J. Membrane Biol. 196, 95–103 (2003)
DOI: 10.1007/s00232-003-0628-9

The Journal of
Membrane
Biology

Suppression of Calcium Sparks in Rat Ventricular Myocytes and Direct Inhibition of Sheep Cardiac RyR Channels by EPA, DHA and Oleic Acid

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Received: 8 April 2003/ Revised: 10 September 2003

Abstract. The anti-arrhythmic effects of long-chain polyunsaturated fatty acids (PUFAs) may be related to their ability to alter calcium handling in cardiac myocytes. We investigated the effect of eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) on calcium sparks in rat cardiac myocytes and the effects of these PUFAs and the monounsaturated oleic acid on cardiac calcium release channels (RyRs). Visualization of subcellular calcium concentrations in single rat ventricular myocytes showed that intensity of calcium sparks was reduced in the presence of EPA and DHA (15 μM). It was also found that calcium sparks decayed more quickly in the presence of EPA but not DHA. Sarcoplasmic vesicles containing RyRs were prepared from sheep hearts and RyR activity was determined by either [^{3}H]ryanodine binding or by single-channel recording. Bilayers were formed from phosphatidylethanolamine and phosphatidylcholine dissolved in either *n*-decane or *n*-tetradecane. EPA inhibited [^{3}H]ryanodine binding to RyRs in SR vesicles with K_I = 40 μM. Poly- and mono-unsaturated free fatty acids inhibited RyR activity in lipid bilayers. EPA (cytosolic or luminal) inhibited RyRs with K_I = 32 μM and Hill coefficient, n_1 = 3.8. Inhibition was independent of the n-alkane solvent and whether RyRs were activated by ATP or Ca^{2+}. DHA and oleic acid also inhibited RyRs, suggesting that free fatty acids generally inhibit RyRs at micromolar concentrations.

Key words: Ion channels/membrane transport — Calcium cycling/excitation-contraction coupling — Artificial lipid bilayers — Contractile function — Calcium release channels

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Introduction

There is now a convincing body of evidence that a diet high in omega-3 polyunsaturated fatty acids (PUFAs) can confer protection against cardiovascular disease (Kromhout et al., 1985; Burr et al., 1989), protection that extends to an anti-arrhythmic effect and protection of myocytes from the consequences of ischemia (Hock et al., 1990). In confirmation of this, studies in animals have demonstrated that omega-3 PUFAs can exert direct anti-arrhythmic effects, particularly against ischemia-induced arrhythmias (Billman et al., 1994, 1997; Leaf et al., 1999). In studies on isolated myocytes, free PUFAs can block the ionic currents that underlie the cardiac action potential (Honore et al., 1994; Xiao et al., 1995; Bogdanov et al., 1998; Leifert et al., 1999).

However, as an alternative explanation to the block of sarcolemmal ionic currents, many groups have speculated that the antiarrhythmic effect of PUFAs may be related to changes in intracellular calcium handling. Effects on calcium handling may be particularly important in ischemic arrhythmias (Kass et al., 1978). During and following ischemia the sarcoplasmic reticulum (SR) becomes overloaded with calcium, and spontaneous calcium release events occur (Wier et al., 1987; Daniels et al., 1991). The rise in intracellular calcium concentration during these events activates the Na^+-Ca^{2+} exchanger and, since this exchanger is electrogenic, an inward current is produced (Lipp, Mechmann & Pott, 1987; Lipp et al., 1990). If sufficiently large, this current can produce afterdepolarizations and initiate arrhythmias.

One way of ameliorating the damaging alterations in calcium handling that occur in ischemia would be to reduce the calcium influx through the L-type calcium channel, and indeed omega-3 PUFAs have been shown to block this channel (Xiao et al., 1997a). This reduction of calcium influx into the cell,

and hence the amount of calcium available for storage in the SR, would presumably reduce the calcium overload of the SR in ischemia.

In addition, it has been demonstrated that the supplementation of the diet of rats with fish oil reduces the activity of the SERCa2 pump, thereby reducing SR calcium uptake and subsequently the likelihood of SR calcium overload (Croset et al., 1989; Taffet et al., 1993). However, an important action of PUFAs has been suggested: the inhibition of calcium release from the SR (Negretti et al., 2000; O'Neill et al., 2002). Until now, this inhibition of calcium release has been inferred, rather than directly demonstrated, and, somewhat in contradiction, Xiao et al. (1997a) reported, *inter alia*, that eicosapentaenoic acid (EPA) did not affect the properties of calcium sparks, which suggests that calcium release is not reduced by EPA.

Therefore, the aim of this study is to determine if free fatty acids directly inhibit the calcium release channels in the SR membrane. We focus on two PUFAs (EPA and docosahexaenoic acid, DHA) and the monounsaturated oleic acid. We examined their effects on the properties of (1) individual calcium sparks in isolated rat myocytes (single spark properties are believed to reflect the kinetics of the calcium release channels; (Xiao et al., 1997b); (2) single calcium-release channels (RyRs) from sheep heart incorporated into artificial lipid bilayers and (3) the binding of ^{3}[H]ryanodine to RyRs in cardiac SR vesicles. We found that mono- and polyunsaturated fatty acids reduced the open probability of the RyR channel, without affecting its conductance, consistent with a reduction in calcium release from the SR. We also found that EPA but not DHA reduces calcium-spark duration and size.

Materials and Methods

Isolated Rat Cardiac Myocytes

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich Chemicals. Adult rat cardiac myocytes were isolated using enzymatic digestion based on a method published previously (Saint, Ju & Gage, 1992). Briefly, Sprague-Dawley rats were injected with 2000 units of heparin IP and 30 min later anaesthetized with sodium pentobarbitone (1 ml/kg) IP. The heart was removed and perfused with calcium-free Tyrode's solution (in mM: 134 NaCl, 10 N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES), 4 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 11 glucose, adjusted to pH 7.4 with 1.0 M NaOH) containing collagenase (0.1 mg ml^{-1}, Yakult), protease (0.04 mg ml^{-1}) delipidated bovine serum albumin (0.8 mg ml^{-1}) and 2,3-butane-dionemonoxime (BDM) (2.5 mg ml^{-1}) until flaccid. Following digestion, the right ventricle was removed and placed into a Tyrode's solution containing 30 mM BDM and delipidated bovine serum albumin (1.5% w/v) and then gently teased apart to separate the cells. The calcium concentration of the cell suspension was gradually increased to 1 mM in 4 steps over a 40-minute time span. Isolated myocytes were plated onto laminin (50 μg ml^{-1})-coated cover slips and were allowed to settle for 40 minutes prior to use. The cells were isolated at 37°C, and experiments performed at room temperature (23–25°C).

The plated ventricular myocytes were incubated at 37°C (in 95% O_2, 5% CO_2) in either a control culture medium containing 1 mM $CaCl_2$ or in a culture medium containing 1 mM $CaCl_2$ and either 15 μM EPA or DHA for two hours. Fatty acids were added from a stock solution dissolved in 100% ethanol and were stored in nitrogen at −80°C before use. Culture medium contained: Dulbecco's Modified Eagle Medium (DMEM) supplemented with (in mM) 1 $CaCl_2$, 10 HEPES, 25 $NaHCO_3$, 2 carnitine, 5 creatine, 5 taurine and adjusted to pH 7.2 with 1 M NaOH.

Recording of Calcium Sparks

Isolated ventricular myocytes were loaded with 30 μM of the acetoxymethyl (AM) ester of Fluo-3 (Molecular Probes) in conjunction with 2.5 μl ml^{-1} of 10% (w/v) pluronic acid (Molecular Probes) (dissolved in dimethylsulfoxide) for 30 minutes at room temperature (22–25°C). Following this, the dye-containing solution was removed and replaced with Tyrode's solution containing 1 mM $CaCl_2$. The myocytes remained in the dark at room temperature for a further 30 minutes to allow for the de-esterification of Fluo-3 AM. The myocytes were placed in a superfusion bath fitted with platinum field-stimulation electrodes (made in-house) and positioned 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 system in fluorescence mode with excitation at 488 nm and emission at 522 nm set for Fluo-3. The objective lens was a × 40 Nikon water immersion lens with a numerical aperture of 1.15. Images were collected using Bio-Rad CoMOS and MPL software.

Myocytes were stimulated using a Grass SD9 stimulator at 1 Hz for 4 minutes to normalize the SR calcium concentration to a steady state prior to the collection of sparks. Sparks were recorded while cells were bathed in the control Tyrode's solution containing 1 mM $CaCl_2$. All experiments were performed at room temperature (22–25°C).

Analysis of Calcium Sparks

Spark duration and width were measured using the computer program Scion Image (Scion, Frederick, MD) as previously described by Honen & Saint (2002). Sparks were analyzed by setting the threshold level so that the background was completely removed just before the spark. For images with variable background brightness, this was performed separately for each spark. Spark width and duration were measured at the level of threshold. Spark width and duration were determined by the fitting of an ellipse to each event at the level of threshold using a built-in Scion image macro; the minor axis was considered to be width and the major axis, duration.

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 intensity was calculated from the profile of the spark. Peak intensity (F) was determined as a percentage of the background immediately prior to the spark (F_o). 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.

The width, duration and intensity of the calcium sparks were averaged for each cell. At least 10 sparks were recorded per cell and 10 cells were used for each treatment. Control, EPA and DHA data were compared using a one way ANOVA and Tukey's Post Hoc analysis.

[^{3}H]Ryanodine Binding

Cardiac SR vesicles (50 μg/ml) were incubated for 3 h at 37°C with 10 nM [^{3}H]ryanodine in 0.5 ml media containing Complete®

protease inhibitor cocktail (containing 1 mM EDTA; Roche Diagnostics)and (in mM) 250 KCl, 50 HEPES, pH 7.2, 5 ATP, 0.1 $CaCl_2$ (10 nM free Ca^{2+}) or 1 $CaCl_2$ (10 μM free Ca^{2+}) and the required concentration of EPA. EPA was added from ethanol stock solutions to produce a final ethanol concentration of 0.5% in all samples, including controls. Nonspecific binding was determined using a 1000-fold excess of unlabeled ryanodine (Sigma Aldrich). Samples were diluted with 10 volumes of ice-cold wash buffer containing 250 mM KCl, 10 mM TES, pH 7.4 and placed on Whatman GF/C filters soaked with 2% polyethyleneimine. Filters were washed with three 5 ml volumes of ice-cold wash buffer. [^{3}H]ryanodine that was bound to the SR vesicles was determined from the radioactivity remaining on the filters using liquid scintillation counting.

Single RyR Channels in Artificial Bilayers

RyRs from sheep hearts were isolated and reconstituted with lipid bilayers as previously described (Laver et al., 1995). Briefly, sheep hearts were excised from anaesthetized ewes (5% pentobartitone (IV) followed by oxygen/halothane). Cardiac muscle was minced and differentially centrifuged to yield a crude microsomal fraction. SR vesicles were snap-frozen and stored at −70°C. Lipid bilayers were formed from phosphatidylethanolamine and phosphatidylcholine (8:2 wt/wt, Avanti Polar Lipids, Alabaster, AL) in n-decane or n-tetradecane, (50 mg/ml, ICN Biomedicals) across an aperture of 150–250 μm diameter in a Delrin cup. During SR-vesicle incorporation the cis (cytoplasmic) solution contained 250 mM Cs^+ (230 mM $CsCH_3O_3S$, 20 mM CsCl) with 1.0 mM $CaCl_2$, while the trans (luminal) solution contained 50 mM Cs^+ (30 mM $CsCH_3O_3S$, 20 mM $CsCl_2$), and 1 mM $CaCl_2$. The osmotic gradient across the membrane and the Ca^{2+} in the cis solution aided vesicle fusion with the bilayer. The cesium salts were obtained from Aldrich Chemical Company and $CaCl_2$ from BDH Chemicals. Unless otherwise stated, solutions were pH-buffered with 10 mM N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES, ICN Biomedicals) and solutions were titrated to pH 7.4 using CsOH (optical grade, ICN Biomedicals). Free [Ca^{2+}] was buffered with 4.5 mM BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, tetra potassium salt, Molecular Probes) and titrated with $CaCl_2$. Free [Ca^{2+}] was measured using a Ca^{2+} electrode (Fluka). The composition of the cis solution was altered either by addition of aliquots of stock solutions or by local perfusion of the bath. The local perfusion method allowed solution exchange, within 1 s, between twelve available solutions in random sequence (O'Neill et al., 2003). EPA or DHA was added to the bath from a 10 mM stock in ethanol, which was stored under N_2 at −70°C.

Data Acquisition and Analysis

Data acquisition and analysis were carried out as described previously (Laver et al., 2000). Electrical potentials are expressed using standard physiological convention (i.e., cytoplasmic side relative to the luminal side at virtual ground). Single-channel recordings were obtained using a bilayer potential difference of 40 mV. For measurements of unitary current and open probability, the current signal was low-pass filtered at 1 kHz with a Gaussian digital filter. Unitary current and time-averaged currents were measured using Channel2 software (P.W. Gage and M. Smith, Australian National University, Canberra). Open and closed dwell-times were compiled from channel records using 50% threshold detection method.

Results

The Effects of EPA and DHA on Calcium Sparks in Rat Ventricular Myocytes

Treatment of the myocytes with either EPA or DHA (15 μM) significantly reduced the calcium spark intensity in comparison to the control values (Fig. 1). Their intensity was reduced from 105 ± 3% greater than background intensity in the control myocytes to 81 ± 6% greater than background intensity in EPA-treated myocytes and 80 ± 5% greater than background intensity in DHA-treated myocytes (Table 1).

The width of calcium sparks treated with EPA was found to be reduced (2.16 ± 0.07 μm compared to control of 2.96 ± 0.16 μm: $P < 0.05$), as was spark duration (40.3 ± 1.8 ms in comparison to 53.6 ± 3.2 ms in the controls: $P < 0.05$). Sparks from myocytes treated with DHA did not significantly differ in their width (2.88 ± 0.14 μm) or duration (57.9 ± 3.9 ms) compared to the controls (Fig. 2).

The Effects of Fatty Acids on Cardiac Ryanodine Receptors

When single-channel properties of sheep RyR channels were examined in lipid bilayers, it was found that 50 μM of free fatty acids in the baths reduced the open probability (P_o) of the cardiac ryanodine receptor. Figure 3 shows examples of single-channel current traces, illustrating that addition of 50 μM EPA (plus 0.5% ethanol) to either the cytosolic or luminal baths and the addition of 50 μM DHA to the cytosolic bath reduced P_o. In four control experiments, the addition of 0.5% ethanol produced no significant change in P_o, so it is unlikely to contribute to the fatty acid-induced reduction in P_o observed in these experiments.

In the presence of 2 mM ATP and 100 nM Ca^{2+}, EPA (50 μM) in the cytosolic bath reduced the mean P_o from 0.31 ± 0.07 to 0.009 ± 0.008 ($P < 0.05$), while in the luminal bath it reduced P_o from 0.32 ± 0.07 to 0.15 ± 0.04 ($P < 0.05$, Fig. 4*A*). Cytosolic application of 50 μM DHA also reduced the mean P_o from 0.20 ± 0.05 to 0.05 ± 0.01 ($P < 0.05$), while oleic acid reduced P_o from 0.32 ± 0.07 to 0.15 ± 0.04 ($P < 0.05$). This effect occurred within 30 s for both cytosolic additions of EPA and DHA, luminal addition of EPA, and within 5 mins for cytosolic application of oleic acid. The effects of EPA over a range of concentrations show inhibition with a half-inhibiting concentration, $K_I = 32 \pm 2$ μM and a Hill coefficient $n_I = 3.8 \pm 0.7$ (Fig. 4*B*).

The fact that EPA inhibits RyR from both sides of the membrane suggests a site of action within the bilayer itself. If this is the case then it is possible that the nature of the bilayer hydrophobic interior is important in determining the effect of fatty acids on RyR function. Artificial bilayers used for single-channel studies are nearly always made from solutions of lipids with

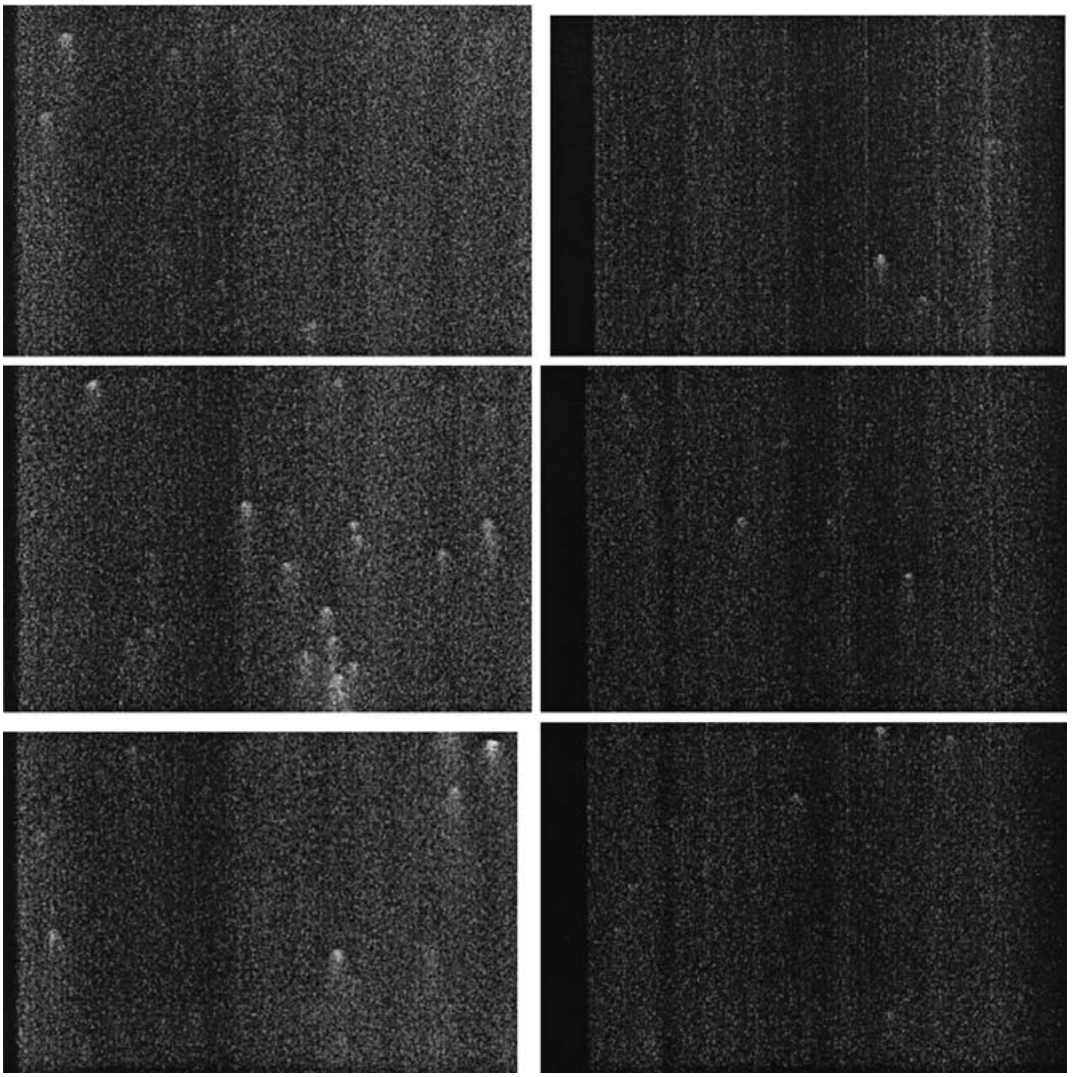

Fig. 1. Representative line-scan images, without background correction, as described in the text, for control myocytes (*left column*) and myocytes incubated in 15 μM EPA (*right column*). Total image width is 96 μm and the height is 1.05 s for all images.

n-decane, which is retained in the lipid bilayer and is not present in biological membranes. Therefore, bilayers were also made here using an *n*-tetradecane solvent because it is retained to a much lesser extent than *n*-decane at room temperature (Coster & Laver, 1986). In bilayers formed from *n*-tetradecane solutions, the addition of EPA to the cytosolic face caused a similar reduction in RyR P_o to that seen in *n*-decane-containing bilayers (Fig. 4*B*). The cytosolic addition of EPA reduced RyR P_o from 0.69 ± 0.08 in the controls to 0.04 ± 0.02 in 50 μM EPA.

To test if EPA inhibits RyRs in the native SR membrane, we used a ^{3}[H]ryanodine binding assay to measure RyR activity. Figure 5 shows that EPA significantly inhibited ^{3}[H]ryanodine binding and presumably the activity of ATP-activated RyRs with half inhibition (K_I) at 40 μM. The effect of EPA on ^{3}[H]ryanodine binding is similar to its effect on RyR open-probability in artificial lipid bilayers shown in Fig. 4.

Table 1. Effect of EPA and DHA on calcium-spark width, duration and intensity

Treatment	Width (μm)	Duration (ms)	Intensity (%)	*n*
Control	2.96 ± 0.16	53.63 ± 3.2	105 ± 3	10
EPA	2.16 ± 0.07	40.30 ± 1.78	81 ± 6	10
DHA	2.88 ± 0.14	57.93 ± 3.94	79 ± 5	10

Mean width, duration and intensity from control myocytes and from myocytes collected in a control solution but treated with EPA or DHA; n = number of myocytes.

Figure 6 shows that inhibition of RyRs by EPA and DHA under a variety of experimental conditions was associated with similar changes in T_c and T_o. The reduction in P_o induced by 50 μM cytosolic EPA and DHA, and luminal EPA, was due to a reduction in the RyR mean open time (T_o) and an increase in the mean closed time (T_c). Under control conditions (100

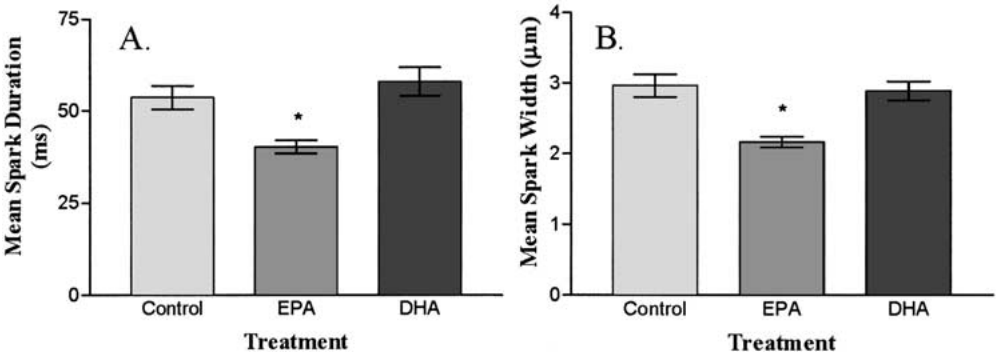

Fig. 2. Effect of EPA on spark properties. Bar graphs show the effect of incubation of the cells in 15 μM EPA or DHA on spark duration (panel *A*), spark width (panel *B*), measured as described in the text. Error bars show ± SEM. * indicates significant difference between control and EPA at $P < 0.05$.

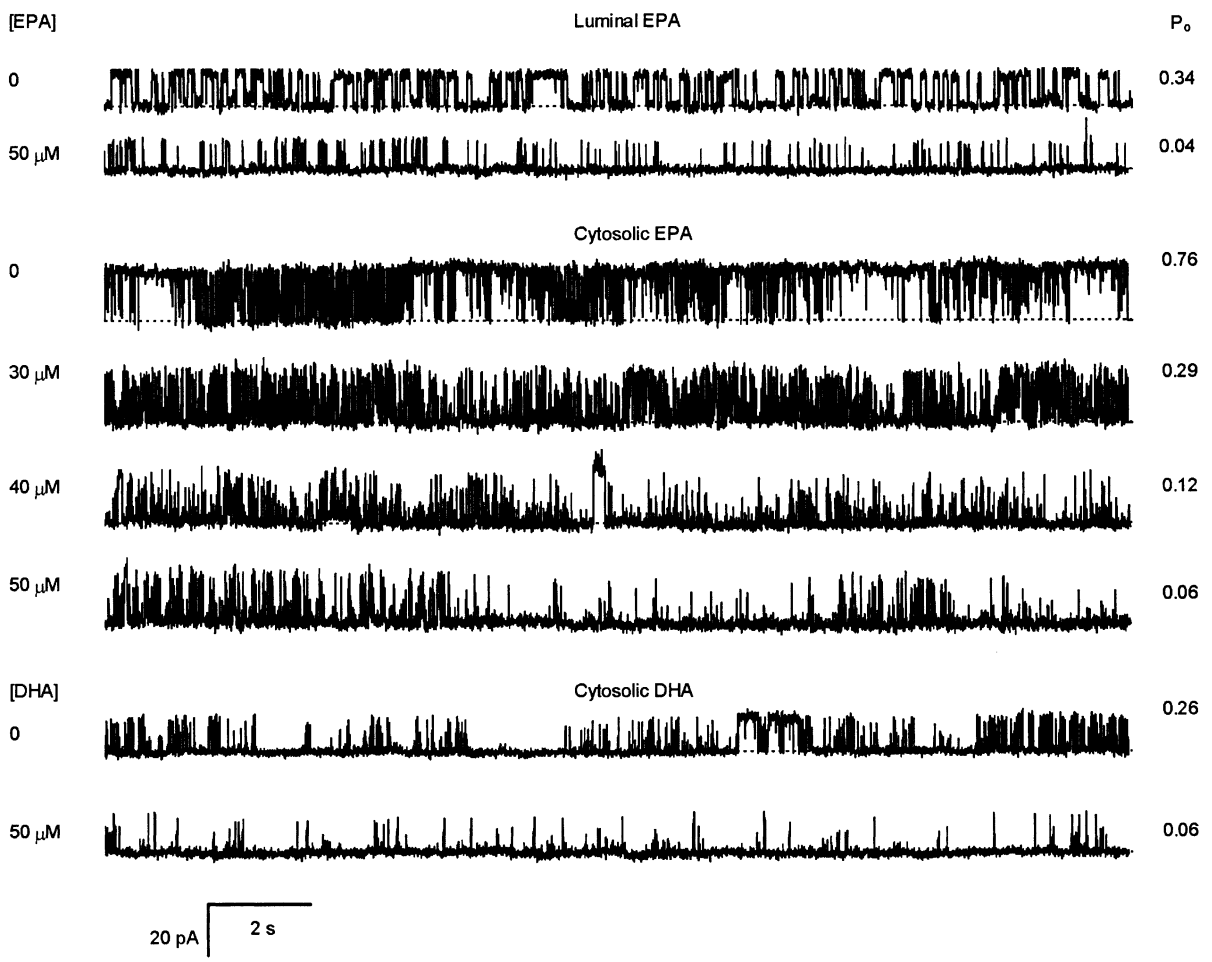

Fig. 3. Representative recordings of cardiac RyRs (membrane potential = +40 mV) before and after the addition of EPA to the luminal or cytosolic baths, and DHA to the cytosolic bath. Channel openings are indicated by upward current transitions from the baseline (*dashed lines*). RyRs were activated by the presence of 100 nM Ca^{2+} and 2 mM ATP. The records shown were taken after 60 s exposure to the EPA or DHA at concentrations shown at the left of each trace. The open-probabilities of the RyRs calculated from these traces are shown at the right.

nM Ca^{2+} plus 2 mM ATP) T_o = 7.8 ± 3.5 ms, T_c = 7.9 ± 1.5 ms (n = 9). RyRs inhibited to ~20% of control activity showed 3-fold increases and decreases in T_c and T_o, respectively.

Cardiac RyRs are generally Ca^{2+}-activated with a $K_a \sim 1$ μM. Here, too, the P_o of the cardiac RyRs depended on cytosolic [Ca^{2+}] in both the absence (K_a = 1.5 ± 1.0 μM) and presence of EPA (K_a = 2.8 ±

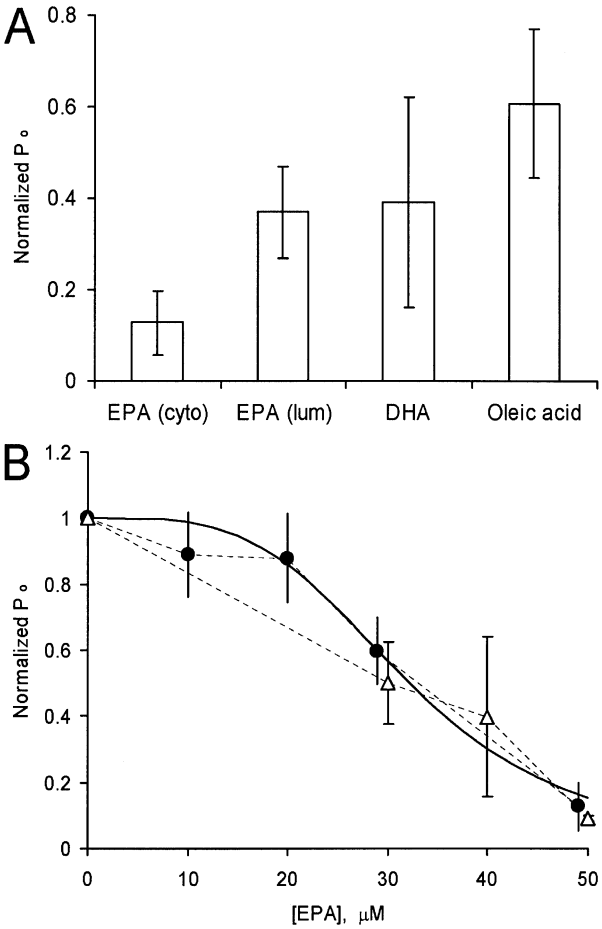

Fig. 4. The effect of free fatty acids on the open-probability (P_o ± SEM) of ATP-activated cardiac RyRs in lipid bilayers. (*A*) P_o with respect to control in the presence of 50 μM EPA (applied to cytoplasmic and luminal sides of the membrane obtained from 5 & 6 experiments, respectively), DHA (8 RyRs from 6 experiments) and oleic acid (16 RyRs from 8 experiments), The cytosolic solution contained 2 mM ATP and 100 nM free Ca^{2+}. (•) Bilayers contained *n*-decane. P_o at 0 EPA was 0.34 ± 0.07 (n = 17). Replicates for 10–50 μM EPA are 5, 5, 9 and 7, respectively. (Δ) Bilayers contained n-tetradecane. P_o at 0 EPA was 0.68 ± 0.08 (3 RyRs from 2 experiments). (*Solid curve*) Hill fit to the cytosolic [EPA] dependencies where K_I = 32 ± 2 μM and n_I = 3.8 ± .0.7. (*B*) The effect of cytoplasmic EPA on RyRs in bilayers containing phosphatidylethanolamine and phosphatidylcholine plus *n*-alkane hydrophobic solvents for bilayer formation.

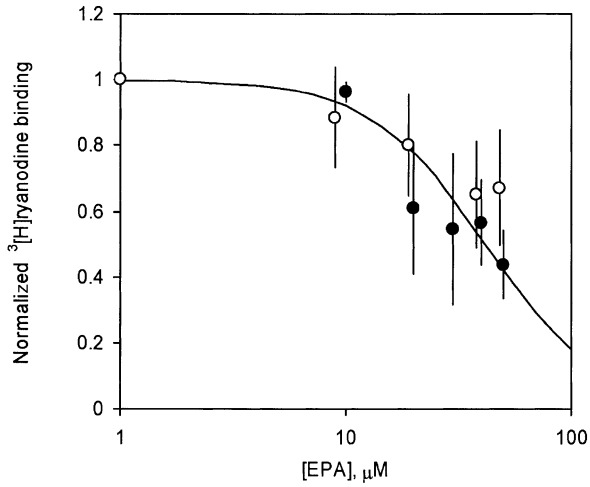

Fig. 5. Effect of EPA on [^{3}H]ryanodine binding to SR vesicles as determined according to Materials and Methods. (O) 10 μM free cytoplasmic [Ca^{2+}], means ± SEM (n = 5). (•) 10 nM free cytoplasmic [Ca^{2+}] plus 5 mM ATP, means ± range (n = 2). Data are normalized to [^{3}H]ryanodine binding under control conditions. The solid line shows a Hill fit to the data with K_I = 40 μM and H_1 = 1.7. Media contained 250 mM KCl, 50 mM HEPES (pH 7.2) 5 mM ATP, 1 mM EDTA, adjusted to a final free [Ca^{2+}] with $CaCl_2$. All experiments were performed in duplicate.

1.6 μM) (Fig. 7). Data were normalized to the P_o of the RyR collected in 150 μM Ca^{2+} between each test concentration. Although EPA had no significant effect on K_a, it did cause a significant reduction in RyR P_o.P_o declined from 0.54 ± 0.05 to 0.36 ± 0.06 within 2 min following the application of 50 μM EPA.

Discussion

A large part of the myocardial cellular damage that occurs in ischaemia is a consequence of calcium overload (Kusuoka et al., 1987). This calcium overload is also arrhythmogenic, since spontaneous calcium-release events can more easily become regenerative and generate propagating calcium waves in the cells (Capogrossi et al., 1987). PUFAs have been shown to be beneficial against these types of ischaemia-induced arrhythmias at the level of the whole heart (McLennan, Abeywardena & Charnock, 1988), and at the cellular level, PUFAs have been shown to reduce the frequency of spontaneous release from the SR (Kang & Leaf, 1996; Negretti et al., 2000). However, it has been difficult to separate the different effects of PUFAs on store load, calcium release and calcium pumping, a difficulty highlighted by Negretti et al. (2000) who note that at least two mechanisms are likely to be responsible for the reduction in spontaneous SR activity: a reduction in resting [Ca^{2+}] and an inhibition of calcium-induced calcium release (CICR). Separating these two effects can be problematical. In principle, calcium sparks should provide such information, since the kinetic properties of individual calcium sparks are independent of SERCa2 activity, sarcolemmal ion currents or pumps, and hence dependent almost exclusively on the properties of the RyR channel (Xiao et al., 1997b). However, information on the effects of PUFAs on calcium spark properties is sparse. Xiao et al. (1997a) reported that calcium sparks were somewhat briefer in the presence of EPA, but this effect was not significant in their experiments. Here, we show that calcium sparks are briefer in the presence of 15 μM EPA but not DHA, suggesting that RyR channel kinetics are altered by EPA. We also show that the application of

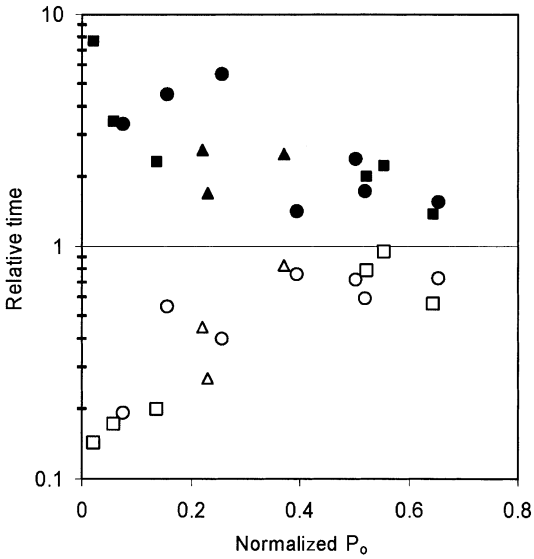

Fig. 6. Effect of EPA (30–50 μM) inhibition of cardiac RyRs on mean open (*empty symbols*) and closed times (*filled symbols*). Mean times and P_o of inhibited channels are shown normalized to control values. The effects of cytosolic ($n = 5$) and luminal ($n = 4$) EPA and cytosolic DHA are shown by circles, squares and triangles, respectively.

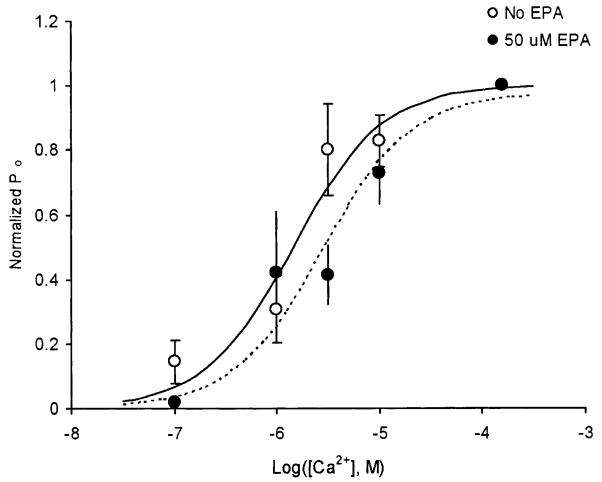

Fig. 7. Calcium dependence of open probability P_o (± SEM, n = 5–9) for cardiac RyRs in the absence of ATP. Data was normalized to control data at 150 μM Ca^{2+} collected between each $[Ca^{2+}]$ tested. P_o of control in the absence of EPA was 0.54 ± 0.05 (n = 28) and with 50 μM EPA it was 0.36 ± 0.06 (n = 23). The curves show Hill fits to the data with $n_a = 1$ and where K_a = (1.5 ± 1.0 μM, *solid curve*) and K_a = (2.8 ± 1.6 μM, *dashed curve*).

both EPA and DHA reduce the intensity of calcium sparks. indicating that less calcium is released over a given area. However, these observations could still be confounded by the interplay of changes in RyR channel properties and SR store load. An unequivocal answer, therefore, lies in the direct examination of RyR channel properties.

Mechanism of Fatty-Acid Inhibition

We show here that oleic acid, EPA and DHA produce a concentration-dependent reduction in RyR-channel open-probability with no discernable change in the channel conductance. The fall in open-probability is due to both a fall in mean open time and a rise in mean closed time. Arachidonic acid has also been shown to inhibit RyRs in SR membrane, using the ryanodine binding assay (Uehara et al., 1996). Taken together, these results indicate that free fatty acids (> 10 μM) have a general inhibitory effect on RyRs. This is in marked contrast to the case of IP_3-sensitive calcium-release channels (Striggow & Ehrlich 1997), which could be inhibited by 100 nM arachidonic acid but not by 100 μM oleic acid.

The inhibitory effect of fatty acid is not peculiar to the artificial bilayer model used here, since the ryanodine binding assay also shows that fatty acids inhibit RyRs in their native SR membranes. The fact that EPA had the same effects on RyRs in bilayers made from *n*-decane and *n*-tetradecane solutions suggests that *n*-alkane content of the bilayer is not an important factor in the mechanism of fatty-acid inhibition (these alkanes have very different solubilities in lipid bilayers (Coster & Laver 1986)).

The reduction in P_o is not associated with a decrease in pH due to fatty-acid ionization. Addition of 50 μM EPA reduced pH of the bath solutions from 7.4 to 7.34, which, in itself, does not significantly inhibit RyRs (Laver et al., 2000). The effects of EPA seemed to be nonspecific since it equally inhibited RyRs activated by ATP at 100 nM $[Ca^{2+}]$ or by Ca^{2+} alone and had no effect on the K_a for Ca^{2+} activation.

The fact that EPA has similar effects when added from either side of the membrane suggests that its site of action is within the bilayer. The fatty acids might bind directly with the channel within the bilayer, as is thought to be the case for local anesthetics (Hille, 1977). Alternatively, fatty acids might modulate RyR activity via changes in the bilayers' physical properties, such as membrane fluidity. Previously it has been demonstrated that the application of EPA increases fluidity of cell membranes (Leifert, McMurchie & Saint, 2001). This is probably not the inhibitory mechanism for the sarcolemmal ion channels where the inhibitory fatty-acid concentrations are too low to affect membrane fluidity (~1% mole fraction in the membrane; Pound, Kang & Leaf, 2001). However, fatty acids inhibit RyRs at higher concentrations where they could be acting via membrane fluidity change (~10%, estimated from K_I = 30 μM and the partition coefficient for arachidonic acid in blood cell membranes determined by Pound et al., 2001).

Physiological Importance of Fatty Acid-induced RyR Inhibition

Although it has been suggested that a reduction in the gain of CICR may be an important facet of the effects of PUFAs on calcium handling, it has been difficult to clearly demonstrate this. Here we have demonstrated direct inhibition of calcium release channels by fatty acids. However, this effect occurs at concentrations (~30 μM) 30-fold higher than those used to block the sarcolemmal ion currents in whole-cell acute studies (Xiao et al., 1995). This raises the question of whether RyR inhibition provides an additional protective mechanism in vivo. Negretti et al. (2000) argue that addition of 10–30 μM produces fatty-acid levels in membranes similar to those attained in vivo. This was based on experiments by Connor, Lin & Colvis (1996) in which rabbits released fatty acids into their blood serum following hormone-induced lipolysis. The upper end of this range coincides with the fatty-acid concentrations in our bathing media where RyRs showed significant inhibition. Thus it is possible that free fatty-acid levels during an ischemic event in the heart are high enough to inhibit RyR activity.

Even within this study, the effects of EPA on sparks occurred at half the EPA concentrations at which RyR inhibition was observed. The reasons for this are not clear. Differences could stem from the effects of cell metabolism (*see* below) or the different fatty-acid profiles in different experimental membrane preparations. Intact cells will have higher levels of endogenous PUFAs than artificial bilayers and isolated SR vesicles, so that more PUFAs would need to be added to these preparations to observe a similar channel-inhibitory response.

Although oleic acid, arachidonic acid, EPA and DHA have very different arrhythmic activity (Nair et al., 1997), they all have inhibitory actions on RyRs (*see* above), although oleic acid was less inhibitory than the PUFAs. Therefore, if fatty-acid inhibition of RyRs underlies the anti-arrhythmic properties of dietary PUFAs, then there must be a mechanism that confers a specific benefit to the polyunsaturated carbon chains. Such a mechanism could be the specific release of PUFAs in the membrane by phospholipases (the phospholipase A_2 family) that are upregulated during ischemia (Connor et al., 1996; Capper & Marshall, 2001; Ford, 2002; Strokin, Sergeeva & Reiser, 2003). Therefore, even though free monounsaturated fatty acids could produce inhibition of RyRs, they are not released in response to ischemia because they are not cleaved by phospholipase A_2.

Even among PUFAs, the similar effect of EPA and DHA on RyRs in bilayers does not correlate with their different action on spark properties in intact cells. It might be that EPA and DHA undergo different metabolic pathways within a cellular environment, which is absent in an artificial bilayer system. DHA is more readily incorporated within the phospholipids than EPA when administered through the diet. In addition, DHA can be converted to EPA, but the reverse reaction is not possible (Grimsgaard et al., 1997). It is therefore possible that when incubated with the myocytes, DHA is effectively "mopped up" by the cell to a greater extent than EPA, so that there is insufficient DHA present in the cytosol to alter the calcium sparks.

In summary, we show that cardiac calcium-release channels are directly inhibited by fatty acids. It is interesting to note that while PUFAs reduce RyR activity and protect against arrhythmias, mutations in the human cardiac RyR that cause exercise-induced ventricular arrhythmias cause increased basal channel activity (Jiang et al., 2002). During cardiac ischemia when free fatty-acid concentrations are relatively high, inhibition of RyRs might, indeed, provide a protective mechanism against arrhythmias. It is yet to be determined if fatty acid concentrations in the SR membrane are high enough for this mechanism to be effective in vivo. We also show that RyRs are inhibited by acute addition of fatty acids, whether or not they have an effect on sparks or have a protective affect against arrhythmias. We argue that the specific effects of the different fatty acids in vivo stem from the different ways in which they are handled within the cell.

We thank Dr. Manohar Garg for helpful discussions and Melanie Laver for her assistance with the DHA experiments. Also our thanks to Dr. Angela Dulhunty, Suzy Pace and Joan Stivala for supplying SR Vesicles. This work was supported in part by grants from the National Health and Medical Research Council of Australia (to DAS) and the Australian Research Council (to DRL).

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