Partitioning of polyunsaturated fatty acids, which prevent cardiac arrhythmias, into phospholipid cell membranes

Eric M. Pound, Jing X. Kang, and A. Leaf[1]

Departments of Medicine, Massachusetts General Hospital, and the Harvard Medical School, Boston, MA 02114

Abstract **It has been demonstrated in animal studies that polyunsaturated fatty acids (PUFA) prevent ischemia-induced malignant ventricular arrhythmias, a major cause of sudden cardiac death in humans. To learn how these PUFA, at low micromolar concentrations, exert their antiarrhythmic activity, we studied their effects in vitro on the contractions of isolated cardiac myocytes and the conductances of their sarcolemmal ion channels. These fatty acids directly stabilize electrically every cardiac myocyte by modulating the conductances of specific ion channels in their sarcolemma. In this study, we determined the molar ratio of PUFA to the moles of phospholipid (PL) in cell membranes to learn if the ratio is so low as to preclude the possibility that the primary site of action of PUFA is on the packing of the membrane PL. [^{3}H]-arachidonic acid (AA) was used to measure the incorporation of PUFA, and the inorganic phosphorous of the PL was determined as a measure of the moles of PL in the cell membrane. Our results indicate that the mole percent of AA to moles of phospolipid is very low ($\leqslant$1.0) at the concentrations that affect myocyte contraction and the conductance of voltage-dependent Na^+ and L-type Ca^{2+} channels in rat cardiomyocytes and in α-subunits of human myocardial Na^+ channels. In conclusion, it seems highly unlikely that these fatty acids are affecting the packing of PL within cell membranes as their way of modulating changes in cell membrane ion currents and in preventing arrhythmias in our contractility studies.**—Pound, E. M., J. X. Kang, and A. Leaf. **Partitioning of polyunsaturated fatty acids, which prevent cardiac arrhythmias, into phospholipid cell membranes.** ***J. Lipid Res.*** **2001.** 42: **346–351.**

Supplementary key words eicosapentaenoic acid • docosahexaenoic acid • arachidonic acid • fish oil • sudden cardiac death

In the course of our exploration of the effects of free nonesterified polyunsaturated fatty acids (PUFA) on ion channel conductances in the sarcolemma of cardiomyocytes, it appeared that PUFA blocked many specific ion currents. Previously, Xiao (1) tested only a few ion currents in these cells, mainly the voltage-dependent Na^+ current (I_{Na}) and the L-type Ca^{2+} ($I_{Ca,\,L}$), which we think are the major channels affected by PUFA in preventing malignant cardiac arrhythmias. Potassium channels have also been measured. The two repolarizing potassium currents (I_{to}), the initial fast outward repolarizing and the delayed rectifier (I_K) currents are inhibited, whereas the inward I_{K1} channel, which may stabilize the resting membrane potential, is unaffected. However, Xiao (1) found that the chloride current (I_{Cl}) and the ligand-activated acetylcholine potassium channel (I_{Kachol}) also are inhibited by low micromolar concentrations of PUFA .

In our search for the primary site of action of the PUFA on ionic membrane currents in the heart, the fact that so many diverse ion channels appear to be affected has been puzzling. Beyond the voltage-gated ion channels, there would be little amino acid and structural homology, suggesting that the PUFA may not be binding separately and specifically to each different type of ion channel. Another possible primary binding site might be the phospholipid (PL) cell membrane of the myocytes. It has been shown (2) that the same PUFA, when applied at much higher concentrations than those we have used to modify conductances of ion channels, can affect the structure of the PL membranes, which could, in turn, possibly affect all transmembrane ion channels so as to inhibit their respective conductances.

This is a report of studies performed to learn how much of the PUFA partition into plasma membranes when applied in the bathing medium at low micromolar concentrations at which we studied the in vitro effects of PUFA on the contractions of isolated cardiac myocytes and the conductances of their sarcolemmal ion channels.

MATERIALS AND METHODS

Although our intent in this study was to understand how adding individual fatty acids to the bathing medium affected cul-

Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PL, phospholipid; PUFA, polyunsaturated fatty acids; RBC, red blood cells.

[1] To whom correspondence should be addressed at Massachussetts General Hospital–East, Bldg. 149, Room 4001, 13th St., Charlestown, MA 02129.

e-mail: aleaf@partners.org

tured neonatal or adult cardiomyocyte ion channels, these cells were not appropriate models in which to determine the molar ratios of the fatty acids to the PL of the plasma membranes. These cells have very extensive intracellular PL lipids that would also bind the fatty acids, and perceivably differently than would occur in the sarcolemma alone. Only some 3% of the PL in cardiac myocytes have been reported to be in the sarcolemma of cardiac myocytes (3). Synthetic PL bilayers might be used, but lacking the transmembrane proteins, the uptake of the fatty acids might differ from that in an actual cell membrane.

Preparation of the red blood cell ghosts

Because we could not obtain pure sarcolemmal membranes from our cardiac myocytes, we decided to use human red blood cell (RBC) ghosts (outdated blood from Hospital Blood Bank, used in accordance with Hospital regulations) carefully washed three times with a 1.7 mM Tris buffered saline (BS) and spun down at 18,000 rpm for 20 min after each wash. When cells were free of hemoglobin, they were resuspended in BS solution and lightly homogenized to permit later division into equal aliquots of membrane fragments. The fragments were then incubated at 37°C for 30 min with 1.5 mg of trypsin to remove any protein on the inner and outer cell membrane while leaving the transmembrane protein moieties intact. The conditions for removing extramembrane protein were tested at 15, 30, and 60 min of incubation with trypsin, and no further protein was removed beyond 30 min at 37°C.

An equal amount of the deproteinated ghosts still in the trypsin-containing medium was placed in separate Eppendorf tubes and centrifuged at 18,000 *g* for 20 min. The supernatant was discarded and the pellets resuspended and washed once with the BS, centrifuged again, and the excess liquid was removed from the membrane pellet and sides of the tube with a fine-tipped Pasteur pipette. The membrane fragments were then frozen at −20°C and used within 3 days to assure no microbial growth.

After thawing the frozen membrane pellets, 500 μl of the BS solution was added to each tube and the PL fragments were carefully resuspended with a Pasteur pipette. Five hundred microliters of the [^{3}H]arachidonic acid ([^{3}H]AA)/AA/bovine serum albumin (BSA) solutions, prepared as described below, was then added to the tubes, gently mixed, and the PL fragments were spun down at 18,000 *g* for 20 min. The supernatant was removed without disturbing the membrane pellet. The tubes were then spun again for 5 min at 18,000 *g* to collect any small droplets of liquid that were adhering to the inside of the Eppendorf tubes, which were then removed with a fine-tipped pipette. Two hundred and ten microliters of the incubation medium (free of labeled and unlabeled AA) was added to the PL pellet and the sample was then sonicated to ensure complete homogenization of the suspension. The sample was then divided into four 50-μl parts: two for the inorganic phosphate analysis and two for liquid scintillation counting of the [^{3}H]AA.

Moles of PL in ghosts

The PL in the RBC ghosts were quantified by a slight modification of the method of Mrsny, Volwerk, and Griffith (4) that involved measuring the moles of phosphate after digesting the organic components of the ghosts; this was accomplished by heating the cell fractions with 70% perchloric acid in a sand bath at 140°C, and allowing the sample to reflux overnight or for a minimum of 6 h. After digestion, 3.0 ml of water, 1.0 ml of 2.5% ammonium molybdate, and 0.5 ml of 10% ascorbic acid were added to each tube, the latter being prepared with each use. The mixture was vortexed and placed in an incubator at 37°C for 90 min. The inorganic phosphate was quantified colorimetrically with a Shimadsu UV160U ultraviolet-visible recording spectrophotometer (Kyoto, Japan) at 820 nm, and the quantity of inorganic phosphate in the PL sample read from a standard curve.

Incubation solutions

To determine the ratio of moles of AA to moles of PL, the following solutions and additions were prepared. The incubation medium contained (in mM) NaCl, 133; KCl, 3.6; $CaCl_2$, 1.2; $MgCl_2$, 0.3; glucose, 10; and HEPES, 10 titrated to pH 7.40. To this basic incubation medium was added *a*) high specific activity [^{3}H]AA to yield optimal counting levels at the final dilution; *b*) BSA to yield final concentrations of 33.3, 16.7, 8.3, 4.2, 2.1, 1.5, 1.0, or 0 μM; and *c*) unlabeled AA to yield final concentrations of 27, 22.5, 18, 13.5, 9.0, 4.5, and 1.25 μM. To keep the final volume and the concentration of [^{3}H]AA constant, the volume of unlabeled AA in solution was slightly varied to compensate for the different volumes of BSA solution. Because of the variations in the volume of unlabeled AA required, the reported final concentrations were corrected for the slight adjustments in the different dilutions. All determinations were run in duplicate for each experiment. The molar ratios of AA to PL were calculated and reported as such.

Calculation of the partitioning of AA between albumin and PL

The partition coefficient is defined as the moles of free AA per mole of PL divided by the moles of free AA bound per mole of albumin. This describes the molar equilibrium distribution of AA between the binding sites on RBC ghost membrane PL and delipidated BSA. The mean quantity of PL for each concentration of albumin was calculated and that value was used as the moles of PL in the calculation of the partition coefficients at that concentration of albumin. For the concentrations of albumin tested (1.0, 1.5, 2.1, 4.2, 8.3, 16.7, and 33.3 μM), the mean PL values ± SD were 160 ± 34 nM (n = 28), 164 ± 21 nM (n = 21), 158 ± 19 nM (n = 21), 192 ± 34 nM (n =21), 115 ± 11 nM (n = 8), 205 ± 5 nM (n = 8), and 168 ± 30 nM (n = 8), respectively. The volume of the aqueous phase was 1.0 ml in every experiment.

PUFA effects on cardiomyocyte contractility

Contractility experiments were performed with neonatal rat cardiomyocytes to correlate the effects of varying molar ratios of AA to PL on the function of the myocytes. The cells were prepared and cultured on glass coverslips as reported (5). Effects on the spontaneous beating rate of the myocytes were examined while perfusing the cells at 0.33 ml/min with 5:1, 4:1, 3:1, 2:1, or 1:1.2 ratios of fatty acid to BSA. Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and AA (all 10 μM) were separately studied in these experiments with varying amounts of delipidated BSA to obtain the stated ratios. With the AA experiments, 20 μM indomethacin was added to the perfusion medium prior to adding the AA. Indomethacin alone has no effect on the beating rate of the myocytes, but it will block the arrhythmogenic effects on the beating rate of prostaglandins generated by the action of cyclo-oxygenase on the AA (5, 6).

The contractility experiments were conducted by first adding the PUFA (10 μM) to the fluid perfusing the cultured cells as soon as their rate of contractions became regular. When the characteristic slowing of the beating rate was evident, the perfusing solution was changed to one containing the PUFA (10 μM) with delipidated BSA to produce the desired ratio of PUFA to BSA. If the ratio of PUFA to BSA returned the beating rate to its control rate prior to addition of the PUFA alone, such a result indicated that the concentration of BSA was sufficient to withdraw the PUFA from the myocytes reversing the slowing effect of

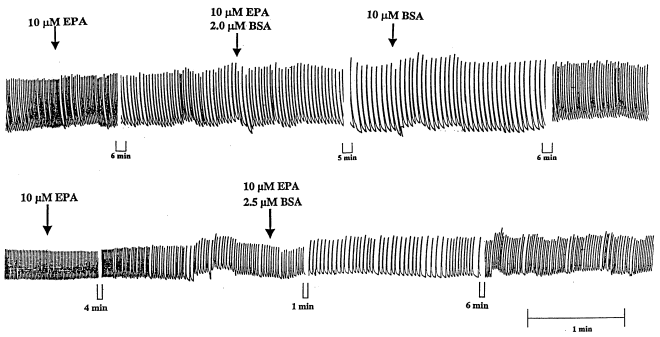

Fig. 1. Tracings of representative contractility experiments with different molar ratios of EPA to BSA were tested to determine at which ratio the typical slowing effect on the rate of spontaneous myocyte contractions changed from "no return" to the control rate of beating prior to the exposure to EPA (top tracing), and the ratio at which there is a "return" to or toward the control beating rate. Each tracing shows the rate and amplitude of spontaneous contractions recorded with an edge monitor from a single myocyte within a small clump of some 10 to several hundred myocytes growing in a syncytium adherent to a microscope coverslip. Following a brief recording of the control rate of contractions, EPA (10 μM) was added to the perfusate bathing the cultured neonatal rat cardiomyocytes. When the slowing effect on the beating rate was manifest, the perfusate solution was changed to the same 10 μM EPA, but with BSA added. The top tracing shows that when the slowing effect persisted, a large concentration of BSA was added with increase in the beating rate toward the control rate. This indicates that the persistent slow beating rate following the exposure to EPA with BSA at a ratio of 5:1 was due to sufficient free EPA still partitioned in the phospholipid sarcolemma to maintain the slow beating rate. By contrast, the lower tracing shows, in another myocyte on a different coverslip, that at an EPA to BS ratio of 4:1, the beating rate returned toward the faster control rate without any additional increase in BSA. This indicates that at this ratio, the BSA extracted the free EPA from the sarcolemma of the myocytes so that the slowing effect on the beating rate of the EPA could not be sustained. The variations in the amplitudes of contraction and of the baseline are apparent spontaneous changes and are not explained by the experimental protocol. The bracketed gaps in the tracings were necessary to allow showing of the salient features of the experiment within a permissible length of tracing.

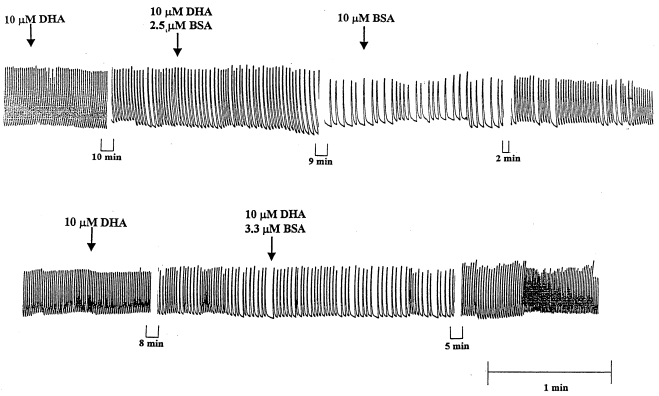

Fig. 2. A representative experiment with DHA showing results as depicted in Fig. 1 for EPA. At the end of the lower tracing, there is an abrupt increase in the beating rate, returning it close to the rate at the start of the lower tracing. This abrupt increase was spontaneous. Note that the shift from "no return" to "return" occurred with DHA at ratios of DHA to BSA between 4:1 and 3:1.

the free PUFA on the myocyte. If that ratio of PUFA to BSA failed to return the slowed beating rate back to the control rate after 10 min of recording, another 10 μM BSA was added to the perfusion fluid. This large excess of BSA would then return the beating rate to the control level, indicating that the slowing had been the result of free PUFA partitioned into the sarcolemma of the myocyte. **Figures 1** and **2** show representative experiments testing EPA and DHA, respectively, at stated ratios with BSA in this manner.

RESULTS

The average of all experiments performed with concentrations of delipidated BSA varying from 0 to 33.3 μM for each of a number of concentrations of free AA varying from 1.1 to 27.0 μM is shown in **Table 1** and in **Fig. 3**. The values recorded in Table 1 are the ratios of moles of free AA per mole of PL in the lipid membrane. **Table 2** shows the partition coefficient defined as the ratios from Table 1 divided by the moles of free AA per mole of albumin. This is the equilibrium distribution of AA between albumin and RBC ghost PL. All experiments performed with ⩽4 μM BSA were done at least in triplicate.

Two things are notable from this data. First, in our prior in vitro experiments on the contractility of neonatal rat cardiomyocytes, on the whole-cell patch clamp studies of the rat sodium and calcium channels, and on the human myocardial sodium channels, 10 μM PUFA was the maximal concentration used, with rare exceptions. At 10, 5, and 1.0 μM concentrations in the absence of BSA, there were only 3, 1.6, and 0.35 mol% of AA per mole of PL, respectively. At the two highest concentrations of BSA tested (16.7 and 33.3 μM), the percent AA to PL ratios were essentially zero. The concentration of BSA we have regularly used to reverse effects of the PUFA on the myocytes by extracting the free fatty acids from the cardiac myocytes is 33.3 μM (equal to 2 mg/ml). The virtual absence of AA in the PL membranes at this concentration of albumin confirms the adequacy of this usage of delipidated BSA.

Second, at 4.2 μM BSA, the concentrations of AA in the PL rose to 1% of PL. With lower concentrations of BSA, increasing amounts of AA were found in the lipid membranes. With 2.1 μM BSA and 8–9 μM AA, AA is seen to

TABLE 1. Molar ratios of AA to PL of RBC ghosts on exposure to varying concentrations of AA and delipidated BSA

	BSA							
AA	0	1	1.5	2.1	4.2	8.3	16.7	33.3
	$\mu M \times 10^{-4}$							
1.1	38	6.03			1.78	1.67	0.51	0.03
4.5	138	38.7	22.6	13.3	6.05	7.54	1.15	0.96
9.0	282	115	91.7	49.7	11.4	18.8	2.77	2.20
13.5	383	238	182	118	21.9	17.6	5.27	3.45
18.0	531	380	291	244	41.7	30.6	8.63	4.69
22.5	730	556	449	348	74.3	35.6	12.9	5.94
27.0		759	568	491	139	46.1	18.0	7.18

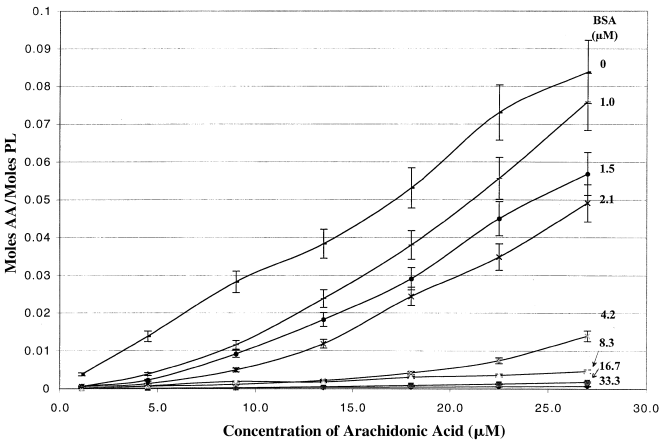

Fig. 3. The ratio of moles of AA to moles of delipidated BSA is shown for increasing concentrations of AA. The molar ratios are indicated on the ordinate and the concentrations of free AA are indicated on the abscissa. Each drawn line shows the ratios at increasing concentrations of AA to the single concentrations of BSA indicated for each line at the right. The standard error vertical bars are shown at the concentrations of AA actually tested.

partition into the PL and its concentration rises significantly above the baseline. Similar results occur at 4–5 μM AA with 1.0 μM BSA. This occurs at ratios of AA to BSA of approximately 3:1 or 4:1.

Having found what ratios of fatty acid to BSA were associated with a significant increase of AA in the PL membrane fragments, it became of interest to learn what ratio would provide sufficient free fatty acid in the cardiomyocyte membranes to effect a slowing of the beating rate. We have found in spontaneously beating neonatal rat cardiomyocyte cultures that all the antiarrhythmic PUFA characteristically slow the spontaneous beating rate of these cells (5). This correlates very highly with the antiarrhythmic effects of these same PUFA, whereas at similar concentrations, monounsaturated or saturated fatty acids are not antiarrhythmic and do not reduce the contraction rate of the myocytes. Therefore, effects of AA, EPA, and DHA on the spontaneous contractions of neonatal rat cardiomyocytes in the presence of varying amounts of BSA were tested to learn at what ratio of free fatty acid to albumin the characteristic slowing of the rate of contractions would be returned to its control rate. Figure 1 illustrates a representative experiment with EPA of such a test, and Fig. 2 illustrates a similar experiment with DHA. The results are summarized in **Table 3**. With AA as the test PUFA, it is apparent that the typical slowing of the beating rate was not returned to its control rate by the addition of albumin to ratios of 8:1 and 5:1 of AA to BSA. This indicates that at such high ratios of AA to BSA, sufficient free AA partitioned into the PL membranes to exert its functional effect on contractions. At 4:1, however, the albumin bound such a high proportion of the 10 μM AA that insufficient free AA accumulated in the membrane PL to sustain the slowing action of the AA. The beating rate, therefore, returned to its faster control rate that existed prior to the addition of AA. It can be seen in Table 3 that with EPA and DHA, respectively, as the ratio of the fatty acid to albumin was reduced, there was a transition from "slowing with no return" to "slowing with return," as occurred with AA. It is of interest to note that with these three PUFA of both n-6 and n-3 classes, the transition occurs at ratios of approximately 3:1 to 4:1. The lower transition ratio for DHA than for the other two PUFA suggests that DHA may be more potent than AA, or that EPA may not be as tightly bound to albumin.

We may now estimate the molar ratio of PUFA to PL just sufficient to produce the functional consequences on cardiomyocytes in which we are interested. This ratio should be that at which the effect on myocyte contractility shifts from "no return to control beating rate" to "return to control beating rate." For both AA and EPA, this change occurred between ratios of 5:1 and 4:1 of PUFA to PL. From Table 1, we can estimate that with 10 μM AA and 2 μM BSA (a ratio of 5:1 in the bathing medium), the molar ratio of AA to PL was 0.008 (0.8% with respect to PL). At an AA to BSA ratio of 4:1, the AA to PL ratio would be even smaller: 0.0046 (0.46% with respect to PL). From the data in Table 1, several sets of ratios of 5:1 and 4:1 may be

TABLE 2. Partition coefficient[a] for AA between albumin and ghost PL

	BSA						
AA	1	1.5	2.1	4.2	8.3	16.7	33.3
	$\mu M \times 10^{-4}$						
1.1	5.68			6.84	12.5	7.59	0.80
4.5	10.0	8.22	6.52	5.80	14.2	4.29	7.13
9.0	16.1	18.3	12.7	5.47	17.7	5.17	8.19
13.5	24.6	26.0	21.4	7.02	11.0	6.57	8.54
18.0	31.9	33.0	36.2	10.2	14.4	8.09	8.72
22.5	40.9	44.6	43.0	14.8	13.4	9.67	8.83
27.0	51.1	48.2	53.5	24.0	14.5	11.3	8.90

[a] Partition coefficient = $(AA^{PL}/PL)/(AA^{Alb}/Alb)$, where PL is the moles of phospholipid in the ghost cell membrane, Alb is the moles of BSA, and AA is the moles of AA in the PL and Alb, respectively.

TABLE 3. Effects of AA, EPA, and DHA on the spontaneous rate of contractions of cultured neonatal rat cardiomyocytes before and after the addition of increasing concentrations of delipidated BSA

	BSA	Molar Ratio of Fatty Acid to BSA	Effects of Fatty Acid on Beating Rate	n[a]
	μM			
AA (10 μM)	1.3	8:1	Slowing with no return[b]	2
	2.0	5:1	Slowing with no return	2
	2.5	4:1	Slowing with return	4
EPA (10 μM)	2.0	5:1	Slowing with no return	5
	2.5	4:1	Slowing with return	7
	3.3	3:1	Slowing with return	4
	5.0	2:1	Slowing with return	1
	12.5	0.83:1	Slowing with return	2
DHA (10 μM)	2.5	4:1	Slowing with no return	3
	3.3	3:1	Slowing with return	5
	5.0	2:1	Slowing with return	3

[a] Number of experiences.

[b] Slows with the addition of fatty acid and doesn't return to control rate with the addition of the respective ratio of fatty acid to BSA.

calculated. The average of four such paired calculations at BSA concentrations of 1.1, 1.5, 2.1, and 4.2 gave mean ratios of 4.7×10^{-3} and 3.7×10^{-3}, respectively, with $P < 0.01$ for difference of means. The estimates for DHA would be even lower. The free fatty acid concentrations at such low ratios would be attained in the absence of BSA at about 1.0 μM EPA or DHA.

The partition coefficients in Table 2, which describe the equilibrium distribution of AA between BSA and RBC ghost PL, are all <1.0. This indicates a stronger affinity of albumin for the AA than of the PL membranes.

DISCUSSION

The aim of this study was to quantify partitioning of the antiarrhythmic PUFA into the PL plasma membranes of cardiac myocytes when bathed in the concentrations at which we studied their in vitro effects. This information will help evaluate the mechanism by which these fatty acids modulate ion channels in the sarcolemma in a manner that prevents ischemia-induced malignant arrhythmias in animals (1, 7–9) and humans (10–12). The primary site of action of these fatty acids has not yet been identified; it could be directly on the channel proteins, or it could be on the PL of the cell membrane. It has been shown that these same fatty acids can alter the physical structure of PL membranes (2) that secondarily might affect the transmembrane ion channels.

Our data show that the molar ratios of fatty acid to PL at the concentrations generally used in our in vitro studies remain very low: generally, 0.01 or less. Furthermore, Xiao et al. (13, 14) obtained classic inhibitory sigmoidal curves relating the concentration of the applied PUFA to the inhibition of ion currents starting at low nanomolar concentrations of the PUFA. Such low concentrations would be unlikely to change the physical state of sarcolemmal PL membranes in the manner demonstrated by Klausner and associates (2). They showed that to the extent they were tested, these same PUFA affect the physical state: the packing of PL in the membrane PL bilayers. However, the applied concentrations of fatty acids were ≥10-fold of those we have shown to suppress currents through the membrane ion channels. Also, we have found that the methyl or ethyl esters of the PUFA have no antiarrhythmic effects (5), although their effects on the membrane PL packing might be expected to be similar to that of the free fatty acids. Cholesterol, which compresses PL membranes, or phytanic acid, which expands them, also had no effect on the action of PUFA when added to the myocyte perfusate (1). Our present finding of very low ratios of PUFA to PL allow more confidence in rejecting the possibility that a primary action of the n-3 PUFA is on the physical state of the sarcolemma. This does not, however, exclude the possibility of a primary effect on the packing of membrane PL in limited domains of the sarcolemma in which are embedded the transmembrane ion channel proteins, changing the latter allosterically.

Because of the extensive intracellular PL membranes within cardiac myocytes, we chose to use human RBC ghosts as a model for the plasma membrane. We determined the molar ratios of PUFA to PL in carefully washed fragments of erythrocyte ghosts to quantify the extent of partitioning that occurs just in the plasma membrane of a cell. This is, of course, based on the assumption that for this kind of inquiry, the RBC ghost and the sarcolemma of a cardiac myoctye would be comparable. We appreciate that the class of PL comprising the membrane (15, 16), their structural fatty acids (17, 18), and possibly the distribution of the PL in the membranes (19) may affect the affinity of the PL for free fatty acids. We think, however, that such factors would not affect the order of magnitude of the values herein reported.

It is of interest to us that at 33.3 μM delipidated BSA in the bathing medium, the concentrations of AA partitioned into the PL were barely detectable by the methods used. This was the case even when initial concentrations of 25 μM free AA were present in the aqueous phase prior to the addition of the PL. This indicates the adequacy of our use of 33.3 μM (2 mg/ml) of delipidated BSA to reduce free PUFA in our cultured rat cardiomyocytes during studies on myocyte contraction or on ionic currents to such low levels that these functional effects of the fatty acids were lost. However, as the ratio of the fatty acid to albumin was increased, there was a transition in the contractility studies (Table 3 and Figs. 1 and 2) from "slowing, no return" to "slowing, with return" as occurred with AA, EPA, and DHA. It is also of interest that with these three PUFA, both n-6 and n-3, that the transition occurs at ratios of approximately 4:1 or 3:1 molecules of fatty acids per molecule of albumin. This we would interpret as signifying the presence of at least three fatty acid binding sites per molecule of albumin, which have higher affinity for the PUFA than do the membrane PL. This finding from our simplistic experiments fits surprisingly well with the more sophisticated studies performed by Cistola, Small, and Hamilton (20) of the number of high-affinity binding sites determined by nuclear magnetic resonance (NMR) using nonperturbing ^{13}C-carboxy-labeled fatty acids, as well as those of Kamp and Hamilton (21) by fluorescence spectroscopy. These studies showed three binding sites with high and nearly equal affinities at room temperature for three fatty acids per albumin molecule.

In conclusion, the major finding of this study is that the ratios of free fatty acid to PL attained at the PUFA concentrations we have been studying for their effects on cardiac arrhythmias are so low (<1.0%) that it seems unlikely that PUFA are affecting the packing of PL within cell membranes as their way of modulating changes in cell membrane ion currents. This finding does not preclude the possibility that PUFA may concentrate within domains of the membrane through which the transmembrane segments of ion channels penetrate and, thereby, affect the ion channel conductances. Also, in accord with much more direct and precise NMR and fluorescence measurements, our functional studies are in accord with a number of highest binding affinities for free fatty acids per molecule of albumin of 3.

We thank James Hamilton, Frits Kamp, and Klaus Gawrisch for helpful discussions. This study was supported by grants DK38165 and HL62284 of the National Institutes of Health of the United States Public Health Service (A.L.).

Manuscript received 31 July 2000 and in revised form 26 October 2000.

REFERENCES

1. Leaf, A., J. X. Kang, Y-F. Xiao, G. E. Billman, and R. A. Voskuyl. 1999. The antiarrhythmic and anticonvulsant effects of dietary n-3 fatty acids. *J. Membrane Biol.* **172:** 1–11.
2. Klausner, R. D., A. M. Kleinfeld, R. L. Hoover, and M. J. Karnovsky. 1980. Lipid domains in membranes. *J. Biol. Chem.* **255:** 1286–1295.
3. Gross, R. W. 1992. Myocardial phospholipases A_2 and their membrane substrates. *Trends Cardiovasc. Med.* **2:** 115–121.
4. Mrsny, R. A., J. J. Volwerk, and O. H. Griffith. 1986. A simplified procedure for lipid phosphorus analysis shows that digestion rates vary with phospholipid structure. *Chem. Phys. Lipids.* **39:** 185–191.
5. Kang, J. X., and A. Leaf. 1994. Effects of long-chain polyunsaturated fatty acids on the contraction of neonatal rat cardiac myocytes. *Proc. Natl. Acad. Sci. USA.* **91:** 9886–9890.
6. Li, Y., J. X. Kang, and A. Leaf. 1997. Differential effects of various eicosanoids on the production or prevention of arrhythmias in cultured neonatal rat cardiac myocytes. *Prostaglandins.* **54:** 511–530.
7. McLennan, P. L., M. Y. Abeywardena, and J. S. Charnock. 1985. Influence of dietary lipids on arrhythmias and infarction after coronary artery ligation in rats. *Can. J. Physiol. Pharmacol.* **63:** 1411–1417.
8. Billman, G. E., J. X. Kang, and A. Leaf. 1997. Prevention of ischemia-induced cardiac sudden death by n-3 polyunsaturated fatty acids. *Lipids.* **32:** 1161–1168.
9. Billman, G. E., J. X. Kang, and A. Leaf. 1999. Prevention of ischemia-induced cardiac sudden death by pure n-3 polyunsaturated fatty acids. *Circulation.* **99:** 2452–2457.
10. Burr, M., J. F. Gilbert, R. M. Holliday, P. C. Elwood, A. M. Fehily, S. Rogers, P. M. Sweetnam, and N. M. Deadman. 1989. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet.* **334:** 757–761.
11. De Lorgeril, M., S. Renaud, N. Mamelle, P. Salen, J-L. Martin, I. Monjaud, J. Guidollet, P. Touboul, and J. Delaye. 1994. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. *Lancet.* **143:** 1454–1459.
12. GISSI-Prevenzione Investigators. 1999. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. *Lancet.* **354:** 447–455.
13. Xiao, Y-F., S. N. Wright, G. K. Wang, J. P. Morgan, and A. Leaf. 1998. Fatty acids suppress voltage-gated Na^+ currents in HEK293t cells transfected with the α-subunit of the human cardiac Na^+ channel. *Proc. Natl. Acad. Sci. USA.* **95**: 2680–2685.
14. Xiao, Y-F., A. M. Gomez, J. P. Morgan, W. J. Lederer, and A. Leaf. 1997. Suppression of voltage-gated L-type Ca^{2+} currents by polyunsaturated fatty acids in adult and neonatal rat ventricular myocytes. *Proc. Natl. Acad. Sci. USA.* **94:** 4182–4187.
15. Kamp, F., J. A. Hamilton, and H. V. Westerhoff. 1993. Movement of fatty acid analogues, and bile acids across phospholipid bilayers. *Biochemistry.* **32:** 11074–11086.
16. Gross, R. W. 1984. High plasmalogen and arachidonic acid content of canine myocardial sarcolemma. *Biochemistry.* **23:** 158–165.
17. Daniels, C., N. Noy, and D. Zakim. 1985. Rates of hydration of fatty acids bound to unilamellar vesicles of phosphatidylcholine or to albumin. *Biochemistry.* **24:** 3286–3292.
18. Hamilton, J. A., and D. P. Cistola. 1986. Transfer of oleic acid between albumin and phospholipid. *Proc. Natl. Acad. Sci. USA.* **83:** 82–86.
19. Bevers, E. M., P. Comfurius, W. C. Dekkers, and R. F. A. Zwaal. 1999. Lipid translocation across the plasma membrane of mammalian cells. *Biochim. Biophys. Acta.* **1439:** 317–330.
20. Cistola, D. P., D. M. Small, and J. A. Hamilton. 1987. Carbon-13 NMR studies of saturated fatty acids bound to bovine serum albumin. I. The filling of individual fatty acid binding sites. *J. Biol. Chem.* **262:** 10971–10979.
21. Kamp, F., and J. A. Hamilton. 1992. pH gradients across phospholipid membranes caused by fast flip-flop of fatty acids. *Proc. Natl. Acad. Sci. USA.* **85:** 11367–11370.