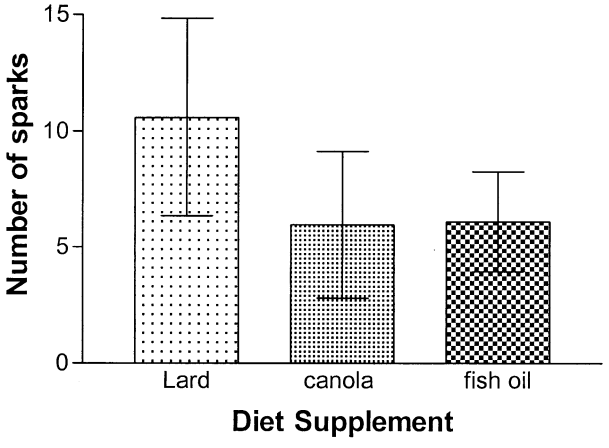

Fig. 4. Mean frequency of sparks in atrial cells from animals in the different dietary groups. Error bars show ± s.e.m. (n = 6 for lard group, n = 5 for canola and fish oil groups, where n= number of rats).

Apart from store load, the other factor influencing calcium flux is the properties of the ryanodine receptors RyR. Either an increase in conductance or in mean open time of the release channels will produce a greater flux of calcium. There is very little data on whether different fatty acids, either as free acids or incorporated into the phospholipid, can alter the properties of RyR channels and, from inspection of sparks, one cannot easily tell whether RyR channel conductance or kinetics is changed. However, the "aberrant" spark decay profiles that we see may give an insight into the behavior of the RyRs. Since the calcium flux required to generate a spark appears to be considerably higher than that flowing through a single RyR under physiological

conditions, sparks are thought to arise from the concerted action of a cluster of RyR channels [32]. Decay of the spark then depends critically on the number of RyRs which open together, and the gain of the CIRC release process:- too many RyR receptors, or too high a gain of CICR, and calcium release will become regenerative. This has been called the "local control" theory of calcium release [33]. Since the major effect of dietary supplementation appears as a change in the proportion of sparks with "aberrant" decay characteristics, we hypothesize that the effect is mediated via a change in this local control, leading to a tendency for sparks to become regenerative.

In conclusion, we show that dietary intervention in the form of supplementation with either saturated or polyunsaturated fats produces a change in cardiac cellular calcium handling, manifest as a change in the morphology of calcium sparks. Sparks from rats fed fish oil had a shorter duration, and a lower incidence of "aberrant" decay profiles. This change in calcium handling may underlie the part of the antiarrhythmic actions of dietary n-3 PUFAs, in that this would make the cells more resistant to the induction of regenerative calcium release.

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