Inhibition of sarcoplasmic reticulum function by polyunsaturated fatty acids in intact, isolated myocytes from rat ventricular muscle

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1. We have studied the effects of two polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on spontaneous and electrically stimulated contractions in single, isolated ventricular myocytes from rat hearts.

2. The frequency of spontaneous waves of calcium release and contraction (induced by elevation of the bathing calcium concentration) is reduced in the presence of EPA. At the same time the resting level of intracellular calcium falls, the resting cell length increases and the amplitude of shortening decreases. All these effects are reversed on removal of EPA.

3. Imaging of the waves of calcium release shows that the amplitude and the rate of propagation of the wave is increased in EPA. Consistent with the increased amplitude, integration of the caffeine-induced Na^+–Ca^{2+} exchange current (a measure of the sarcoplasmic reticulum (SR) calcium content) is increased by both EPA and DHA.

4. EPA has a maintained negative inotropic effect on voltage clamped myocytes. This seems to be entirely due to inhibition of the L-type calcium current. Smaller depolarising pulses in control conditions that elicit the same calcium current as in EPA also activate the same level of contraction. This is in spite of the increased SR calcium content in EPA.

5. It is concluded that PUFAs have two effects on the SR; they reduce the availability of calcium for uptake and they inhibit the release mechanism. Both of these effects should lower the frequency of spontaneous waves of calcium release. As spontaneous release of calcium can initiate arrhythmias, some of the anti-arrhythmic action of PUFAs must be exerted at the level of the SR.

Polyunsaturated fatty acids (PUFAs) present in fish oils are known to have protective effects against arrhythmias generated post-infarction and when present in the diet, seem to protect against heart disease in general (for review see Leaf *et al.* 1999). Much work has been carried out on the cardiac effects of PUFAs and how protection is effected. PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) inhibit sodium and L-type calcium currents in cardiac myocytes (Xiao *et al.* 1997; Macleod *et al.* 1998). It has also been shown that the delayed rectifier (Honore *et al.* 1994) and transient outward currents (Macleod *et al.* 1998) are inhibited. The net effect of these changes in surface membrane currents is depressed electrical excitability (Kang *et al.* 1995), clearly this will help to reduce the occurrence of arrhythmias and can explain the protective effect. An important cause of cardiac arrhythmias is abnormal functioning of the sarcoplasmic reticulum (SR). Thus far, however, relatively little work has examined possible effects of PUFAs on cardiac sarcoplasmic reticulum function.

Post-ischaemia damaged cardiac myocytes undergo delayed afterdepolarisations that may be large enough to generate an arrhythmogenic action potential (Stern *et al.* 1988). The cause of the depolarisation is spontaneous release of calcium from the sarcoplasmic reticulum (Kass *et al.* 1978). Following ischaemic damage, cellular calcium regulation is compromised and the SR becomes overloaded with calcium and prone to spontaneous release events. This spontaneous release takes the form of propagating waves of calcium-induced calcium release (CICR) and has been demonstrated both in single, isolated cardiac myocytes and in multicellular preparations (Wier *et al.* 1987; Daniels *et al.* 1991). The raised intracellular calcium concentration during propagating waves activates the Na^+–Ca^{2+} exchanger and an inward, depolarising current is generated (Lipp *et al.* 1987, 1990). If large enough, the waves can generate sufficient inward current and depolarisation to initiate an action potential. Although depression of surface membrane excitability may make it more difficult for the wave to cause an action potential, it is

less likely to be of great importance to generation of waves. It is known, however, that modulation of SR function can affect the frequency and amplitude of propagating waves; both important determinants of the likelihood of generating arrhythmias e.g. inhibition of calcium release from the SR leads to less frequent but larger waves (Overend *et al.* 1997). Some evidence that PUFAs affect SR function has recently been reported (Rodrigo *et al.* 1999) in chemically skinned ventricular myocytes. These authors suggested that SR calcium release is inhibited by PUFAs but could not rule out inhibition of calcium uptake.

The purpose of the present study was to determine what, if any, effect PUFAs have on arrhythmogenic, propagating waves of calcium-induced calcium release in intact, ventricular myocytes and whether this may provide additional protection against arrhythmias. We have studied the actions of EPA on electrically stimulated contractions and spontaneous waves in single cardiac myocytes isolated from rat ventricular muscle. Our results indicate that PUFAs do indeed affect SR function in two ways; through inhibition of calcium release and reduced availability of calcium. Therefore, at least part of the anti-arrhythmic action of PUFAs must be at the level of the SR.

METHODS

Rat myocytes were isolated using a collagenase and protease technique as previously described (Eisner *et al.* 1989). Rats were killed by stunning and cervical dislocation. For intracellular calcium measurements, cells were loaded with the membrane permeant form of either indo-1 or fluo-3, 5 μM for 5 min in each case, 20 min was allowed for de-esterification. Cells were placed in a superperfusion chamber on the stage of an inverted microscope. Indo-1 fluorescence was excited at 360 nm and recorded at 400 nm and 500 nm (O'Neill *et al.* 1990) using epi-fluorescence optics. Fluo-3 fluorescence was excited at 488 nm and measured at 515 nm using the Bio-Rad MRC 1024 confocal microscope. All voltage clamp experiments were carried out using the perforated patch clamp technique (Horn & Marty, 1988) using the switch clamp mode of the Axoclamp 2B amplifier (Axon Instruments). Pipettes were filled with the following solution (mM): KCH_3O_3S, 125; KCl, 20; NaCl, 10; Hepes, 10; $MgCl_2$, 5; titrated to pH 7·2 with KOH and a final concentration of amphotericin B of 240 μg ml^{-1}.

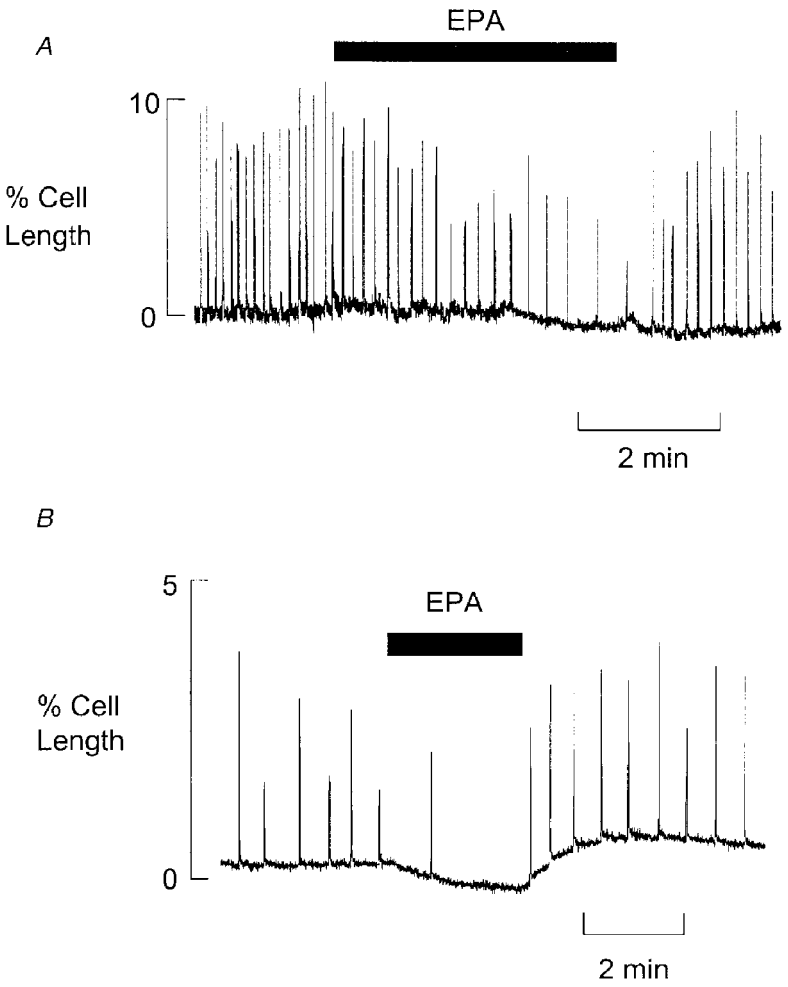

Figure 1. EPA inhibits spontaneous waves of contraction in isolated ventricular myocytes
A and *B*, bathing calcium was 4 mM, to induce spontaneous contractions. EPA (10 μM) was applied as indicated by the bars. On removal of EPA, 2 mg ml^{-1} BSA was added to the bathing solution to assist in removal of EPA.

The bathing solution was as follows (mM): NaCl, 135; KCl, 4; Hepes, 10; glucose, 11; $MgCl_2$, 1; titrated to pH 7·4 with NaOH. Initially cells were bathed in the above solution at 1 mM $CaCl_2$, later this level was altered to between 2 and 8 mM, as indicated in the figure legends, to induce spontaneous waves of calcium release. Eicosapentaenoic acid (EPA) and docosohexaenoic acid (DHA) were prepared in ethanol as 10 mM stock solutions and stored under a nitrogen atmosphere before use. Fresh stock solutions were prepared each week. Fatty acid-free bovine serum albumin (BSA, 2 mg ml^{-1}) was added to the 'recontrol' solution to ensure a rapid and complete removal of fatty acid from the solution (Kang & Leaf, 1994; Kang *et al.* 1995). In voltage clamp experiments the above solution was modified to contain 5 mM 4-aminopyridine (4-AP) and 0·1 mM $BaCl_2$. All experiments were carried out at room temperature (25 °C). Cell length was measured using a video based edge detection system modified from a published circuit (Steadman *et al.* 1988).

The calcium content of the SR was measured from the integral of the caffeine-induced Na^+–Ca^{2+} exchange inward current, as previously reported (Varro *et al.* 1993; Negretti *et al.* 1995). This integral is corrected for non-Na^+–Ca^{2+} exchange efflux and expressed with respect to the volume of the cell.

All statistics quoted are means ± S.E.M., Student's paired *t* tests were used throughout to test significance. All experiments carried out in Venezuela were according to guidelines laid down by the Universidad Central de Venezuela.

RESULTS

It has been clearly demonstrated that PUFAs protect against arrhythmias via reduced electrical excitability of the surface membrane (Kang *et al.* 1995), however, Fig. 1 shows that another mechanism may also be involved. In the records of cell length shown in Fig. 1 a spontaneous wave of contraction is indicated by each upward deflection. In Fig. 1*A* when 10 μM EPA is applied there is a clear reduction in the frequency of these waves. When EPA is removed and BSA added to the bathing medium (BSA binds the fatty acid ensuring rapid and complete removal) the frequency of oscillations returns to the control level, similar effects were observed in 47 of 57 cells studied. As these contractions are not stimulated by action potentials this result suggests the involvement of another mechanism in addition to depressed electrical excitability of the surface membrane. Figure 1 shows two other effects of EPA. In addition to the reduction of wave frequency there is a decrease in the amplitude of waves and an increase of resting cell length, both of which reverse on removal of EPA. On average, wave amplitude decreased by 15·9 ± 3·0% ($n = 41$, $P < 0·001$) and resting cell length increased by 2·0 ± 0·6% ($n = 46$, $P < 0·001$).

We can see in Fig. 2 the probable cause of the increase of resting cell length shown in Fig. 1*B*. The traces shown are

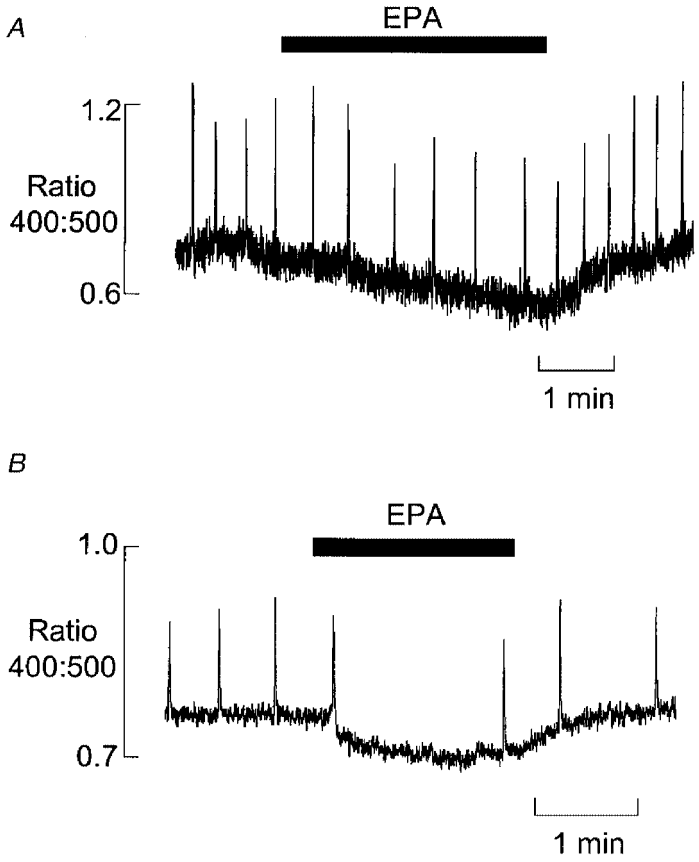

Figure 2. The effect of EPA on $[Ca^{2+}]_i$

A and *B*, the cells were bathed in 8 mM calcium throughout to induce spontaneous activity. In each trace 10 μM EPA was applied as indicated by the bar, recontrol solution contained 2 mg ml^{-1} BSA.

from two different cells loaded with indo-1, each transient increase of the ratio represents the change of $[Ca^{2+}]_i$ during a propagated wave. EPA (10 μM) was applied in each trace as indicated by the bar. Again, we see the frequency of waves is clearly lower in EPA and recovers in the recontrol solution (2 mg ml^{-1} BSA). The range of response is shown in these two examples, in *A* the fall of frequency is clear but moderate, in *B* the frequency is markedly reduced in EPA. It is also clear in both cases that the basal level of $[Ca^{2+}]_i$ falls in EPA, this may allow the relaxation of cell length in Fig. 1*B*. The resting ratio fell from $0{\cdot}62 \pm 0{\cdot}03$ to $0{\cdot}58 \pm 0{\cdot}03$ ($P < 0{\cdot}005$, $n = 46$), this fall was fully reversed on removal of EPA. It may be therefore, that inhibition of oscillations results from the fall of baseline calcium, reducing the availability of calcium to the SR. At lower $[Ca^{2+}]_i$ the rate of SR uptake of calcium will be reduced leading to a lower frequency of spontaneous waves (Díaz *et al.* 1997). Alternatively, inhibition of the SR calcium release mechanism can also lower the frequency of spontaneous release events (Overend *et al.* 1997). These two possibilities will have different effects on the calcium content of the SR. If calcium release is inhibited, we expect an increased SR content; lowered calcium availability will however, either not change, or reduce the SR calcium content (Díaz *et al.* 1997). The following experiments help to distinguish between these two possibilities.

If SR calcium content is increased, we might expect the amplitude of the wave of calcium release to be increased (cf. the effect of tetracaine (Overend *et al.* 1997). In myocytes loaded with fluo-3 we have imaged calcium during

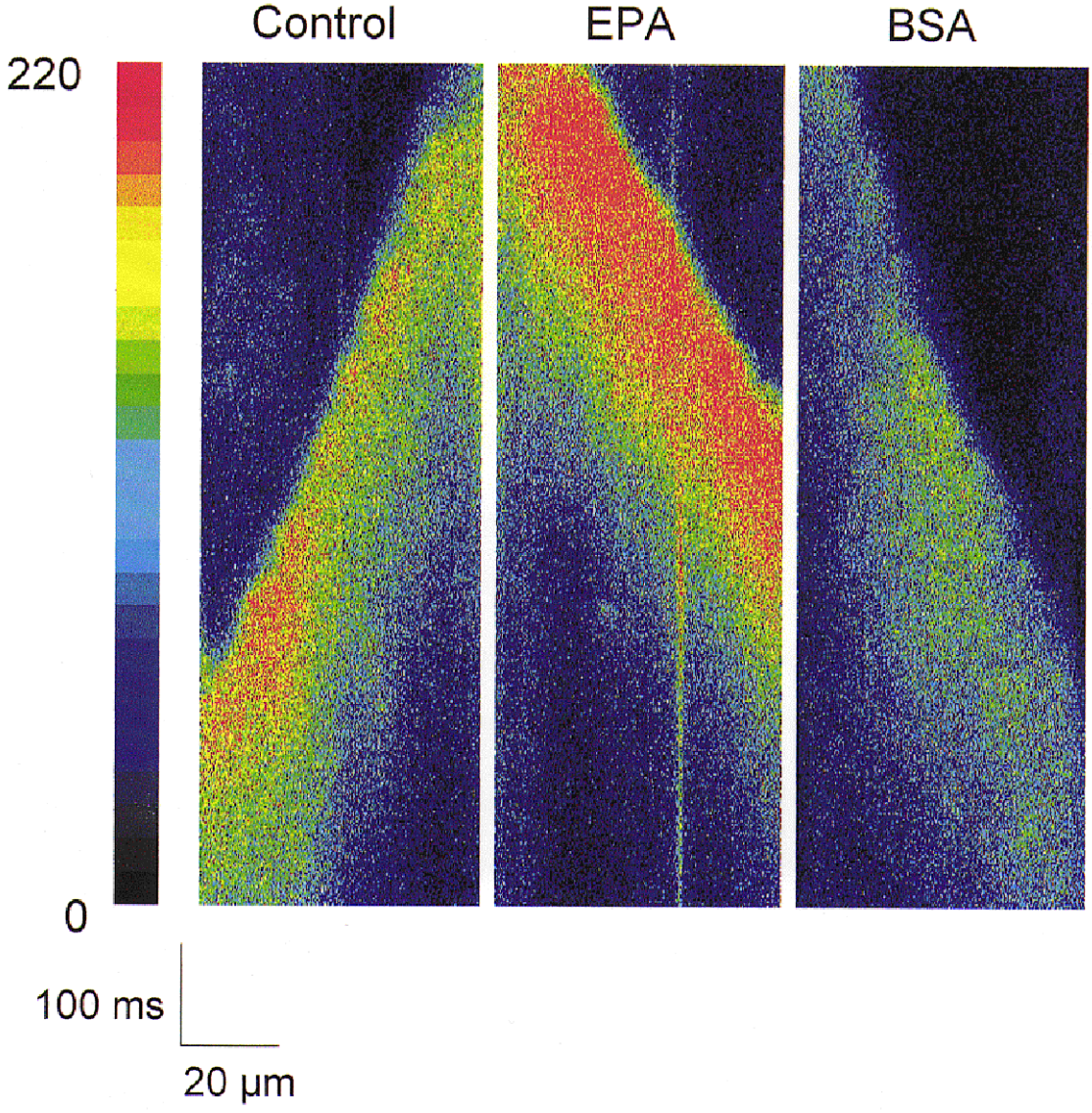

Figure 3. Linescans of a single ventricular myocyte loaded with fluo-3

Each scan shows a wave propagating along the cell in (left to right): control, 10 μM EPA and recontrol (2 mg ml^{-1} BSA). The cell was bathed in 2 mM external calcium to induce spontaneous waves. Fluorescence intensity is indicated by the pseudocolour scale at the left. Wave propagation proceeds from right to left in the control panel and left to right in the other two panels.

propagated waves of calcium release using laser scanning confocal microscopy. The linescan images presented here represent the fluorescence signal recorded on a single line along the length of the cell. The line is scanned repetitively by the laser every 2 ms, each scan is then stacked vertically to give a measure of fluorescence (i.e. $[Ca^{2+}]_i$) with both spatial and temporal information. The sloping increases of fluorescence intensity shown represent waves of calcium release passing along the line described by the laser. Typical linescan measurements from such an experiment are shown in Fig. 3. In the control panel, the propagating wave of calcium release begins at the right of the cell and propagates to the left, the direction of propagation is from the left in the other two panels. In the presence of EPA (10 μM), it is clear that the peak calcium reached in the wave is greater. In EPA the wave also propagates faster, as indicated by the less steep slope of the wavefront, i.e. the wave takes less time to propagate along the cell. Both these effects are reversible, in the right panel the wave is smaller and slower once again after removal of EPA and addition of BSA. In nine cells studied, wave amplitude increased by $22{\cdot}5 \pm 8{\cdot}7\%$ ($P < 0{\cdot}05$) and velocity ($71{\cdot}0 \pm 5{\cdot}4\ \mu m\ s^{-1}$ under control conditions) increased by $18{\cdot}5 \pm 5{\cdot}0\%$ ($P < 0{\cdot}01$). An increase of wave amplitude is consistent with increased SR calcium content and therefore, inhibition of calcium release by EPA.

To determine whether SR calcium content is increased in EPA, we have measured, under voltage clamp conditions, the caffeine-induced Na^+–Ca^{2+} exchange current. The integral of this current can be used as a quantitative measure of the SR calcium content (Varro *et al.* 1993). In Fig. 4*A* are shown the caffeine-induced currents, below which are the integrals converted into SR calcium content. It is clear from the increase of both the current and the integral after 3 min in EPA (5 μM), that there has been an increase in the SR calcium content. In 4 cells 5 μM EPA increased the SR content from $98{\cdot}9 \pm 16{\cdot}5$ to $139{\cdot}4 \pm 18{\cdot}0$ μmol (l cell volume)$^{-1}$ ($P < 0{\cdot}05$). Similar experiments with another PUFA, DHA (5 μM) increased SR calcium content from $102{\cdot}3 \pm 8{\cdot}9$ to $137{\cdot}9 \pm 9{\cdot}7$ μmol (l cell volume)$^{-1}$ ($n = 3$, $P < 0{\cdot}001$). Again, these results are more consistent with inhibition of calcium release from the SR than reduced availability of calcium.

A reduced frequency of oscillations combined with an increase of SR calcium content has been reported previously when SR calcium release is inhibited by tetracaine (Overend *et al.* 1997). We have looked for further evidence of inhibition of calcium release by examining the relationship between calcium current and contraction under voltage clamp conditions. Figure 5 shows that EPA produces a reversible, negative inotropic effect. Figure 5*A* shows a slow timebase record of contraction. EPA (10 μM) was applied as

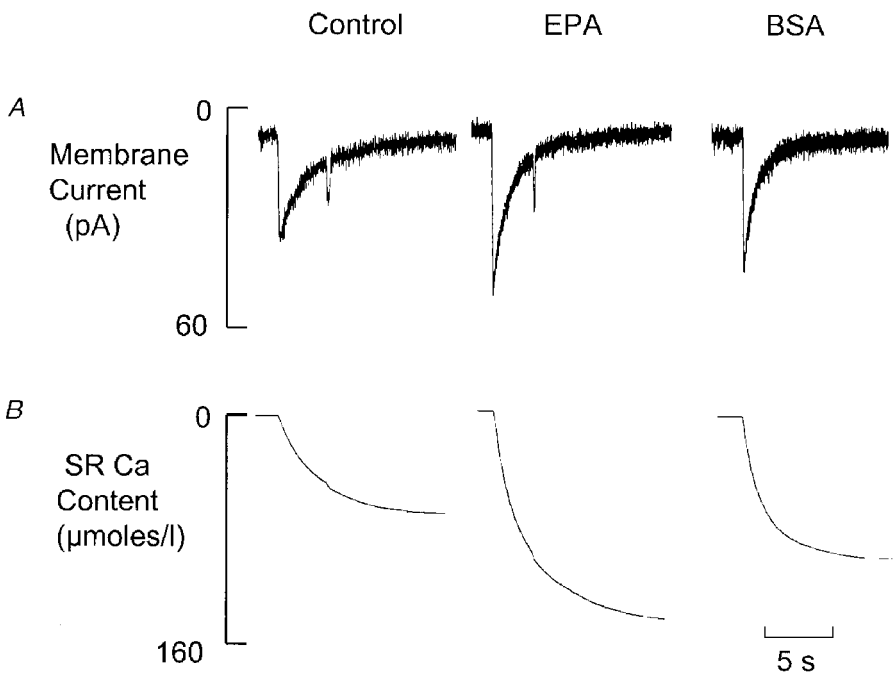

Figure 4. SR calcium content response to EPA

Records of caffeine-induced Na^+–Ca^{2+} exchange currents (*A*) and integrals (*B*) in control, 5 μM EPA and recontrol (2 mg ml^{-1} BSA). The corrected current integrals are expressed in micromoles Ca^{2+} (litre cell volume)$^{-1}$. The cell was bathed in 1 mM calcium. The cell was held at −80 mV and stimulated to contract by depolarisation to 0 mV for 200 ms, stimulation was stopped shortly before caffeine was applied. During measurements of caffeine-induced currents, the membrane potential was held constant at −80 mV. Each current trace was measured after application of 10 mM caffeine to the bathing solution. The bathing solution contained 5 mM 4-AP and 0·1 mM $BaCl_2$ throughout.

indicated by the black bar. Each contraction was elicited by depolarisation from −40 to 0 mV for 200 ms. Figure 5*B* shows records (each the average of 5) of calcium current and contraction taken from the record in *A* at the points indicated by the letters. In EPA calcium current and contraction are reduced in amplitude. The trace labelled d shows a record of calcium current taken after the record in Fig. 5*A*. In trace d the voltage pulse is from −40 to −10 mV, this produces calcium current and contraction similar in size to that in EPA (trace b). It appears from this that the ratio of i_{Ca}: contraction is unchanged in EPA. However, under identical conditions we find an increase of SR calcium content (Fig. 4), therefore, a smaller fraction of the SR content is released in EPA.

DISCUSSION

Polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid, are known to have beneficial effects on the heart; reducing the likelihood of arrhythmias post-ischaemia in animal models (Billman *et al.* 1994, 1999) and being associated with low levels of coronary disease when present in the human diet (Bang *et al.* 1976). Studies of the mechanisms involved in this protection in cardiac muscle have focused on the effects at the surface membrane of the cell. It has been shown that surface membrane sodium, calcium and potassium currents are inhibited by PUFAs (Xiao *et al.* 1997; Macleod *et al.* 1998). The combined effect of this is to reduce electrical excitability (Kang *et al.* 1995), making arrhythmias less likely.

Role of the sarcoplasmic reticulum in generation of arrhythmias

It is well known that when the sarcoplasmic reticulum is overloaded with calcium, as may happen post-ischaemia, it produces spontaneous release events that propagate along the cell as a wave (Kass *et al.* 1978; Cheng *et al.* 1996). Through activation of the Na^+–Ca^{2+} exchanger, spontaneous release of calcium may depolarise the cell membrane sufficiently to generate arrhythmias. The main interest of this study was to examine if any of the protection against arrhythmias afforded by PUFAs is achieved through an effect on SR function.

Can inhibition of SR calcium release account for the effects of EPA?

Previously it has been shown that inhibition of the calcium release mechanism in calcium overloaded cardiac myocytes leads to a lowering of spontaneous oscillation frequency and that associated with this is an increase of SR calcium

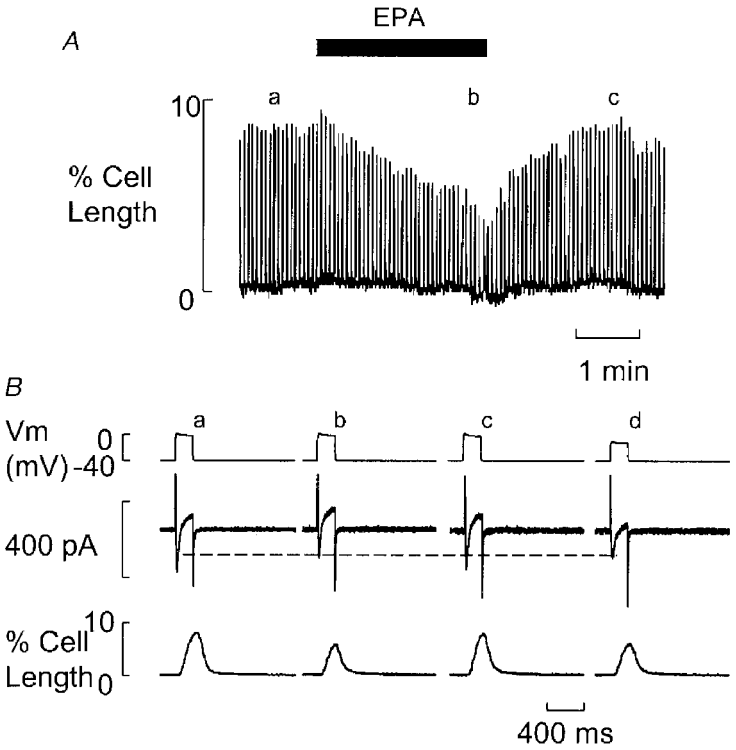

Figure 5. EPA produces a reversible, negative inotropic effect

A, slow timebase record of cell length in a voltage-clamped cell. Membrane potential was held at −40 mV, contraction was stimulated by depolarisation to 0 mV for 200 ms at 0·5 Hz. EPA (10 μM) was applied as indicated by the bar. *B*, sample records, each the average of 5, from the regions of *A* indicated by the letters (a–c), showing membrane potential (top), calcium current (middle) and cell length (bottom). Trace d shows the effect of depolarisation to −10 mV and was taken just after *A*. External calcium was 1 mM, 5 mM 4-AP and 0·1 mM $BaCl_2$ were present throughout. The dashed line shows the level of calcium current in the presence of EPA.

content (Overend *et al.* 1997). A spontaneous wave of calcium release occurs once a set value of SR Ca^{2+} content is reached (Díaz *et al.* 1997), if the gain of calcium-induced calcium release is reduced, the set value of SR Ca^{2+} content required for spontaneous release is increased (Overend *et al.* 1997). With a greater SR content, there is greater release of Ca^{2+} from the SR during the wave and greater efflux of Ca^{2+} from the cell is activated. More time is required to replace this greater loss before another wave is possible, i.e. the frequency of waves is reduced (Overend *et al.* 1997). We report here that EPA (and DHA) increases SR calcium content and reduces the frequency of spontaneous waves of calcium release. The linescans of propagating waves shown in this study confirm that in EPA, as in tetracaine (Overend *et al.* 1997), each wave is larger and will activate more efflux. Therefore, it seems EPA is inhibiting release of calcium from the SR. With a lower frequency of spontaneous waves of calcium release, the risk of an arrhythmic action potential occurs less frequently.

The two measures of calcium release during the wave we have used seem to disagree over the amplitude of the wave: imaging the wave shows an increased release (Fig. 3), but the global mean calcium signal appears to show no change (Fig. 2). Imaging of the wave allows us to measure the change of calcium at the leading edge of the calcium release wave. The global mean, however, takes no account of the spatial characteristics of the wave and so will be influenced by the resting level of calcium outside the region of calcium release, the rate of rise and fall of the wave and also the area of the cell it occupies as it propagates. The ability to image calcium release avoids these complications and allows us to conclude that the amplitude of the wave is increased in EPA. The observed decrease in the extent of shortening activated by the wave of Ca^{2+} release may be explained in similar terms, e.g. if the rise in $[Ca^{2+}]_i$ does not have time to equilibrate with the contractile proteins then the higher propagation velocity, combined with the lower resting level of $[Ca^{2+}]_i$ may lead to less activation of contraction, despite the higher absolute level of $[Ca^{2+}]_i$ reached during release.

A recent study of spontaneous release of calcium in permeabilized ventricular myocytes (Rodrigo *et al.* 1999) has also concluded that EPA has an effect on SR calcium handling. EPA was shown to decrease the frequency of spontaneous contractions in these skinned cells. Obviously surface membrane effects are irrelevant in that case. However, the authors were unable to distinguish between reduced SR uptake and decreased sensitivity of release. Our demonstration of an increase in the calcium content of the SR in the presence of EPA shows that decreased uptake, if present, cannot be the dominant effect of EPA. This leaves inhibition of the release mechanism as the only viable mechanism.

The linescans of Fig. 3 show in addition to EPA increasing the amplitude of the wave, an increase in wave propagation velocity. As yet the factors that control wave propagation velocity are not well understood. If EPA is inhibiting SR calcium release, one might expect propagation to be slowed. On the other hand, increased wave amplitude ought to be a more effective stimulus for release at adjacent SR sites. Although at this stage we cannot rule out other effects of EPA, it appears that increased calcium release has a greater effect on propagation velocity than reduced sensitivity of release.

Availability of Ca^{2+} to the SR

The above argument assumes no change in the availability of calcium to the SR. However, another factor that will contribute to inhibition of waves by EPA is the fall of the baseline intracellular calcium it induces (Fig. 2). This represents reduced availability of calcium for the SR. Each oscillation of $[Ca^{2+}]_i$ activates efflux of calcium from the cell via the sarcolemmal Ca-ATPase and the Na^+–Ca^{2+} exchanger. As a result, not all the calcium released from the SR returns there. Before another oscillation is possible, the SR must replace this calcium. It does this by pumping calcium from the cytoplasm but this takes longer at lower $[Ca^{2+}]_i$ as the SR Ca-ATPase is less activated. A previous study of SR content and frequency of oscillations (Díaz *et al.* 1997) has shown that reducing the availability of calcium to the SR (by lowering external calcium and hence intracellular calcium) does lower the frequency of oscillations. However, if calcium availability is still sufficient to support waves, the SR load is unaffected, i.e. only the time required for refilling the SR after an oscillation is affected when availability of calcium is altered. That lower availability of calcium is not the only mechanism reducing oscillation frequency, therefore, is shown by the increase of SR content despite the reduced availability of calcium.

A recent study in neonatal rat cultured-cardiac myocytes demonstrated that PUFAs can produce a reduction in the resting level of $[Ca^{2+}]_i$ (Kang & Leaf, 1996), however, it was concluded that the anti-arrhythmic effect of PUFAs resulted from reduced electrical excitability of the surface membrane. In the present study, the spontaneous release of calcium from the SR is not the result of surface membrane electrical activity as in neonatal cultured myocytes and so the fall of $[Ca^{2+}]_i$ is likely to be an important determinant of release frequency. The cause of the steady state fall of $[Ca^{2+}]_i$ must be either an increased efflux or a decreased influx of calcium across the surface membrane, as a finite store such as the SR cannot lower $[Ca^{2+}]_i$ in the long term. Some of the reported effects of PUFAs at the surface membrane of the cell e.g. inhibition of sodium channels and L-type Ca^{2+} channels (Xiao *et al.* 1997; Macleod *et al.* 1998) may contribute to this. Both will tend to reduce Ca^{2+} availability to the SR by favouring Ca^{2+} efflux on Na^+–Ca^{2+} exchange and reducing influx, respectively. However, if these were the dominant effects of PUFAs at the level of the SR the Ca^{2+} content would either fall (if spontaneous release is abolished) or not change (if spontaneous release continues at a lower frequency; (Díaz *et al.* 1997) neither effect can explain the increase of SR Ca^{2+} content that we report.

Relationship between calcium current and calcium release

In this study we find that EPA has a negative inotropic effect (Fig. 5), this has also been reported in two recent studies (Xiao *et al.* 1997; Macleod *et al.* 1998). We also find that a given calcium current activates the same level of contraction whether EPA is present or not. From very similar evidence (Xiao *et al.* 1997) it has been concluded that the calcium release mechanism is unaffected by EPA, yet we conclude SR calcium release is inhibited. The key observation, not made previously, is that SR calcium content rises in the presence of EPA (Fig. 4). Thus, although the ratio of i_{Ca}:contraction is unchanged in EPA, the calcium released from the SR is a smaller fraction of the total content, i.e. calcium release is less sensitive to i_{Ca}. It is no coincidence that the ratio of i_{Ca}:contraction is unchanged in EPA. Calcium release from the SR activates efflux from the cell. This efflux must balance influx in the steady state. Therefore, if we match i_{Ca} (influx) in EPA and control, the level of calcium efflux and, therefore, calcium release required is also matched (Eisner *et al.* 1998). The maintained negative inotropic effect in EPA must be due to other factors, the most obvious candidate for this is inhibition of the L-type calcium current (Overend *et al.* 1998). It is worth noting that inhibition of CICR itself will not affect the normal systolic function of the heart as the increase of SR Ca^{2+} content will compensate for the reduced sensitivity of release. At the same time, however, there will be a reduction in the frequency of spontaneous release. Normal function of the heart, therefore, should be preserved while protection against arrhythmias is provided by inhibition of CICR.

The reduced extent of shortening during the propagating wave shown in Fig. 1 might represent a direct effect of EPA on the contractile proteins. It is worth noting, however, that maintenance of the same i_{Ca}:contraction relationship would not be expected if EPA reduced the sensitivity of the contractile proteins to calcium unless calcium release were increased in compensation. It seems unlikely that the level of contraction (as distinct from calcium release) could influence the level of SR filling.

Our results show that PUFAs in the concentration range 5–10 μM have effects on calcium release by the SR. One problem we should consider is, in the presence of serum albumin (which binds fatty acids with high affinity (Ashbrook *et al.* 1975), are such concentrations possible? If we assume that albumin has an affinity for EPA in the same range as those of other long chain fatty acids and that EPA forms between 5 and 10% of the total fatty acid content of the plasma (as is the case following supplementation of the diet with fish oil (Connor *et al.* 1996), for a total EPA concentration of 40 μM (Connor *et al.* 1996) it can be shown that between 20 and 80% of the EPA will not be bound to serum albumin. Thus, between 8 and 32 μM remain unbound, sufficient for the effects reported here.

In conclusion, we find evidence that EPA and DHA inhibit spontaneous waves of propagating calcium release. This is the result of a combination of inhibition of calcium release and reduced availability of calcium to the SR. As these waves are responsible for some kinds of cardiac arrhythmias, at least some of the anti-arrhythmic properties of PUFAs must be exerted at the level of the SR.

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