Effects of eicosapentaenoic acid on cardiac SR Ca\(^{2+}\)-release and ryanodine receptor function

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Abstract

n-3 polyunsaturated fatty acids (PUFAs) can prevent life-threatening arrhythmias but the mechanisms responsible have not been established. There is strong evidence that part of the antiarrhythmic action of PUFAs is mediated through inhibition of the Ca\(^{2+}\)-release mechanism of the sarcoplasmic reticulum (SR). It has also been shown that PUFAs activate protein kinase A (PKA) and produce effects in the cardiac cell similar to \(\beta\)-adrenergic stimulation. We have investigated whether the inhibitory effect of PUFAs on the Ca\(^{2+}\)-release mechanism is caused by direct inhibition of the SR Ca\(^{2+}\)-release channel/ryanodine receptor (RyR) or requires activation of PKA. Experiments in intact cells under voltage-clamp show that the n-3 PUFA eicosapentaenoic acid (EPA) is able to reduce the frequency of spontaneous waves of Ca\(^{2+}\)-release while increasing SR Ca\(^{2+}\) content even when PKA activity is inhibited with H-89. This suggests that the EPA-induced inhibition of SR Ca\(^{2+}\)-release is not dependent on activation of PKA. Consistent with this, single-channel studies demonstrate that EPA (10–100 \(\mu\)M), but not saturated fatty acids, reduce the open probability (Po) of the cardiac RyR incorporated into phospholipid bilayers. EPA also inhibited the binding of \[^3H\]ryanodine to isolated heavy SR. Our results indicate that direct inhibition of RyR channel gating by PUFAs play an important role in the overall antiarrhythmic properties of these compounds.

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1. Introduction

n-3 PUFAs such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids are known to be antiarrhythmic. They depress surface membrane electrical excitability \([1,2]\) and inhibit spontaneous release of Ca\(^{2+}\) from overloaded cardiac SR \([3]\). These effects become important during myocardial ischaemia when cardiac myocytes are likely to be damaged and become less able to control intracellular Ca\(^{2+}\). During the ensuing Ca\(^{2+}\) overload the SR becomes unstable, producing spontaneous waves of Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (CICR). Such waves can trigger arrhythmias by generating a transient inward current on Na\(^+/Ca^{2+}\) exchange. Thus, during myocardial ischaemia, release of n-3 PUFAs by phospholipase A2 should reduce the risk of cardiac arrhythmias by locally increasing the threshold for an action potential and by reducing the likelihood of spontaneous Ca\(^{2+}\)-release \([4,5]\). Consistent with this, dietary fish oils have been shown to protect against ventricular fibrillation during experimental ischaemia \([1,6]\). Epidemiological evidence also shows that a moderate intake of fish can reduce the risk of sudden cardiac death by up to 50% in subjects who have recovered from a first ischaemic event \([7]\).

The mechanisms by which n-3 PUFAs reduce excitability of cardiac cells and inhibit SR Ca\(^{2+}\)-release are poorly understood. More specifically, it is not known whether n-3 PUFAs interact directly and selectively with certain channels to modify function, whether they change the properties of cell membranes and indirectly modify channel behaviour, or whether they activate intracellular pathways to produce antiarrhythmic effects.
Here we investigate the SR-specific mechanisms behind the antiarrhythmic actions of EPA. We consider two ways EPA could affect spontaneous release of SR Ca\(^{2+}\). We examine whether EPA directly modifies single RyR channel function and whether EPA acts indirectly via activation of intracellular PKA. During myocardial ischaemia, fatty acids are mobilized from membrane phospholipids [4,5] and may influence second messenger pathways. A diet containing n-3 PUFAs raises their proportion in membrane phospholipids [6,7] and, subsequently, more are released during ischaemia [8]. One possible consequence of this would be to increase cAMP levels [9] and stimulate protein kinase A (PKA). Evidence for PKA involvement comes from experiments in rat ventricular myocytes, where EPA has a positive lusitropic effect (increased rate of relaxation of electrically stimulated contraction) that can be prevented by H-89, an inhibitor of PKA [10]. If EPA activates PKA, phosphorylation levels of PKA targets should increase. Many of the targets of PKA influence cellular Ca\(^{2+}\) regulation, including RyR channels, L-type Ca\(^{2+}\) channels and phospholamban. If EPA does stimulate PKA, it is difficult to understand how this action alone could prevent cardiac arrhythmias. Phosphorylation of phospholamban would increase the rate of SR Ca\(^{2+}\) uptake and SR Ca\(^{2+}\) content. Phosphorylation of L-type Ca\(^{2+}\) channels would tend to increase influx of Ca\(^{2+}\) and SR content. Phosphorylation of RyR channels would be expected to increase Po [11–13]. Clearly, if n-3 PUFAs are to be effective in the prevention of fatal ventricular arrhythmias there must be some other effect, most likely at the level of the RyR channel, which can overcome any pro-arrhythmic effects of PKA activation.

In this work we demonstrate that EPA produces a reduction in the Po of isolated RyR channels incorporated into phospholipid bilayers and a decrease in the binding of \(^{3}H\)ryanodine to SR vesicles. Additionally, we show that the inhibitory effects of EPA on the Ca\(^{2+}\)-release mechanism of the intact cell are still present when PKA activity is inhibited by H-89, demonstrating that EPA inhibition of SR Ca\(^{2+}\)-release is independent of PKA activation.

Preliminary results have been published in abstract form: Swan et al. [14,15].

2. Methods

2.1. Voltage-clamp experiments

Myocytes were isolated from rat ventricular muscle using a collagenase and protease technique as previously described [16]. Rats (Charles River, UK) were sacrificed by stunning and cervical dislocation. Care and use of animals were in accordance with the Animals (Scientific Procedures) Act 1986 and conforms to the Guide for the Care of Laboratory Animals published by the US National Institute of Health (NIH Publication no. 85-23, revised 1996). Cells were voltage-clamped with the perforated-patch technique using the switch clamp mode of the Axoclamp 2B voltage-clamp amplifier (Axon Instruments). Pipettes were filled with the following solution (mM): KCH\(_3\)O\(_3\)S, 125; KCl, 12; NaCl, 20; HEPES, 10; MgCl\(_2\), 5, titrated to pH 7.2 with KOH and a final concentration of amphotericin B of 240 \(\mu\)g/ml. With pipette resistance of 3–5 M\(\Omega\), the switching frequency set to 1–1.5 kHz, the gain of the amplifier was increased to the maximum achievable such that each transient inward current and caffeine-induced current caused a deflection of the voltage trace of less than 0.5 and 1.5 mV.
respectively. The initial bathing solution was as follows (mM): NaCl, 135; KCl, 4; HEPES, 10; glucose, 11; MgCl₂, 1, titrated to pH 7.4 with NaOH. Ca²⁺ overload was produced by the combined action of high intracellular [Na] (resulting from the 20 mM level in the pipette solution) and raised external [Ca²⁺]. All solutions contained 6 mM Ca²⁺ plus 5 mM 4-aminopyridine and 0.1 mM BaCl₂ to ensure that caffeine-induced and transient inward currents represent only Na/Ca exchange current. The integral of the transient part of the caffeine-induced current (corrected for sarcolemmal Ca²⁺ ATPase) represents the SR Ca²⁺ content leaving the cell on Na/Ca exchange, indicated by its sensitivity to Ni²⁺ [17]. The total Ca²⁺ extruded is expressed per unit cell volume. In the presence of H-89, bathing Ca²⁺ was raised to 10 mM to ensure the continued presence of spontaneous waves of CICR. H-89 was used at

![Graph](https://academic.oup.com/cardiovascres/article-abstract/60/2/337/338864/339)

Fig. 2. SR Ca²⁺ content is increased by EPA in the presence of H-89. (A) Caffeine-induced Na/Ca exchange currents and their integrals (SR Ca²⁺ content) in (from the left) control, EPA (10 μM), H-89 (10 μM) and EPA (10 μM) + H-89 (10 μM) all from the same cell. EPA clearly increases SR content in control and H-89. The membrane potentials for each treatment are shown above each trace. (B) Summary data from six cells (*p < 0.05, **p < 0.02).
10 μM; this is sufficient to inhibit the inotropic effect of 1 μM isoprenaline [18]. Experiments were performed at 20 °C. External calcium was raised in H-89 to ensure the cell remained in calcium overload; this allowed us to examine the effects of EPA on waves in H-89.

2.2. Single-channel experiments

Heavy SR membrane vesicles were prepared from sheep hearts and incorporated into planar phosphatidylethanolamine bilayers as previously described [19]. Vesicles were incorporated in a fixed orientation such that the cis chamber corresponded to the cytosolic side of the channel and the trans chamber corresponded to the SR lumen. After vesicle fusion, the cis chamber was perfused with 250 mM HEPES, 125 mM Tris, 10 μM free [Ca2+]i, pH 7.2, and the trans chamber was perfused with 250 mM glutamic acid, 10 mM HEPES, titrated to pH 7.2 with Ca(OH)2 (free [Ca2+]i, ~ 50 mM). The trans chamber was held at ground and the cis chamber was held at potentials relative to ground. Experiments were performed at 22 °C. The pH and free [Ca2+]i of the cis recording solutions were measured using a calcium electrode and pH electrode as previously described [19], and were not affected by the fatty acids. Current recordings were filtered at 0.5 kHz (3 dB) with an eight-pole Bessel filter and digitised at 2 kHz. Po values were determined from 3 min of recording and lifetime analysis was performed as previously described [13,19,20].

2.3. [3H]Ryanodine binding

Membrane vesicles were diluted to 50–100 μg protein/ml and incubated at 37 °C in a buffered medium containing KCl (250 mM), PMSF (5 μM), PIPES–KOH (25 mM), pH 7.2, with [3H]ryanodine (5 nM) for 90 min. Following incubation, the medium was diluted with 5 ml of ice-cold buffer and filtered through Whatman GF-B filters. Filters were further washed with 3 × 5 ml aliquots of buffer and counted in 10 ml aqueous counting scintillant the following day. Nonspecific binding was determined from incubations

![Fig. 3. Effect of EPA on the gating of representative cardiac RyR channels. Two channels have incorporated into the bilayer. In the top trace, channels are activated by 1 μM cytosolic Ca2+ plus 1 mM ATP. (Adding 1 mM ATP to the cis solution of 10 μM Ca2+ lowers the free [Ca2+]i to 1 μM.) Sequential addition of increasing concentrations of EPA (10, 20 and 100 μM) reduces the Po of the channels. The open and closed channel levels are indicated by O and C, respectively. The holding potential was 0 mV.](https://academic.oup.com/cardiovascres/article-abstract/60/2/337/338864/22january2019)
to which unlabelled ryanodine (5 μM) had been added. Incubations were performed in triplicate.

Mean ± S.E.M. for \( n \geq 4 \) and mean ± S.D. for \( n = 3 \) are given. Student’s t-test or one-way ANOVA with Dunnett’s post test, were used to assess the difference between mean values.

2.4. Materials

All chemicals were obtained from Sigma-Aldrich (Poole, UK). Stock solutions of fatty acids were made up in ethanol, and stored at 4 ± 1 °C. Ethanol (1%) was the highest final concentration used in our experiments. Control bilayer and \([^{3}\text{H}]\text{ryanodine} \) binding experiments demonstrated that 1% ethanol itself had no effect on RyR channel gating.

3. Results

Previously, we have shown that n-3 PUFAs inhibit the Ca\(^{2+}\)-release mechanism of cardiac SR [3]. If this requires activation of PKA, we would expect H-89, the inhibitor of the kinase, to prevent it. Fig. 1 illustrates a cell in which Ca\(^{2+}\) overload was imposed on a voltage-clamped cell by raising external Ca\(^{2+}\) to 6 mM. This leads to spontaneous waves of CICR that activate the Na/Ca exchanger producing transient inward currents (Fig. 1). As we previously reported, EPA (10 μM) reduces the frequency of these waves and increases their size [3]. The transient inward currents, therefore, are less frequent but of greater amplitude, on average wave frequency fell in EPA to 67 ± 10% of control (\( n = 7; \ p < 0.05 \)). In H-89, external Ca\(^{2+}\) was raised further as waves were often abolished by the PKA inhibitor. This ensured SR Ca\(^{2+}\) overload was maintained, allowing waves of CICR to continue so that we could determine changes in sensitivity of the Ca\(^{2+}\)-release mechanism. When the protocol is repeated in H-89 (5 μM for 5 min), the effect of EPA is the same as before; wave frequency is reduced and amplitude increased.

On average, wave frequency was reduced by EPA in the presence of H-89 to 80 ± 9% of control (\( n = 7; \ p < 0.02 \)). This suggests that EPA is inhibiting SR Ca\(^{2+}\)-release without the need to activate PKA.

To confirm that the reduction in wave frequency is due to inhibition of the Ca\(^{2+}\)-release mechanism, SR Ca\(^{2+}\) content must increase as wave frequency is reduced. The transient inward current amplitude activated by waves is only an indirect measure of SR content. We therefore, measured SR Ca\(^{2+}\) using the integral of the Na/Ca exchange current activated on application of caffeine [17]. In Fig. 2A, four such currents are shown, the integrals of each are shown below each trace. EPA (10 μM) increases SR Ca\(^{2+}\) content from, in this case, 70.4–105.2 μmoles/l cell volume. This

![Fig. 4. Open and closed lifetime distributions and probability density functions (pdfs) for a representative single channel activated by (A) 1 μM cytosolic Ca\(^{2+}\) and 1 mM ATP and (B) following addition of EPA (100 μM). The best fits to the data were obtained by the method of maximum likelihood and the resulting time constants and corresponding percentage areas are shown.](https://academic.oup.com/cardiovascres/article-abstract/60/2/337/338864)
increase of SR Ca$^{2+}$ content was associated with a lower frequency of spontaneous waves of Ca$^{2+}$-release and is evidence that the Ca$^{2+}$-release mechanism is inhibited [21]. In the third trace external Ca$^{2+}$ was 10 mM; however, the caffeine integral indicates that H-89 leads to SR depletion (spontaneous waves of Ca$^{2+}$-release still occurred despite this). In a separate series of experiments (not shown), SR Ca$^{2+}$ content was reduced in H-89 (10$^{-3}$ M) from 73.2 ± 3.4 to 54.5 ± 4.5 μmoles/l cell volume ($n$ = 8, $p$ < 0.02). However, Fig. 2A shows that application of EPA still leads to significantly increased SR Ca$^{2+}$ content. Thus, the inhibitory effect of EPA on the Ca$^{2+}$-release mechanism is not dependent on the activity of PKA. These data are summarised in Fig. 2B.

The above results strongly suggest that EPA alters SR Ca$^{2+}$-release by modulating RyR function. Additionally, the use of H-89 indicates that EPA does not alter RyR Po via PKA. We therefore investigated whether EPA could directly modulate cardiac RyR. Fig. 3 illustrates typical effects of EPA on sheep cardiac RyR incorporated into a planar phospholipid bilayer. EPA caused no significant change in single-channel conductance (not shown) but caused a reduction in Po. Channels were initially activated by 1 mM ATP and 1 μM free cytosolic Ca$^{2+}$ (top) and exhibited gating characterised by brief open and closed events and high Po values (0.61 ± 0.05; S.E.M.; $n$ = 4). After addition of 10, 20 and 100 μM EPA to the cytosolic channel side, Po was 0.51 ± 0.10, 0.38 ± 0.08 and 0.19 ± 0.03 ($p$ < 0.01), respectively (S.E.M.; $n$ = 4).

Channels activated by ATP (1 mM) and Ca$^{2+}$ (1 μM) displayed short mean closed lifetimes (2.26 ± 1.05 ms) and slightly longer mean open times (7.02 ± 6.17 ms; S.D.; $n$ = 3). Addition of 10, 20 and 100 μM EPA increased mean closed lifetimes to 27.54 ± 44.12, 72 ± 105 and 118 ± 122 ms (S.D.; $n$ = 3), respectively, and mean open lifetimes were reduced to 4.31 ± 6.35, 1.94 ± 1.80 and 0.51 ± 0.04 ms, respectively. In the presence of ATP (Fig. 4A), Po is high and the open and closed lifetime distributions are best described by three open and two closed exponentials, indicating at least three open and two closed states. EPA profoundly reduces open lifetime duration (panel B); the third long open state is abolished and virtually all events occur to the shortest open state. The figure demonstrates that EPA also shifts closed states towards longer events.

To investigate if the reduction in RyR Po observed with EPA is related to the degree of saturation of this PUFA, we investigated if the saturated fatty acid, stearic acid, could produce similar effects. Using the same conditions as in

Fig. 5. Comparison of the effects of EPA and stearic acid on cardiac RyR open probability (Po). ANOVA and Dunnett’s post test were used to determine a statistical difference between control and test. The histogram shows the mean values ± S.E.M. for $n$ = 4 (*$p$ < 0.05).
Fig. 3, we found stearic acid did not reduce Po at concentrations up to 100 μM. Fig. 5 compares the effects of EPA and stearic acid on the Po of RyR channels and demonstrates that stearic acid had no significant effects at the concentrations used.

As the single-channel experiments involve reconstitution into an artificial membrane, we wanted to investigate how EPA modified the function of RyR channels in their native SR membranes as EPA will undoubtedly enter the membrane. We therefore examined if EPA could affect \(^{[3]H}\)ryanodine binding to heavy SR vesicles. Ryanodine binds only to open RyR channels and therefore can be used as an index of the Po of RyR channels in the SR vesicles. In the presence of 100 μM Ca\(^{2+}\), EPA reduced \(^{[3]H}\)ryanodine binding to SR membrane vesicles (Fig. 6A).

Although a change in cytosolic [Ca\(^{2+}\)] is the primary physiological mechanism controlling cardiac RyR channel gating, other endogenous ligands play a regulatory role. ATP and Mg\(^{2+}\) modulate RyR channel activity and, together with the cytosolic and luminal [Ca\(^{2+}\)], ensure that the channels are closed at resting intracellular [Ca\(^{2+}\)] and open when intracellular [Ca\(^{2+}\)] increases. We therefore investigated if EPA could reduce RyR channel Po in their native SR membranes in the presence of physiological levels of Mg\(^{2+}\) and ATP. Fig. 6B illustrates that 10 μM EPA, the concentration that suppresses spontaneous waves of Ca\(^{2+}\)-release in intact myocytes, also significantly reduces \(^{[3]H}\)ryanodine binding to heavy SR vesicles in the presence of 0.5 mM free Mg\(^{2+}\) and 5 mM ATP.

The \(^{[3]H}\)ryanodine binding data together with the single-channel results, demonstrate that EPA can reduce RyR channel Po under a variety of experimental conditions and that this could explain the results shown in Figs. 1 and 2. EPA is effective even in the presence of H-89, indicating that EPA does not require activation of PKA. Interestingly, H-89 itself alters SR Ca\(^{2+}\)-release and reduces SR Ca\(^{2+}\) content. As H-89 acts by blocking the ATP binding site of PKA, it is possible that H-89 could also bind to either the ATP or caffeine binding sites on RyR. Fig. 7A demonstrates that H-89 is an effective activator of the cardiac RyR. Po increased from 0.15 ± 0.03 in 10 μM cytosolic Ca\(^{2+}\) to 0.20 ± 0.05, 0.38 ± 0.09 and 0.57 ± 0.14 (S.E.M.; n = 4) in 5, 10 and 50 μM H-89. No change in single channel conductance was observed. Fig. 7B demonstrates that in channels where Po has been significantly increased by H-89, 10 μM EPA can still produce a significant reduction in Po.

4. Discussion

n-3 PUFAs are known to be antiarrhythmic. It is thought that, in cases where the diet is sufficiently rich in n-3 PUFAs, they are released from membranes by phospholipase A2 during ischaemia [4,5]. This provides protection against cardiac arrhythmias in animal models [1,6] and may also be responsible for the protection provided in patients following myocardial ischaemia [7]. The antiarrhythmic effects of n-3 PUFAs are mediated, at least in part, by inhibition of the SR Ca\(^{2+}\)-release mechanism [3]. In the rat cardiac myocyte, EPA reduces the frequency of calcium waves and increases wave amplitude (Fig. 1). As these waves are potentially arrhythmogenic, a lower frequency should be beneficial. These effects are associated with an increase in the SR Ca\(^{2+}\) content (Fig. 2) as demonstrated by application of caffeine. Previous publications have provided strong evidence that the buildup of Ca\(^{2+}\) in the SR is caused by an inhibitory effect of EPA on SR Ca\(^{2+}\)-release [3,22]. The regulatory factors which lead to RyR channel opening and the initiation of a Ca\(^{2+}\) wave are suppressed and a higher SR Ca\(^{2+}\) content is required before wave propagation can take place. EPA could produce such an inhibitory effect on SR Ca\(^{2+}\)-release by more than one mechanism; EPA could activate intracellular enzyme pathways and indirectly modify the gating of RyR channels or it could interact more locally with the RyR channel complex itself to cause a decrease in Po. One possible mechanism that may be involved is activation of PKA. Although evidence exists
that EPA can inhibit PKA, it also increases cAMP levels [9,23]. The net result of this on PKA activity is unclear, however, in rat cardiac myocytes EPA leads to faster relaxation of electrically stimulated contraction (positive lusitropic effect); this effect is prevented by H-89, consistent with activation of PKA [10].

In cardiac cells, activation of PKA leads to phosphorylation of the RyR [24] and this would be expected to lead to an increase in Po [12,25,26]. In the present study, we aimed to block any changes in phosphorylation levels that could result from stimulation of PKA by EPA. This was done by applying the PKA inhibitor, H-89 to the cells prior to administration of EPA. We demonstrated that, in spite of evidence that EPA leads to activation of PKA, the ability of EPA to reduce the frequency of spontaneous Ca\(^{2+}\) waves and increase SR content is not reduced by inhibiting PKA activation and therefore is unlikely to depend on PKA-dependent phosphorylation of RyR. In fact we demonstrate that EPA reduces the Po of RyR channels.

EPA reduces RyR Po when channels are incorporated into planar lipid bilayers but is equally effective when channels remain in their native membranes as evidenced from the \[^{3}H\]ryanodine binding studies (Fig. 6). Furthermore, EPA appears to be an effective inhibitor of RyR channel opening irrespective of the ligands controlling channel gating. This is verified by the fact that EPA reduces Po when cytosolic Ca\(^{2+}\) is the sole activator of the channels (Fig. 5), when cytosolic Ca\(^{2+}\) plus ATP activate the channels (Fig. 3) or when channels are activated by Ca\(^{2+}\) plus ATP in the presence of Mg\(^{2+}\) (Fig. 6). Thus, higher levels of channel activators (or lower concentrations of inhibitors) are required to maintain a high Po. In effect, it may be that we observe a reduction in the sensitivity of the channel to cytosolic Ca\(^{2+}\). Importantly, the inhibitory action of EPA is most easily demonstrated in the conditions most similar to

Fig. 7. (A) Direct effects of H-89 on the gating of a typical cardiac RyR channel incorporated into a bilayer. In the top trace the channel was activated by 10 \(\mu\)M cytosolic Ca\(^{2+}\) alone. Sequential additions of H-89 to the concentrations indicated were made to the cytosolic chamber. The holding potential was 0 mV. The closed and open channel levels are indicated by C and O, respectively. (B) Histogram illustrating the effect of EPA on channels activated by H-89. Control channels were activated by 10 \(\mu\)M cytosolic Ca\(^{2+}\) alone. Subsequent additions of H-89 (10 \(\mu\)M) and EPA (10 \(\mu\)M) activated and inhibited channel activity, respectively. The error bars show S.E.M. for \(n=4\) (*\(p<0.05\)).
those in the cell, i.e. in their native membranes, activated by both ATP and Ca\(^{2+}\) and in the presence of Mg\(^{2+}\).

The effects of EPA on RyR Po are not observed with all fatty acids. The saturated fatty acid, stearic acid, has no effect over the range of effective concentration of EPA (Fig. 3) and therefore the degree of saturation does appear to be important. The n-6 PUFA, arachidonic acid, has also been shown to inhibit \[^{3}H\]ryanodine binding to SR [27,28], but the significance of this result is uncertain because Uehara et al. [28] found no effect on the gating of RyR in bilayers. They concluded that arachidonic acid did not reduce RyR Po but affected the binding of ryanodine. As yet, no study has clearly demonstrated for n-6 PUFA the inhibitory effect on CICR that we have shown here and previously [3] for n-3 PUFA. The powerful antiarrhythmic effects in clinical and animal studies of dietary n-3 PUFA could be due to a stronger inhibition of RyR by these PUFA compared with n-6 PUFA or may relate to effects at other sites in the cell, e.g. the surface membrane.

Myocardial ischaemia triggers hydrolysis of membrane phospholipids and release of non-esterified fatty acids into the cell. Incorporation of a higher proportion of n-3 PUFA into cell membrane phospholipids will ensure a higher level of release following myocardial ischaemia. A recent publication investigating the link between PUFA content of the plasma membrane and ion channel activity [29] suggested PUFA concentrations required for antiarrhythmic action are too low to produce a significant change in the overall arrangement of the phospholipids within cardiac membranes. They suggest therefore that n-3 PUFA exert antiarrhythmic effects by direct interactions with sarcolemmal ion channels rather than indirectly by perturbing membrane phospholipid packing. As low concentrations of EPA inhibit RyR Po, it seems likely that a direct effect of the free cytosolic EPA on RyR or a closely associated protein is the cause. The inhibitory effects of EPA on RyR channel gating can be reversed by perfusing the EPA out of the solutions on the cytosolic side of the bilayer (results not shown), thus an irreversible change in RyR channel gating is not induced by EPA. This is to be expected if changes in gating of single RyR channels cause the inhibitory effects of EPA on the SR in cardiac cells as these effects are also reversible. Additionally, it is unlikely that EPA causes dissociation of an accessory protein that controls channel gating, as this too would be washed away.

A complicating factor is that the inhibitor of PKA, H-89, also can activate RyR (Fig. 7) but it has also been shown to inhibit SERCA in cardiac cells [18]. The combination of these two effects may help explain the lower SR Ca\(^{2+}\) content in H-89 and also that the waves of CICR propagate at this lower SR content. Increased Po of RyR and reduced SR Ca\(^{2+}\) uptake rate would both favour wave propagation. In the cell, inhibition of PKA might be expected to lower RyR Po if the channel was already phosphorylated and the action of phosphatases was not counteracted. It is likely, however, that the direct effects of H-89 on the gating of RyR have overridden any indirect effects caused by inhibiting PKA. It is not entirely surprising that H-89 can activate RyR as it inhibits PKA by binding to the ATP site of PKA. Numerous ligands for cell surface purinoceptors activate RyR channels by binding to at least three distinct sets of binding sites on RyR; the ATP, suramin and caffeine binding sites [30,31]. It seems unlikely that H-89 is binding to suramin sites on the channel as we would expect the increase in Po to be accompanied by an increase in conductance [31]. It is therefore likely that H-89 increases Po via either the caffeine or ATP sites. These effects of H-89 do not prevent us from determining the effect of EPA. We have ensured that waves of CICR continue in H-89 by raising external Ca\(^{2+}\) to compensate for the depletion of the SR. This enables us to measure the change in the “threshold” SR content that will support propagation of a wave and indicate inhibition of the Ca\(^{2+}\)-release mechanism.

The antiarrhythmic actions of n-3 PUFA are mediated by at least two mechanisms: reduced surface membrane electrical excitability [1,2] and inhibition of the Ca\(^{2+}\)-release mechanism of the SR [3]. The latter mechanism is responsible for reducing the frequency of possible triggers for arrhythmic action potentials as each wave of Ca\(^{2+}\)-release activates Na/Ca exchange and depolarises the cell membrane. Although individual waves are larger in EPA, this may be less important than lower frequency if more than one cell has to produce a wave simultaneously to generate an action potential. The present work shows that the n-3 PUFA, EPA, exerts part of its antiarrhythmic action by reducing RyR Po and does not need to act as a second messenger or enter a metabolic pathway inside the cell. Specifically, we have shown that activation of PKA is not required for the effects of EPA on the SR even though evidence exists that n-3 PUFA lead to increased PKA activity [10]. We have demonstrated that any pro-arrhythmic action of EPA that involves activation of PKA can be counteracted by the direct effects of EPA on RyR which lead to inhibition of the Ca\(^{2+}\)-release mechanism.

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