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Review

Experimental studies on antiarrhythmic and antiseizure effects of polyunsaturated fatty acids in excitable tissues

A. Leaf,* J.X. Kang,* Y.-F. Xiao,† G.E. Billman,‡ and R.A. Voskuyl§

**Department of Medicine, Massachusetts General Hospital, and †Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA USA; ‡Department of Physiology, The Ohio State University, Columbus, OH USA; and §Department of Physiology, Leiden University Medical Center, and the Stichting Epilepsie Instellingen Nederland, Heemstede, The Netherlands*

It has been shown that in animals, and probably in humans, n-3 polyunsaturated fatty acids (PUFAs) are antiarrhythmic. We discuss our recent studies on the antiarrhythmic actions of PUFAs. PUFAs stabilize the electrical activity of isolated cardiac myocytes by requiring a stronger electrical stimulus to elicit an action potential and by markedly prolonging the refractory period. These electrophysiologic effects are the result of specific modulation of ion currents, particularly of the voltage-dependent sodium current and of the L-type calcium currents across sarcolemmal phospholipid membranes. This appears to be the probable major antiarrhythmic mechanism of PUFAs. However, they also similarly affect neuronal ion channels with potentially important functional effects on the nervous system. (J. Nutr. Biochem. 10:440–448, 1999)

Keywords: omega-3; n-3; omega-6; n-6; eicosapentaenoic acid; docosahexaenoic acid; arrhythmias; seizures

Introduction

The motivation for this study was clinical, and therefore it is necessary that we review a bit of background before explaining our findings that long chain polyunsaturated fatty acids (PUFAs) have the ability to modulate the conductance of ion channels of excitable tissues in a specific and very salubrious manner.

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Address correspondence to Dr. Alexander Leaf, Massachusetts General Hospital, East Bldg 149, 13th Street, Charlestown, MA 02129 USA.

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Although there has been a gratifying reduction in the mortality from coronary heart attacks in the United States since 1960, there remains a consistent recent mortality from cardiac sudden death—death within 1 hour following an acute myocardial infarction—of some 250,000 persons annually in the United States alone.[1] These deaths are almost all due to persistent ventricular arrhythmias, usually ventricular fibrillation (VF). Despite huge expenditures by the pharmaceutical industry, there is no effective and safe drug for either the treatment or prevention of these lethal arrhythmias.

My laboratory (A.L.) was engaged in studying possible effects of PUFAs on the cardiovascular system when studies by two Australian investigators came to our attention. McLennan et al.[2,3] were simply feeding rats diets in which they could vary the major source of fat over periods of 3 months or longer. They then ligated the coronary arteries of the rats and recorded the percent of animals that developed intractable VF. McLennan[4] reported that when the major

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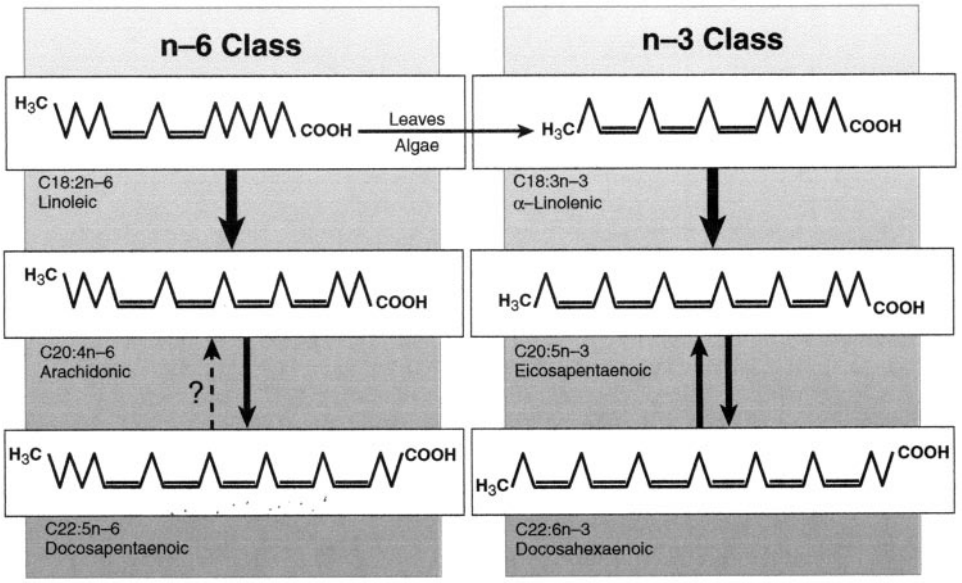

Figure 1 The two classes of dietary essential polyunsaturated fatty acids, n-6 (ω-6) and n-3 (ω-3), the former obtained from plant sources and the latter largely from fish oils. The n-6 and n-3 refer to the position of the first C=C bond from the methyl end of the acyl chain.

source of fat was saturated over 40% of the rats developed VF. When rats were fed monounsaturated olive oil, there was no significant reduction in arrhythmias. When fed a vegetable oil, there was a significant reduction of 70% in fatal arrhythmias. However, when tuna fish oil was the major source of dietary fat, there were no fatal VFs whether the ligature remained on the coronary arteries or whether it was released, allowing reflow. This seemed a remarkable finding that deserved an effort to confirm.

There are two classes of essential PUFAs (*Figure 1*). They are essential because, although we cannot synthesize them in our bodies, they are required for normal growth and development. One class is the n-6 (or ω-6) PUFAs, which are present abundantly in vegetable oils. Linoleic acid (LA; C18:n-6) is the parent compound of this class. Counting back six carbon atoms from the methyl end of this fatty acids is the first C=C unsaturated bond, hence the n-6 appellation to this class. In our bodies LA can be further elongated and desaturated to a 20-carbon fatty acid, arachidonic acid (AA; C20:4n-6), which is the immediate precursor of prostaglandins, leukotrienes, lipoxines, and epoxygenase compounds, many of which are potent cell messengers. In the chloroplasts of green leaves and algae, LA can be desaturated to yield α-linolenic acid (LNA; C18:3n-3), which is the parent compound of the n-3 (or ω-3) class of PUFAs. In our bodies LNA can be further elongated to eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3). EPA and DHA are present in our diets today primarily from fish oils. DHA is conserved and stored in the sn-2 position of phospholipids of cell membranes, especially of heart and brain.

Confirmatory in vivo studies

To see if we could confirm the observations of previous studies,[2,4] we studied the ability of a concentrate of the free fatty acids of fish oils administered first as a phospholipid emulsion intravenously to surgically prepared dogs.[5] Ten of 13 exercising, unanesthetized canines were protected ($P <$ 0.005) by the fatty acid infusion of fish oil just prior to occluding their left circumflex coronary artery, whereas the same dog tested with the same exercise-ischemia test 1 week before and 1 week following the test developed fatal VF within 2 minutes of the coronary occlusion when an infusion of soybean oil emulsion was used instead of the fish oil. We purposely chose to administer the fatty acids intravenously rather than by diet, because with prolonged feeding studies, there are invariably potential confounding factors. For example, in the instance we are citing, it was argued that the beneficial effect of the fish oil might as well have been due to the reduction of other dietary fats to maintain total fat intake constant between the fish oil fed and control animals. However, when we infused the fish oil fatty acids intravenously just before the ischemic stress test, we could be convinced that the prevention of the fatal VF resulted from something contained in the fish oil emulsion. To find which ingredients of fish oil might be active, we did another series of experiments, infusing pure DHA, EPA, or LNA intravenously, primarily carried on albumin. As seen in *Table 1*, each of these major dietary n-3 PUFAs were potently antiarrhythmic.[6]

Table 1 Prevention of ischemia-induced fatal ventricular arrhythmias by n-3 polyunsaturated fatty acids (PUFAs) in a dog model of sudden cardiac death

	Number of Dogs Tested		
n-3 PUFAs	Total	Protected	*P*
Fish oil concentrate*	13	10	<0.005
EPA†	7	5	<0.02
DHA‡	8	6	<0.004

*72% n-3 PUFA with free eicosapentaenoic acid (EPA), 33.9% and docosahexaenoic acid (DHA) 25% (Pronova Biocare as. Lysaker, Norway; EPAX6000FA).
†98.4% free EPA; 1.1% free DHA (Pronova-Biocare a.s.).
‡90.8% free DHA; 0.9% free EPA (Pronova-Biocare a.s.).

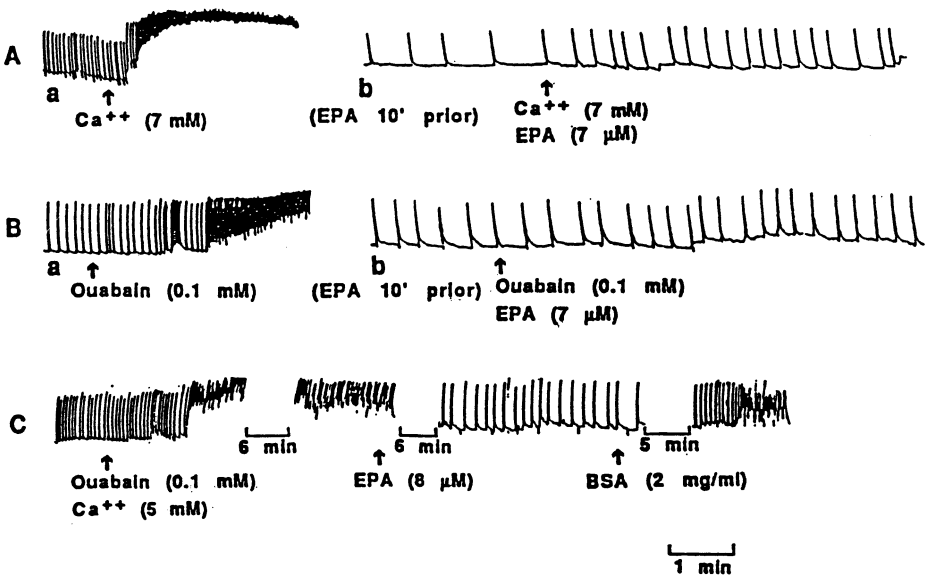

Figure 2 The effects of eicosapentaenoic acid (EPA; 7 μM) on tachyarrhythmias induced by increased concentration of Ca^{2+} *(A)* or the cardiac glycoside ouabain (10^{-4} M)*(B)* in the superfusate of cultured neonatal rat cardiomyocytes. With each additive the cells developed tachyarrhythmias with fibrillation. When EPA was added to the superfusate prior to addition of the toxin, the beating rates slowed and the expected arrhythmia was prevented *(C)*. When both elevated Ca^{2+} and ouabain were added to the superfusate, fibrillation occurred, but the addition of EPA (8 μM) in the presence of elevated Ca^{2+} and ouabain stopped the arrhythmia. When bovine serum albumin (BSA) was added to the superfusate to extract the free EPA, the arrhythmia recurred.

Ex vivo studies on the mechanism of the antiarrhythmic effects

With these surprising, but encouraging results, we decided to see if we could learn the physiologic and biochemical bases for the antiarrhythmic action of these fatty acids. For this we turned to a cultured neonatal rat cardiomyocyte preparation. We wanted to be able to see the contractile activity of heart cells in the absence of neural or humoral influences. By the second day in a primary cell culture, these cardiomyocytes grow in clumps adherent to microscope coverslips. Placing the coverslips in a perfusion set up and using an edge-monitor, we could watch and record a single myocyte in a clump of myocytes contracting spontaneously, synchronously, and rhythmically. When EPA or DHA were added at low micromolar concentrations to the superfusate, within a few minutes the beating rate of the myocytes slowed down, but the amplitude of contractions were unchanged. This slowing was reversible. Adding delipidated bovine serum albumin (BSA; approximately 30 μM) to the superfusate extracted the free fatty acids from the cells and the beating rate returned to normal. Albumin has bindings sites for free fatty acids, three of which have high affinities[7] and can reverse the normal traffic of fatty acids from albumin to the cells. We then tested all the agents we knew to produce fatal arrhythmias in humans to observe their actions on these cultured neonatal rat heart cells and the effects of PUFAs thereon.

Figures 2A and *2B* show the effects of raising the extracellular $[Ca^{2+}]_e$ or adding toxic concentrations of the cardiac glycoside ouabain to the superfusate.[8] Shortly after the additions, the myocytes markedly increased their beating rates, developed contractures and a tachyarrhythmia, and fibrillated. However, when EPA was added first, the beating rate was slowed and when the $[Ca^{2+}]_e$ was raised or the ouabain was added, the expected arrhythmias were prevented. *Figure 2C* shows that when $[Ca^{2+}]_e$ was increased *and* ouabain was added to the superfusate, the myocytes developed a violent fibrillation, but the addition of EPA in the continued presence of the high $[Ca^{2+}]_e$ and ouabain soon stopped the arrhythmia. Finally, the addition of BSA to the superfusate to extract the free EPA from the cells, still in the presence of the high $[Ca^{2+}]_e$ and ouabain, reinstituted the arrhythmia. This taught us two important facts: (1) The PUFA exerts its antiarrhythmic effect simply by partitioning into the hospitable lipophilic environment of the acyl chains of the cell membrane phospholipids. If the EPA had been incorporated into membrane phospholipids or if it had been covalently linked to any constituent in the cell membranes, it could not have been extracted by the BSA. (2) PUFAs are antiarrhythmic in this acute experiment only when administered as the free, nonesterified fatty acid. When the ethyl ester or the triglyceride of EPA were added, neither exerted any antiarrhythmic effect. The negatively charged carboxyl group is essential for the acute antiarrhythmic effects shown in these experiments.

When the PUFAs are ingested in the diet largely as triglycerides, they are also antiarrhythmic, as McLennan and colleagues[2,3] first demonstrated in rats and others have since confirmed.[9–12] The route for the delivery of ingested fatty acids to the heart cell membranes is much more circuitous than when administered intravenously. Free fatty acids absorbed from the intestines are resynthesized so efficiently in gut and liver into triglycerides that the free form is not elevated in the plasma. Lipoprotein lipases in tissues liberate the free fatty acids, which either partition into nearby cell membranes or are carried then by albumin to cells, where they are metabolized or stored in triglycerides or phospholipids. From these stores they can be rapidly liberated by lipases to partition as free fatty acids in cardiac membrane phospholipids to provide the preventive antiarrhythmic actions we are studying.[13] The lipases are rapidly activated by sympathetic innervation of the heart and from stored adipocyte triglycerides under conditions of stress (e.g., ischemia, excessive and unusual physical activity, or emotional stress). If the subject regularly ingests n-3 PUFAs, they will be available when needed.

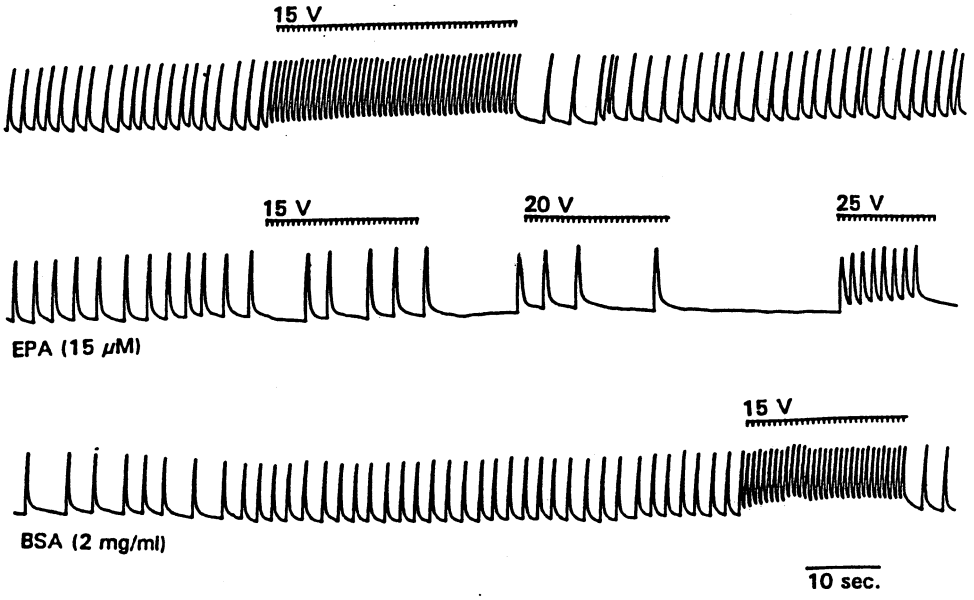

Figure 3 Electrical stabilization of spontaneously contracting neonatal rat cardiomyocytes by eicosapentaenoic acid (EPA). The three tracings were continuous recordings from the same clump of cultured neonatal cardiomyocytes. The top tracing shows that the cells doubled their beating rate in response to stimuli imposed from an external field of 15 volts. After the addition of EPA (15 μM) to the superfusate, the spontaneous beating rate began to slow and the cells were unresponsive to stimuli imposed by fields of 15 and 20 volts; however, at 25 volts, every other stimulus captured a contraction. Adding bovine serum albumin (BSA) and removing the EPA from the superfusate normalized the beating rate and the cells again responded to stimuli delivered at 15 volts external field strength.

Intravenous administration of PUFAs simply bypasses the need for lipase action on stored n-3 PUFAs. Unfortunately, most North Americans today have little, if any, of these protective n-3 PUFAs in their phospholipid or triglyceride stores and, therefore, with acute myocardial infarctions they are likely to contribute to the high mortality statistics from sudden cardiac death. Once the PUFAs are delivered into the cell membranes by either route of administration, their mechanism of action and effects will be the same.

In addition to EPA, we found that, using the cultured rat neonatal cardiomyocytes as our assay, all the other main dietary n-3 PUFAs [LNA, DHA, and docosapentaenoic acid (C20:5n-3; DPA)] are also antiarrhythmic, as are the n-6 PUFAs. AA (C20:4n-6), however, was anomalous. In one third of 48 experiments, AA, like EPA and DHA, was antiarrhythmic; in another third it had no effect, but in a final third it caused violent arrhythmias. These arrhythmias proved to be caused not by the AA per se, but rather by cyclooxygenase oxygenated metabolites of AA. When AA was added to the cultured myocytes together with indomethacin (20 μM), which is a potent cyclooxygenase inhibitor, the AA was antiarrhythmic in 28 of 30 experiments. Furthermore, eicosatetraynoic acid,[14,15] a nonmetabolizable, acetylenic analog of AA and a potent inhibitor of cyclooxygenase, lipoxygenase, and epoxygenase, was only antiarrhythmic. We have subsequently tested most of the available cyclooxygenase metabolites of AA and all except prostacyclin are arrhythmogenic, whereas none of the comparable cyclooxygenase metabolites of EPA are arrhythmogenic.[16] By contrast, monounsaturated oleic acid and saturated stearic, palmitic, and lauric acids had no antiarrhythmic effects.

Because of the potential arrhythmogenic effects of cyclooxygenase metabolites of AA when only n-6-containing vegetable oils are present in the diet, as is becoming the general case in the diets of Western industrialized nations, we have focused our subsequent studies primarily on the antiarrhythmic effects of the n-3 class of PUFAs, which lacks that risk, for clinical usage.

From these findings the structure-function relations for an agent that is antiarrhythmic in the manner of these PUFAs would seem to require only a free carboxyl group attached to a long acyl chain or hydrocarbon with two or more unsaturated C=C bonds. With this rule to guide us, we found that all-*trans*-retinoic acid is similarly antiarrhythmic, whereas retinal and retinol are not.[17]

Electrophysiologic effects of n-3 PUFAs

We found that with all the cardiotoxic agents we tested, in addition to elevated $[Ca^{2+}]_e$ and ouabain (e.g., β-adrenergic agonists,[18] lysophosphatidyl choline, acyl carnitine,[19] thromboxane,[16] and even the calcium inophore A23187[19]), if the fatty acid was added to the superfusate first, then subsequent addition of the cardiotoxin would fail to elicit its expected arrhythmia. If the arrhythmias were established, subsequent addition of the PUFAs would terminate the arrhythmia. This suggested that these fatty acids must somehow be affecting the basic excitability/automaticity of the heart. Therefore, we examined the effects of the PUFAs on the electrophysiology of the individual cardiomyocyte and found two major physiologic effects[20]: (1) In the presence of 2 to 10 μM EPA or DHA, an average increase in the strength of a depolarizing stimulus of some 40 to 50% was required just to elicit an action potential. Without an action potential, there is no contraction of the myocyte. (2) The presence of the n-3 PUFAs prolongs the refractory period of the cardiac cycle by some two- to threefold, with no prolongation of the action potential duration. We believe that these two physiologic effects of the PUFAs, which directly affect every myocyte in the heart, stabilizes them, making them resistant to arrhythmias.

This electrical stability induced by the PUFAs can readily be demonstrated with the neonatal rat cardiomyocyte preparation (*Figure 3*).[19] A myocyte within a clump of

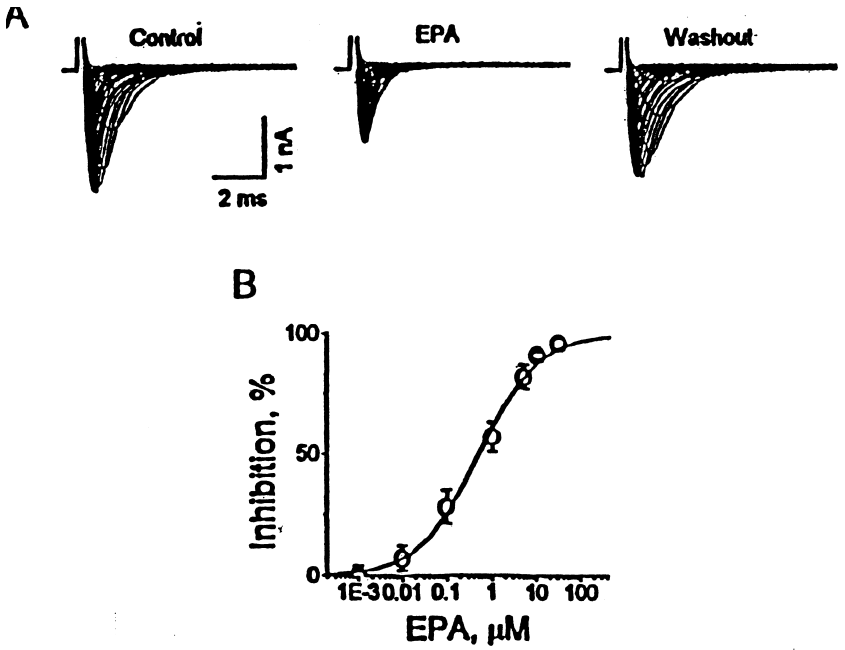

Figure 4 Inhibitory effects of eicosapentaenoic acid (EPA) on $I_{Na\alpha}$ of hH1α channels transiently expressed in HEK293t cells. *(A)* Whole cell voltage clamp traces are superimposed. They were elicited by 10 ms test pulses from −90 mV to 55 mV with 5 mV decrements at 0.2 Hz for control, 5 μM EPA, and washout. The cells were held at −80 mV and hyperpolarized to −160 mV for 200 ms before a test pulse. *(B)* Suppression of $I_{Na\alpha}$ is concentration dependent with an IC_{50} of 0.51 ± 0.06 μM. $I_{Na\alpha}$ was elicited by single voltage pulses from −120 to −30 mV. Each value represents 6 to 12 individual preparations exposed to different concentrations of EPA.

cultured myocytes on a microscope coverslip was shown to be beating regularly (*Figure 3*, top tracing). We could see just the amplitude and rate of contractions of the myocyte. Two platinum electrodes placed across the coverslip were connected to an external voltage source. When a field potential of 15 volts was present across the two platinum electrodes, the beating rate of the myocytes could readily be doubled by the external stimuli. Turning off the external field potential promptly returned the myocytes to their endogenous, spontaneous beating rate. When 15 μM EPA were added to the superfusate of the same cells, the beating rate began to slow, and when an external electric field was again turned on, the myocytes did not respond to the imposed electrical stimuli at 15 or 20 volts. At 25 volts the myocyte responded but only to every other stimulus. After extracting the EPA from the same cell with delipidated BSA, the myocyte responded to stimuli imposed by an external field potential of 15 volts (*Figure 3*, bottom tracing), as they had initially. When one considers that this electrical stabilizing effect of the PUFAs is directly affecting every individual myocyte in the heart, both atrial and ventricular, in the absence of humoral or neural controls, one can sense the potent electrical stabilizing and antiarrhythmic actions of these n-3 PUFAs.

Effects of PUFAs on the fast voltage-dependent sodium current I_{Na}

The voltage-gated sodium current I_{Na} elicits action potentials in most cardiac myocytes. Our finding that the PUFAs increased the magnitude of a depolarizing stimulus required to elicit an action potential made it likely that the PUFAs were affecting the I_{Na}. Thus our exploration of the effects of the PUFAs on membrane ion currents and channels began with I_{Na}.[21] The PUFAs inhibited the I_{Na} in a concentration-dependent manner, with a IC_{50} of 4.8 μM in neonatal rat cardiomyocytes[21] but only 0.51 ± 0.06 μM in a human embryonic kidney cell line (HEK293t) transiently expressing human myocardial sodium α-subunits ($hH1_{\alpha}$[22]; *Figure 4*). Inhibition occurred within seconds of application of the PUFAs to the myocytes. It was voltage dependent, but not use dependent, consistent with the lipophilic nature of the PUFAs.[23] In both preparations—I_{Na} in the rat cardiomyocyte and $I_{Na\alpha}$ in the human myocardial α-subunit transiently expressed in HEK293t cells—the PUFAs caused a large voltage-dependent shift of the steady state inactivation potential to more hyperpolarized values; the shift at $V_{1/2}$ = −19 mV with 10 μM EPA in the neonatal rat cardiomyocyte[21] (*Figure 5*) and a further −27.8 mV with 5 μM EPA in the $hH1_{\alpha}$.[22] There was no effect of the PUFAs on the activation of the Na^+ channels, only on the inactivated channel (*Figure 5*). The transition from the resting state to the inactivated state was accelerated in the presence of 5 μM EPA (the time constant for the development of inactivation in the control was shortened from 26.2 ± 0.78 ms, $n = 6$ to 3.67 ± 0.22 ms, $n = 6$, $P < 0.01$ with 5 μM EPA). Recovery of $I_{Na\alpha}$ from inactivation was markedly delayed; $t_{1/2}$ in the presence of 5 μM EPA was 34.8 ± 2.1 ms compared with 2.2 ± 0.1 ms before EPA was present ($n = 5$, $P < 0.001$). Thus PUFAs prolonged the inactivated state of the hH1α channels by speeding the transition from the active to the inactivated state and retarding the slow inactivation phase of the channel. In more recent studies (Xiao et al., unpublished data), the β1 subunit has been transiently coexpressed with the α-subunit in HEK293t cells and this shifted the steady-state inactivation potential to the right (to more depolarized potentials), returning the electrophysiology of the hH1α channels back almost to identity with what we had observed for the neonatal rat cardiomyocytes. EPA was found to have no effect on the activation but only on the inactivation of $I_{Na\alpha\beta}$, $I_{Na\alpha}$, and $I_{Na,rat}$. Consistent with the effects of these fatty acids solely on the

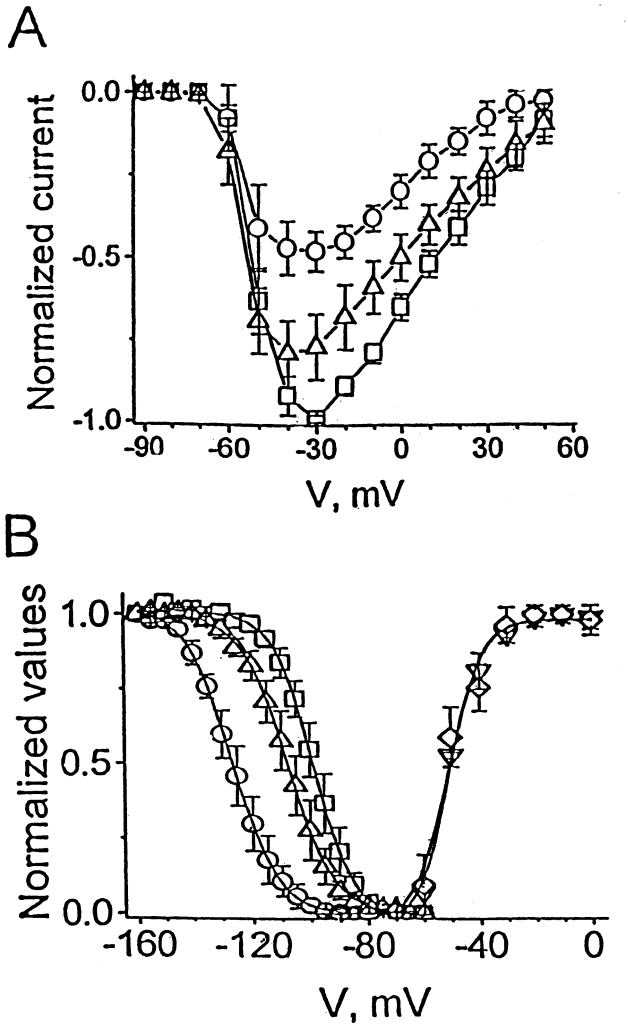

Figure 5 The activation and inactivation of $I_{Na\alpha}$ of human cardiac Na^+ channel α-subunits, $hH1_\alpha$, expressed in human embryonic kidney cells, HEK293t, in the presence (□), absence (○), and washout (Δ) of eicosapentaenoic acid (EPA; 5 μM). *(A)* Averaged and normalized current-voltage relationships ($n = 6$) of $I_{Na\alpha}$ are plotted, showing the inhibition of the peak Na^+ current in the presence of EPA and partial recovery following washout of EPA. *(B)* The averaged relative activation of $I_{Na\alpha}$ (right) was unaffected by EPA, and the three curves—control, EPA, and washout—of normalized activation were superimposable. By contrast (left), EPA produced an impressive shift of the steady-state inactivation to more hyperpolarized potentials and this was largely reversible on washout of the EPA. The same unchanged activation curves were also found for the complete $hH1_{\alpha\beta}$ sodium channel with both α and $\beta1$ units coexpressed, and for the neonatal rat cardiac myocyte. The shift of the steady-state inactivation potential to more negative potentials also occurred with $hH1_{\alpha\beta}$ and for the rat myocyte. The shifts were similar for both but not as large as seen in $hH1_\alpha$.

inactivated state of the Na^+ channel is the finding that the binding or interaction of these fatty acids to the inactivated state of the Na^+ channels displayed a 265-fold higher affinity for EPA than channels in the resting, but activatable, state of $hH1_{\alpha\beta}$.

We believe that these effects of the n-3 PUFAs (and DHA and LNA do the same as EPA) are pertinent to the antiarrhythmic actions of these fatty acids. Our current hypothesis is that this voltage-dependent shift of the steady state inactivation potential to more negative, hyperpolarizing voltages is important to the demonstrated antiarrhythmic action of the PUFAs in ischemia-induced fatal arrhythmias. In coronary thrombosis there occurs a gradient of depolarizations of cardiomyocytes within the ischemic tissue. Cells in the central core of the ischemic tissue quickly depolarize and die due to lack of oxygen and metabolic substrates. Depolarization results from the dysfunctional state of Na,K-ATPase and the rise of interstitial K^+ concentrations in the ischemic tissue. However, at the periphery of the ischemic zone myocytes may be only partially depolarized. They become hyperexcitable because their resting membrane potentials become more positive, approaching the threshold for the gating of the fast Na^+ channel. Thus, any further small depolarizing stimulus may elicit an action potential that, if it occurs out of phase with the electrical cycle of the heart, may initiate an arrhythmia. In the presence of the n-3 PUFAs, however, a voltage-dependent shift of the steady-state inactivation potential to more hyperpolarized resting potentials occurs. The consequence of this voltage-dependent, hyperpolarizing shift is that the negative potential necessary to return these Na^+ channels from an inactive state to a closed resting, but activatable state requires a physiologically unobtainable hyperpolarized resting membrane potential. In addition, these partially depolarized cells have Na^+ channels that can slip into "resting inactivation" from the closed resting state within milliseconds without eliciting an action potential.[22] The result of these two effects of the n-3 PUFAs is that these partially depolarized myocytes are quickly eliminated from function, and their potential arrhythmic mischief is aborted. By contrast, myocytes in the non-ischemic myocardium with normal resting membrane potential will not be so drastically affected by this voltage-dependent action of the PUFAs and continue to function normally (Xiao et al., unpublished data).

However, not all fatal cardiac arrhythmias are caused by dysfunction of the Na^+ channel. Many serious arrhythmias can be triggered by excessive cytosolic free Ca^{2+} fluctuations. In clinical practice these may be seen in patients with bone metastases, hyperparathyroidism, immobilization of extremities (which have in common hypercalcemia), and cardiac glycoside toxicity. The effects of the n-3 PUFAs on arrhythmias induced by some cardiac toxins shown in *Figure 2* are examples of arrhythmias induced by excessive Ca^{2+} fluctuations. *Figure 6* is another example in which the cytosolic free Ca^{2+} fluctuations were recorded simultaneously with the contractile activity of the neonatal cardiomyocytes.[19] In this experiment lysophophatidylcholine (LPC), an amphiphile, was the toxic agent. It has been incriminated as one of the endogenous chemical mediators of ventricular arrhythmias in ischemic myocardium, which accumulates very early in the ischemic heart.[24,25] Perfusion of the isolated heart or myocyte with LPC had been shown to produce arrhythmias and contractures associated with cytosolic free Ca^{2+} overload. It produces a number of arrhythmogenic electrophysiologic derangements including depolarization of the resting membrane potential,[26,27] increases in automaticity and occurrence of delayed afterdepolarizations with triggered activity.[28] Furthermore, a correlation has been established between the elevated levels of LPC and the production of arrhythmias in the ischemic

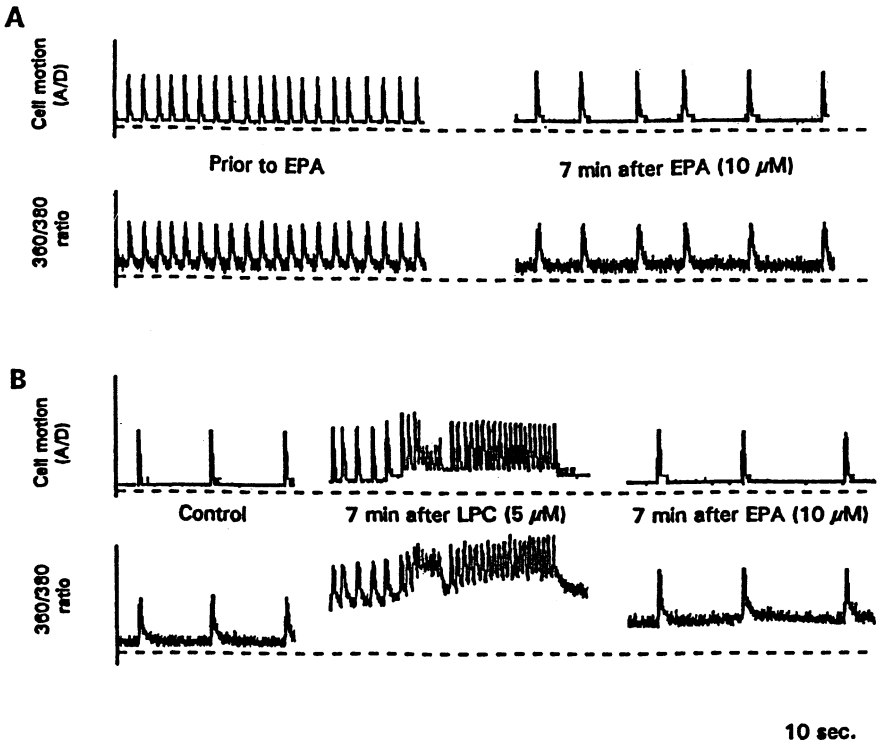

Figure 6 Simultaneous measurements of $[Ca^{2+}]_i$ (as indicated by 360/380 fluorescence ratio of Fura 2) and cell contractions showing the effects of EPA and arrhythmogenic lysophosphatidylcholine (LPC) in cultured neonatal rat cardiomyocytes. *(A)* A representative recording illustrates the $[Ca^{2+}]_i$ transients (lower tracing) and cell contractions (upper tracing) before and after perfusion of eicosapentaenoic acid (EPA; 10 μM) in the absence of LPC ($n = 6$). *(B)* In another cell, tracings show that LPC (5 μM) induces an elevation of basal $[Ca^{2+}]_i$ levels with chaotic transients as cell contracture or tachyarrhythmias occur. Addition of EPA (10 μM) results in return to the initial slow control beating rate and $[Ca^{2+}]_i$ transients with the basal level reduced, but not to normal.

heart.[29,30] *Figure 6A* shows the simultaneous tracings of myocyte contraction (top tracing) and cytosolic free Ca^{2+} levels as estimated by 360/380 nm fluorescence intensity ratio of Fura 2 (lower tracing) in a spontaneously contracting control myocyte before and after the addition of EPA (10 μM) to the superfusate. The contraction of the myocyte results from the spike in cytoslic free Ca^{2+}, which precedes the contraction spike by some 50 ms. The time-averaged cytosolic Ca^{2+} levels remain very low, normally circa 100 nM. EPA reduced the beating rate without altering the amplitude of contractions, as previously reported.[8] On another myocyte that had a slow endogenous beating rate, *Figure 6B* shows the effect of LPC (5 μM) on increasing the cytosolic free Ca^{2+} concentrations and fluctuations and the resulting tachyarrhythmia. The presence of EPA (10 μM) added to the superfusate reduced the cytosolic $[Ca^{2+}]i$, sufficiently to terminate the tachyarrhythmia, though not to normal concentrations in this experiment.

Such excessive cytosolic free Ca^{2+} fluctuations after LPC (*Figure 6B*) can induce delayed after-potentials, which may trigger fatal arrhythmias if the after-potential occurs at a vulnerable moment in the electrical cycle of the heart. Because both $I_{Ca,L}$ and sarcoplasmic reticulum (SR) Ca^{2+}-release underlie many cardiac arrhythmias, Xiao et al.[31] examined the effects of the PUFAs on $I_{Ca,L}$ and Ca^{2+}. Whole-cell voltage clamp techniques and confocal Ca^{2+} imaging were used to determine the effects of PUFAs on the voltage-gated L-type Ca^{2+} current ($I_{Ca,L}$), elementary SR Ca^{2+}-release events (Ca^{2+} sparks), and $[Ca^{2+}]_i$ transients in isolated adult rat ventricular myocytes. Extracellular application of EPA and the other antiarrhythmic PUFAs, but not saturated or monounsaturated fatty acids, produced a prompt and reversible concentration-dependent inhibition of $I_{Ca,L}$. The concentration of EPA to produce 50% inhibition of $I_{Ca,L}$ was 0.8 μM in neonatal rat heart cells and 2.1 μM in adult ventricular myocytes. Although the EPA-induced suppression of $I_{Ca,L}$ did not significantly alter the shape of the current-voltage relation, it did produce a small but significant negative shift of the steady-state inactivation curve ($\Delta V_{0.5} = -3$ to -5 mV). The suppression of the $I_{Ca,L}$ by the PUFAs was voltage and time dependent but not use dependent. This is consistent with the lipophilic nature of these fatty acids, which allows them to enter the hospitable lipophilic environment of the membrane phospholipid bilayer and diffuse laterally to their site of interaction with the transmembrane ion channel protein. This is in contrast to hydrophilic agents, which can only enter the ion channel to reach their active sites via the aqueous pore when the channel is in its open or activated state and therefore, is "use-dependent." The more action potentials, the more total time the channels are in the open configuration permeable to hydrophilic agents.[32] Thus the effects of the PUFAs on $I_{Ca,L}$ resembles their effects on I_{Na}, except that the steady-state inactivation potentials for $I_{Ca,L}$ were shifted to the left to a much lesser degree.

When heart cells become "overloaded" with Ca^{2+}, they become arrhythmogenic[8,19] and produce arrhythmogenic I_{TI} currents and waves of elevated $[Ca^{2+}]i$ that propagate within the heart cell.[31] Also during the Ca^{2+} overload the ryanodine receptors (RyRs) become more sensitive to the triggering process, produce an increased number of spontaneous Ca^{2+} sparks, and produce propagating waves of elevated Ca^{2+}, all of which can be viewed with the confocal microscope while measuring membrane current. This allows examination of the subcellular links between $I_{Ca,L}$, the SR, and cellular Ca^{2+} signaling. There was no reduction or

change in the individual sparks; the time-constant of decay of the calcium sparks was unchanged by the presence of EPA, and there were no effects on the spatial spread of the Ca^{2+} sparks. These findings favored an efficient and unchanged coupling by EPA between the L-type channels in the sarcolemma and the RyRs in the SR. When the number of Ca^{2+} sparks per unit of $I_{Ca,L}$ was determined before and after addition of EPA, the ratio of sparks to $I_{Ca,L}$ was unchanged. This suggests that PUFAs reduce SR Ca^{2+} release only by decreasing the $I_{Ca,L}$ that triggers the SR Ca^{2+} release. Thus it seems our finding that the n-3 PUFAs are potent inhibitors of $I_{Ca,L}$ and that this prevents the cytosolic Ca^{2+} overload[31] appears to be the mechanism by which this cause of triggered arrhythmias evoked by ischemia or cardiac toxins are prevented by the PUFAs.

It has been reported that fish oil feeding inhibits the SR Ca^{2+}-ATPase.[33] Another report indicates that dietary EPA stimulates enhanced microsomal Ca^{2+}-ATPase activity.[11] In this study the activity of NADH-dependent cytochrome C reductase, which is a marker of SR, was the same in the EPA and control groups. Both the purported reduction in SR Ca^{2+}-ATPase activity and the increased sarcolemmal Ca^{2+}-ATPase activity would tend to reduce cytosolic Ca^{2+} fluctuations and constitute additional actions of the PUFAs to influence the SR Ca^{2+} release. Our study, however, did not reveal such additional effects of the n-3 PUFAs.

Although at present we believe that inhibitory effects of the PUFAs on I_{Na} and I_{CaL} seem to be the major effects preventing most arrhythmias, we recognize that other ion channels and currents are also affected by the PUFAs. Both the major repolarizing outward K^+ currents I_{to}, the initial fast outward K^+ current,[34,35] and I_K, the delayed rectifier current,[36] are both inhibited in cardiomyocytes by the PUFAs, whereas I_{K1}, the inward K^+ current, which is activated by hyperpolarization and acts to stabilize the resting membrane potential of the myocyte, is not affected by the PUFAs (Xiao et al., unpublished data). I_{Cl} and the ligand-gated $I_{cAMP,K}$ are also suppressed by EPA (Xiao et al., unpublished data) and perhaps other membrane ion channels, as well.

Effects of PUFAs on the brain

A potential bonus for pursuing the mechanism of the antiarrhythmic effects is the obvious corollary that arose from our findings on the heart, an excitable tissue. Because all excitable tissues in mammals utilize very similar ionic currents and channels for their inter- and intracellular communications, we suspected that other excitable tissues, namely muscle and brain, would also be affected by these PUFAs—and so they are. There is major homology between the ion channel proteins in muscles and nervous tissue with that in heart. To test our expectation, we collaborated with colleagues in Amsterdam and Leiden, Holland, who are part of a large institute for epilepsy research. They found that the PUFAs did indeed modulate both the voltage-dependent Na^+ and L-type Ca^{2+} currents in freshly isolated hippocampal neurons of rats in a manner quite similar to that which we had found in the heart (i.e., no effect of EPA or DHA was found on the activation of either channel, but there was a significant shift of the steady state inactivation potential to more hyperpolarized potentials[37]). As with the antiarrhythmic effects, there were no effects of saturated palmitic acid or of the monounsaturated oleic acid. To learn more about what possible functional effects might result in the brain from these electrophysiologic actions, infusion of EPA or DHA intravenously into rats increased the threshold for localized and generalized seizure activity significantly, using the cortical stimulation model. The threshold remained elevated for over 6 hours but returned to control levels by the following day.[38] Whether the rather modest but significant ($P < 0.001$) effect will have any practical application in epilepsy in humans, we do not know. However, this confirmation of an expectation or prediction based on the antiarrhythmic effects of the PUFAs is supportive of our findings on the heart.

Summary

In summary, it seems apparent that these PUFAs exert an important control of a basic property of the heart and of other excitable tissues, which has been largely overlooked. As we have suggested in the case of the sodium channel, the strong voltage dependence of the shift of the steady-state inactivation to more negative resting membrane potentials has a therapeutically desirable effect. Cells with normal resting membrane potentials will not be functionally affected, whereas partially depolarized cells will be functionally suppressed and, therefore, unable to elicit arrhythmias in the heart or seizure activity in the brain. The suppressing effect on the L-type Ca^{2+} will reduce cytosolic overload of Ca^{2+} fluctuations and triggered after-potential discharges.

With some 250,000 sudden cardiac deaths annually in the United States,[1] and millions more globally, these fatty acids provide a potentially effective and safe preventive agent. The finding that these same PUFAs similarly and specifically modulate neuronal ion currents provides an important physical effect that may underlie the present interest and reports of therapeutic benefits of these n-3 PUFAs in the clinical management of depression[39] and bipolar behavioral disorders.[40]

Our findings may seem bizarre to many today, but interestingly, physicians and biologists accept that over at least some 2 to 4 million years during which these fatty acids have been common ingredients of the human diet of our forebearers, Mother Nature adapted the n-6 PUFAs via the AA cascade to provide many important cell messengers. It was during this period of human evolution that our genes were being adapted to our environment. Until some 10,000 to 15,000 years ago, our forebearers were hunter-gathers and both n-6 and n-3 PUFAs were part of the human diet, comprising perhaps some 4% and 3% of calories, respectively.[41] Perhaps we should not be surprised that during this long evolutionary phase of human existence Mother Nature has adapted the n-3 PUFAs to important cellular functions, some of which have been designed to counter potential deleterious effects of an unopposed excess of n-6 fatty acids that exists in the diets of most Western industrialized countries, even as the n-3 PUFAs have been decreasing or even disappearing from the Western diets.[41] We may be just beginning to scratch the surface of the important biological effects of these interesting fatty acids.

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