

**CHEM*452 Metabolic Processes
Term paper**

**Dietary supplementation of n-3 polyunsaturated fatty acids (PUFAs):
examining the evidence in support of a role for n-3 PUFAs
in the prevention of fatal cardiac arrhythmias**

**Jeff Sharom 0082710
Jeff Haines 0047401**

**Department of Chemistry and Biochemistry
College of Physical and Engineering Science
University of Guelph**

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1. The n-3 fatty acids

1.1 Nomenclature

The conventional chemical nomenclature for fatty acids (FAs) is to begin numbering of the carbons from the carboxyl end of the molecule. The position of double bonds is designated by the symbol Δ , followed by a number. However, an alternative nomenclature accepted in the nutritional sciences is to number a FA's carbons from the methyl end of the molecule, using the ω - or n- system (1). Thus, whereas the designation Δ^6 indicates the presence of a double bond between carbons 6 and 7 from the carboxy terminus, the designation n-6 indicates the presence of a double bond between carbons 6 and 7 from the methyl terminus. ✓

In addition to saturated FAs, biological membranes require unsaturated FAs in order to maintain their structure and fluidity. Unsaturated FAs are subdivided into 3 families depending on the site of the first double bond in the molecule: either n-3, n-6 or n-9. Most of the n-9 FAs are monounsaturated FAs (MUFAs), whereas the n-3 and n-6 FAs are polyunsaturated FAs (PUFAs). The n-9 FAs are typified by oleic acid (18:1, n-9), the n-6 FAs by linoleic acid (LA; 18:2, n-6), and the n-3 FAs by α -linolenic acid (LNA; 18:3, n-3) (see Figure 1) (1).

1.2 Metabolism

Saturated FAs can be synthesized *de novo* from acetyl coenzyme A, and the 18-carbon saturated FA stearic acid is used as the substrate for unsaturated FA biosynthesis. The introduction of a double bond between C9 and C10 of stearic acid to form oleic acid is catalyzed by Δ^9 desaturase, an enzyme that is found in all plants and animals. Consequently, oleic acid can be synthesized by humans and is not required in the diet. However, only plants contain the enzymes necessary to insert additional double bonds into oleic acid between the double bond at

n-9 and the methyl terminus. Thus, n-3 and n-6 PUFAs cannot be synthesized *de novo* in humans and other mammals, and must be obtained from the diet (so-called essential FAs) (2).

Once LA (18:2, n-6) and LNA (18:3, n-3) are obtained in the diet, however, they can be used as substrates to synthesize all further required PUFAs. LA and LNA are metabolized to longer-chain PUFAs by the addition of more 2-carbon units and the insertion of additional double bonds between n-9 and the carboxy terminus of the molecule. The primary site of this synthesis in humans is the liver. LNA (18:3, n-3) is converted to eicosapentaenoic acid (EPA; 20:5, n-3) and docosahexaenoic acid (DHA; 22:6, n-3), while LA (18:2, n-6) is converted to γ -linolenic acid (20:3, n-6) and arachidonic acid (AA; 20:4, n-6) (see Figure 2). Interestingly, the n-3 and n-6 classes of PUFAs compete for the same set of elongase and desaturase enzymes to catalyze their respective synthetic pathways, with the n-3 FAs being the preferred substrates. Thus, increasing dietary consumption of n-3 FAs results in decreased synthesis of AA from LA due to competition, the significance of which will become apparent in the discussion of the eicosanoids (see below) (1).

1.3 Functions *in vivo*

1.31 Modulation of eicosanoid biosynthesis

The eicosanoids are a family of 20-carbon molecules derived from γ -linolenic acid, EPA or AA. Eicosanoids are synthesized in various cell types, and act as chemical messengers for a variety of intracellular and extracellular signals. They are autacoids (local hormones) that have a short half-life and exert their biological effects in close proximity to the cell in which they are synthesized. Various eicosanoid classes include the prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), lipoxins (LXs), hydroxyeicosatetranoic acids (HETEs), and hydroperoxyeicosatetranoic acids (HPETEs). Eicosanoids like the PGs control the intensity of

the immune response by causing fever, increased vascular permeability, vasodilation, pain, edema, and other pro-inflammatory effects (1).

The first step in eicosanoid synthesis is the release of the 20-C FA precursor from plasma membrane phospholipids by the action of phospholipase A₂ (see Figure 3) (2). Although AA usually serves as the 20-C precursor for eicosanoid synthesis, dietary consumption of n-3 FAs leads to partial replacement of the AA (20:4, n-6) in plasma membrane phospholipids with EPA (20:5, n-3). This is a direct result of the aforementioned competitive inhibition that exists between the n-3 and n-6 FAs for cellular elongases and desaturases. Importantly, the eicosanoids derived from EPA generally have 10- to 100-fold less biological activity than the analogous eicosanoids derived from AA. Thus the net effect of the substitution of n-6 FAs with n-3 FAs in eicosanoid biosynthesis is down-regulation the inflammatory response, which can have far-reaching effects on human disease (see later) (3).

1.32 Alteration of membrane fluidity, structure and function

The FA composition of the phospholipids within biological membranes is clearly influenced by FA dietary intake. The PUFAs are typically esterified to the second carbon in the glycerol backbone, and their presence increases overall membrane fluidity due to the disordering effect of their irregular acyl chain. This increase in fluidity can increase the lateral diffusion rate of membrane proteins and receptors in the lipid bilayer. In addition, membrane proteins and receptors are known to be sensitive to their surrounding FA environment – many retain one or more concentric rings of closely-associated membrane lipids, known as a lipid annulus. Lipid-protein interactions can thus influence the formation of lipid-raft signaling complexes, the binding of ligands to their receptors, and signal transduction to the cell interior (3).

ω_3 5 7

1.33 Signal transduction and gene expression

In addition to their effects on signal transduction via plasma membrane receptors and eicosanoid biosynthesis, many lipids also act as important second messengers in intracellular signaling pathways. For instance, many receptors in the plasma membrane transduce signals via G protein complexes to phospholipase C, which acts to cleave phosphatidylinositol-4,5-bisphosphate (PIP₂) to inositol triphosphate (IP₃) and diacylglycerol (DAG). IP₃ causes release of Ca²⁺ from the endoplasmic reticulum, whereas DAG acts in association with Ca²⁺ to activate protein kinase C (PKC). In turn, PKC goes on to phosphorylate and alter the activity of numerous protein targets within the cell. The presence of PUFAs in lipid messengers like DAG is known to influence their effects, although the details are not well understood at this time (3).

*PC-specific release
of DAG also
occurs.*

2. Dietary supplementation with n-3 fatty acids

2.1 Sources of n-3 FAs in the human diet

The primary sources of n-3 FAs in the human diet are vegetable oils and fish (4). Soybean and canola oils are important dietary sources of n-3 FAs in the form of LNA, which is the major FA in chloroplast lipids. Flaxseed oil is an especially rich source of LNA, but is not commonly consumed. Fatty fish (halibut, salmon, mackerel, herring, etc) are rich sources of n-3 FAs in the form of EPA and DHA. The abundance of n-3 FAs in fish oils is a result of their transfer through the food chain from phytoplankton and other marine plants that carry out the elongation and desaturation of LNA (2).

2.2 Meeting dietary recommendations for n-3 fatty acids

Health Canada recommends a total n-3 FA intake of 1.2-1.6 g/d but does not distinguish between LNA, EPA and DHA (5). A group of U.S. nutrition scientists recommends that intake of

LNA be 2.2 g/d and combined intake of DHA and EPA be 0.65 g/d (6), but calculations show that achieving such levels will require an approximate 4-fold increase in n-3 FAs in the U.S. diet. This can theoretically be achieved by consuming 20-62 g/d of fatty fish along with 22-32 g/d of a LNA-rich vegetable oil such as canola (4). As an alternative to such a radical change in eating habits (which may not be realistically attainable), meeting the recommended intake of n-3 FAs could be partially fulfilled by the consumption of dietary supplements, several of which are currently available to consumers. Supplements derived from fish oils typically contain 120 mg DHA and 180 mg EPA and per capsule, while those derived from algae typically contain 100 mg DHA per capsule (4). Note that these should be consumed with care due to the inclusion of large quantities of vitamin A and D. The n-3 FAs themselves do not produce any clinically significant adverse effects at doses up to 3 g/d (7). Clinical deficiency of the n-6 FAs is more common than that of the n-3 FA's, and both are rare. In humans, high levels of DHA are found in the cerebral cortex, retina and testis, and n-3 FA deficiency in infancy has been associated with impaired cognitive and visual function (1). ✓

2.3 Modulation of the n-3: n-6 fatty acid ratio

There are indications that the dietary n-3: n-6 FA ratio is more important than the absolute levels of these 2 types of FAs (4). This is once again a direct result of the competition that exists between the n-3 and n-6 FAs for cellular elongases and desaturases. High amounts of LA in the diet can thus decrease the endogenous conversion of LNA to EPA and DHA *in vivo*. Interestingly, it appears as if the intake of n-6 versus n-3 FAs has undergone a dramatic shift over the course of human evolution (8). In the Paleolithic era, the ratio of n-6: n-3 FAs in the human diet is believed to have been close to 1:1. In present day industrialized countries, this ratio is estimated to be closer to 10:1, while for some individuals it may be as high as 25:1 (4). The } ?

optimum n-3: n-6 ratio – calculated to maximize the endogenous conversion of LNA to EPA DHA – is 2.3: 1 (9). The substantial increase in the consumption of n-6 FAs in the North American diet has mostly occurred in the last 100 years, and is due to a combination of modern agricultural methods and the large-scale production of vegetable oil for cooking (4). In contrast to wild game animals, modern beef cattle contain virtually no n-3 FAs as a result of being fed n-6 FA-rich grains. Modern fish-farming techniques also produce fish that contain less n-3 FAs than wild fresh- or salt-water fish. In addition, the partial hydrogenation of soybean oil that is used in many food-manufacturing processes results in a decrease in levels of n-3 FAs, while leaving high levels of n-6 FAs. ✓

2.4 Food enrichment with n-3 fatty acids

2.41 Conventional approaches

The recommended daily intake of n-3 FAs can be attained through dietary means by the consumption of about 4 fatty fish meals per week (4). However, it appears highly improbable that the majority of the population would be willing to comply with such a dietary modification. Furthermore, it should be noted that the global implementation of such a strategy is impossible. Calculations indicate that there is not enough fish in the biosphere to supply the global population with an n-3 FA dose of 0.5 g/d (10). Both of these factors have provided the impetus for the development of alternate methods for the introduction of n-3 FAs into the human food chain. /

One solution, which circumvents the problem of consumer non-acceptance of fish, is the incorporation of n-3 FAs into products such as meat, eggs, and dairy products (4). Such enrichment can be accomplished by the addition of fish oil, fishmeal, or algae into animal feed. However, in addition to the added expense associated with this strategy, it is also hampered by

the tendency of the n-3 FAs to oxidize and thus produce an unpleasant flavour in food products. This oxidation can be partially prevented by increasing the α -tocopherol content of the animal feed, but this further increases costs. To date, the only n-3 FA-enriched animal product to be marketed is the n-3-enriched egg, which has met with partial success. ←

✓
taste
fishy

Another option, which addresses the unfeasibility of meeting the recommended n-3 FA intake through fish consumption alone, is the expansion of plant sources of n-3 FAs. For instance, the vegetable purslane - consumed extensively in the Mediterranean and Middle East but largely unknown in North America – is the richest source of LNA of any known leafy green plant, and one of the few that contains significant quantities of DHA (11). Purslane grows well in most parts of the U.S. and Canada, and its cultivation could provide a convenient supply of n-3 FAs of plant origin that would also be suitable for vegetarians.

2.42 Recombinant DNA techniques

The most powerful approach to incorporating n-3 FAs into the human food chain is via genetic manipulation of agricultural plant species. The modification of genes that mediate n-3 FA synthesis in traditional crops such as soybean and canola could be used to increase the n-3 FA content of their oil (12). In addition, EPA and DHA, which are not commonly synthesized by land plants, could be produced in a plant species of choice by introduction of the appropriate elongase and desaturase enzymes. The use of recombinant DNA technology has the potential to be an efficient and cost-effective way of introducing n-3 FAs into the diet of the general population. At the same time, such an approach would no doubt be severely hampered by widespread consumer non-acceptance of transgenic organisms and food products derived from them. ✓

2.5 Effects of n-3 fatty acids on human health and disease in general

The n-3 FAs are essential components of human cells, where they comprise structural components of membranes, serve as sources of energy, and act as precursors for a variety of biologically-active signaling molecules (3). This class of PUFA has been shown to have a beneficial effect in a staggering array of human disease states, including cardiovascular disease (atherosclerosis, hypertension, cardiac arrhythmias), cancers (of the breast, colon, prostate), renal disease, rheumatoid arthritis, inflammatory bowel disease, transplant rejection, susceptibility to post-surgical infection, and behavioral disorders like clinical depression (13). Indeed, this class of PUFA has been the subject of thousands of research articles in the last 2 decades. A discussion of all of these potential health benefits of n-3 FAs is clearly beyond the scope of this paper. In order to narrow this extremely broad subject, the effects of n-3 FAs on cardiovascular disease (CVD) were considered in more detail. The link between n-3 FAs and CVD is widely believed to have the most solid experimental and epidemiological data in its favour as compared to some of the other disease associations mentioned above, for which the evidence not yet well-established (13).

2.6 Effects of n-3 fatty acids on cardiovascular disease

The first indication of a role for n-3 FAs in CVD came with a pioneering study in the 1970's that showed that the Greenland Inuit had a low rate of CVD despite the consumption of a diet which was very high in fat (14). The key observation was that this fat was from marine vertebrates like seals, whales and fish, and was thus rich in n-3 FAs. Since then, the effects of n-3 FAs on CVD have been documented in hundreds of studies, including tissue culture experiments, animal studies, and well-controlled clinical trials (13). They are believed to prevent heart disease via a variety of mechanisms, including preventing cardiac arrhythmias, acting as

eicosanoid precursors, inhibiting atherosclerosis, inhibiting cytokine synthesis, reducing blood pressure, and having anti-inflammatory, anti-thrombotic, and hypolipidemic properties (13). A discussion of all these mechanisms of action is again beyond the scope of this paper. In order to further narrow this extensive subject area, the effects of n-3 FAs on cardiac arrhythmias (ventricular tachycardia and fibrillation) were chosen for more in-depth study. About 60% of deaths from myocardial infarction are classified as “sudden deaths”, and are almost all attributable to ventricular fibrillation. The remainder of this paper will thus focus on experimental evidence for a role of the n-3 FAs in making the heart less vulnerable to fatal arrhythmias.

3. The role of n-3 fatty acids in the prevention of cardiac arrhythmias

Research in this area has been dominated by the laboratory of Leaf (Massachusetts General Hospital, and Harvard medical School, Boston, Mass., U.S.A), who has been studying the link between n-3 FAs and cardiac arrhythmias for over a decade. The period 1990-2001 was a prolific time for Leaf's group, with 10 ground-breaking publications appearing in the high-profile journal P.N.A.S. alone (15-24). Two of the more interesting experimental systems will be analyzed below.

3.1 Effects of n-3 fatty acids in a dog model of fatal cardiac arrhythmias

3.11 Experimental system

Following some intriguing reports linking n-3 FAs and ventricular fibrillation in rats (25) and marmosets (26), Leaf's group sought to examine the effect of n-3 FAs in a canine model of fatal cardiac arrhythmias (17). In this experimental model, dogs underwent a surgical procedure in which their left anterior coronary artery was ligated to produce a large myocardial infarction.

Also, a hydraulic occluder was fitted around their left circumflex coronary artery, allowing the researchers to cut off circulation in this artery at will. After allowing the animals 1 month to recover, they were subjected to an exercise/ischemia test. With the dogs running on a treadmill at a fixed heart rate, the researchers occluded the left circumflex coronary artery using the hydraulic cuff. In 60% of the dogs, this resulted in unconsciousness and ventricular fibrillation within 2 minutes (from which they were resuscitated), while the remaining 40% of the dogs did not exhibit any arrhythmias. The "susceptible" subset of 60% of the dogs was subsequently used as the experimental group for the n-3 FA studies. Repeated testing showed that the treadmill exercise/ischemia test reproducibly caused ventricular fibrillation in these animals every time it was carried out.

3.12 Summary of results

The authors monitored the effect of administering an emulsion of partially-purified n-3 FAs (mainly free EPA and DHA), which was released into the blood over a period of 50-60 min just before the exercise/ischemia test. In 7 of the 8 animals tested, this treatment completely prevented the appearance of ventricular fibrillation. In contrast, controls in which the n-3 FA emulsion was replaced by saline solution or an emulsion of soybean oil (containing a small amount of LNA, but no EPA or DHA) resulted in ventricular fibrillation as per usual (see Figure 4). The n-3 FA emulsion also caused an overall reduction in heart rate in 6 of 8 animals, both before and after artery occlusion. Although the n-3 FAs actually caused an increase in heart rate in the remaining 2 dogs, these animals were still protected from ventricular fibrillation when their coronary arteries were occluded in the exercise/ischemia test.

} relevant dose ?

mechanism ?

3.13 Discussion of strengths and methodological limitations

The exercising dog model of fatal cardiac arrhythmias was a useful system for illustrating the anti-arrhythmic properties of the n-3 FAs. Leaf's group became interested in this area after some other animal models yielded interesting results. One study in rats found that ventricular fibrillation could be prevented during coronary artery ligation and reperfusion by the dietary intake of tuna oil (25). Another study in adult marmoset monkeys found that consumption of a fish oil supplement raised the amount of electrical current needed to induce ventricular fibrillation during ischemia (26). Note that the dog study by Leaf's group (17) is different in principle from both of these earlier animal studies, which were based on long-term dietary intake of n-3 FAs in the form of triglycerides. In contrast, this exercising dog study sought to examine the effect of administering n-3 FAs in free form simultaneously with the experimental induction of ventricular fibrillation.

This dog model study has both strengths and weaknesses as compared to the previous animal studies. The use of a concentrate containing 70% n-3 FAs instead of the crude fish oil used in the previous studies makes it easier to attribute the observed effects to the presence of the n-3 FAs rather than other unknown compounds in the oil. Note that later studies using this dog model confirmed this explicitly by employing pure EPA, DHA and LNA – all of which were found to have a similar effect (27). By administering the n-3 FAs intravenously just prior to the exercise/ischemia test, the authors could also be more confident that the observed effects were due to the free FAs on the cells of the heart. Other secondary effects from the long-term dietary consumption of n-3 FAs - such as inhibition of atherosclerosis - could be excluded. ✓

Although the above design made the results simpler to interpret, the question arises as to whether the infusion of 2-5 g of an n-3 FA emulsion directly into the bloodstream represents a

physiologically-relevant situation. The amount of n-3 FAs in the circulation – even directly after a fatty fish meal – would typically be substantially less than this. Also, although the finding that 7 of 8 dogs avoided ventricular fibrillation is statistically significant ($P < 0.05$), it is still a rather small sample size on which to base the conclusions of this study. It should be noted that this series of experiments ultimately involved the physiology of a different species, and under a somewhat unnatural set of experimental conditions. Whether this study's results can be reasonably extrapolated to physiological conditions in humans is an open question. These criticisms aside, there is still no doubt that these findings were exciting and merited further investigation. ✓

3.2 Effects of n-3 fatty acids in a neonatal rat myocyte cell culture model

3.21 Experimental system

In the wake of the above studies in their exercising dog model, Leaf's group sought to elucidate the cellular and molecular basis for the apparent anti-arrhythmic properties of the n-3 FAs. To this end, they employed a cell culture system consisting of neonatal rat myocytes (18). Interestingly, these cells grow in culture to form syncytia of several hundred cells that contract simultaneously and rhythmically as if they were part of an intact heart. Arrhythmias could be induced in these contracting cells by the addition of various arrhythmogenic agents to the tissue culture medium bathing the cells, and were interpreted as being the equivalent of ventricular fibrillation in the whole heart.

In their first study (18), Leaf's group used the cardiac glycoside ouabain and high extracellular Ca^{2+} to induce arrhythmias in the myocytes. The induction of cardiac arrhythmias has been linked to an increase in cytosolic Ca^{2+} levels that occurs in myocytes during ischemia, and ouabain is known to cause a similar increase in cytosolic Ca^{2+} by reversing the Ca^{2+}/Na^+

Ouabain: Na-K ATPase Inhibitor ?!

antiport system. In later studies, similar results were obtained with arrhythmias induced by agents such as cAMP, isoproterenol, lysophosphatidylcholine, acylcarnitine, thromboxane, prostaglandin E₂, and the Ca²⁺ ionophore A23187 (28-30). In order to explore the possible anti-arrhythmic effects of n-3 FAs, they were added to the tissue culture medium in the absence and presence of arrhythmogenic agent, and the electrical activity of the myocytes was monitored. In all experiments, the authors were careful to use concentrations of FAs in the low μM range, thus ensuring that their results would be physiologically relevant.

3.22 Summary of results

The effects of n-3 FAs on this system were intriguing. In every case, if free n-3 FAs were present in the medium before the arrhythmogenic agent was added, the arrhythmias were completely prevented. Alternatively, if the arrhythmias were already in progress, addition of n-3 FAs caused their termination within a short period of time (see Figure 5). The n-3 FAs also caused a decrease in the normal rate of contraction of the myocytes under control conditions in the absence of any arrhythmogenic agents (see Figure 6).

Some important properties of this anti-arrhythmic effect were characterized with a series of experiments using delipidated bovine serum albumin (BSA). Albumin contains 5-8 FA-binding sites and can normally serve as a carrier molecule for FAs in the bloodstream *in vivo*. Delipidated BSA has 3 binding sites with high-enough affinity to actually remove free FAs from cell membranes. If arrhythmias were induced in the myocytes and then terminated by addition of n-3 FAs, subsequent withdrawal of the FAs from the cells with delipidated BSA caused the arrhythmias to recur within a short time (see Figure 5). The decrease in the normal myocyte contraction rate caused by the n-3 FAs could also be reversed by BSA (see Figure 6). These observations demonstrated that the anti-arrhythmic effects of the n-3 FAs were a result of their

partitioning into the lipid bilayer rather than their covalent attachment to any membrane constituents or their incorporation into phospholipids. It also implied that the effects were due to the free n-3 FA themselves and not downstream metabolites like eicosanoids. ✓

By systematically testing different FAs, the authors were able to deduce the structural requirements for a FA to be able to exert these anti-arrhythmic effects. The n-3 FAs LNA, DHA and EPA were all found to be anti-arrhythmic, as were the n-6 FAs LA and AA. All the saturated and monounsaturated FAs examined had no effect on the myocytes, indicating that the presence of at least 2 double bonds was also required. AA, however, displayed complex behaviour: in 1/3 of replicate experiments it was anti-arrhythmic, in another 1/3 it had no effect, and in a final 1/3 it actually induced arrhythmias. This variability was found to be due to the arrhythmogenic effects of AA cyclooxygenase metabolites interfering with the anti-arrhythmic effects of AA itself. For instance, a non-metabolizable analogue of AA was consistently anti-arrhythmic, as was AA when added in conjunction with a cyclooxygenase inhibitor. Interestingly, the ethyl ester and triglyceride forms of the n-3 and n-6 FAs had no effect on the contraction of the myocytes, indicating that the carboxyl group of a free FA was a requirement for activity. !

The authors also showed that the observed anti-arrhythmic effects of the n-3 FAs were most likely due to specific interactions with ion channels or other membrane proteins. They controlled for the non-specific increase in membrane fluidity due to partitioning of the PUFAs into the lipid bilayer by the use of phytanic acid (which increases membrane fluidity) and cholesterol (which decreases membrane fluidity). Neither of these compounds produced any effect on the contraction of the myocytes, suggesting that the n-3 FAs do not exert their effects via a disordering effect on the membrane. Since the excitability of myocytes is regulated by

changes in the activity of Ca^{2+} , Na^+ , and K^+ ion channels, the n-3 and n-6 PUFAs were hypothesized to mediate their effects by interaction with one or more of these proteins.

Subsequent follow-up studies by Leaf's group (19-24) demonstrated that interaction with cellular ion channels was indeed the basis for the anti-arrhythmic effects of the n-3 FAs. During the ischemia of the myocardium that accompanies an acute myocardial infarction, the resting membrane potential of cardiac myocytes is known to become partially depolarized due to reduced output of the Na^+/K^+ ATPase. These myocytes are hyperexcitable due to the close proximity of their resting membrane potential to the threshold for gating the inward Na^+ current that triggers the action potential, and are thus vulnerable to the induction of an arrhythmia. Interestingly, the n-3 FAs were shown to exert a stabilizing effect on the electrical excitability of the neonatal rat myocytes by hyperpolarizing their resting membrane potential (19). The depolarization required to elicit an action potential was increased by ~50% and the refractory period between action potentials was increased by ~150%.

Further work elucidated the basis for this electrical stabilization at the molecular level. The n-3 and n-6 PUFAs were shown to inhibit the conductance of voltage-gated Na^+ and Ca^{2+} channels and to increase the duration of their closed + inactivatable state (19, 20, 22). By using the patch clamp technique on neonatal rat myocytes, it was found that EPA and DHA blocked the Na^+ current with an IC_{50} of 4.8 μM , and the L-type Ca^{2+} current with an IC_{50} of 0.8 μM . The blocking of Ca^{2+} entry into the myocytes was thought to account for the prevention of arrhythmias due to elevated extracellular Ca^{2+} and toxic concentrations of the cardiac glycoside ouabain. Leaf's group also used a radiolabeled-ligand binding assay (21) to show explicitly that the n-3 and n-6 PUFAs block the Na^+ current by binding to and stabilizing an inactivated state of the Na^+ channel. The PUFA-binding site on the channel was originally theorized to be the S4

“voltage sensor” segments of the α -subunits. As well as playing a key role in the function of the channel, these S4 voltage sensors are positively charged and hydrophobic - perfect for interaction with the hydrophobic tail and negatively-charged carboxyl group of the PUFAs. Although an elegant hypothesis, experimental data acquired in a later study (23) was found to be incompatible with this mode of interaction, causing the authors to concede that the PUFAs must be binding at a different site.

Most recently, Leaf's group has demonstrated the inhibitory effect of PUFAs on the human cardiac Na^+ channel, expressed in isolation in transfected HEK293t human embryonic kidney cells (23). A study published this year targeted various residues of the human cardiac Na^+ channel for site-directed mutagenesis (24). Results demonstrated that mutation of an Asn at position 406 significantly decreased the inhibitory effect of PUFAs on the channel, implicating this residue as part of a putative binding site.

Wow

3.23 Discussion of strengths and methodological limitations

The spontaneously contracting neonatal rat myocyte cell culture system was a useful tool for dissecting the effects of n-3 FAs on cardiac arrhythmias. It allowed the study of isolated cardiac cells without the interference of outside electrical and hormonal influences that would otherwise complicate the interpretation of results. This series of experiments was also characterized by a rigorous and elegant set of controls. The structural requirements for a FA to have anti-arrhythmic effects were deduced from parallel experiments with n-3 PUFAs, n-6 PUFAs, n-9 MUFAs, saturated FAs, as well as the ethyl ester and triglyceride forms of the PUFAs. Washout experiments with delipidated BSA were convincing in demonstrating the direct causal relationship between partition of the PUFAs into the myocyte cell membrane and the

prevention of arrhythmias. The non-specific disordering effect of the PUFAs on the lipid bilayer was also controlled for with the use of phytanic acid and cholesterol.

At the same time, the limitations of this cell culture model should be considered. There are well-documented inter-species differences in myocyte electrical excitability and ion channel expression – consequently, extrapolating these effects to human cardiac cells should be done with caution. It is also possible that some properties of the neonatal myocytes used in these studies are significantly different from those found in adult animals and humans - for instance, adult rat myocytes do not exhibit the same spontaneous contraction in cell culture (20). It is thus prudent to keep in mind that the generalizability of the results may be limited. These criticisms aside, it should be noted that Leaf's group later succeeded in observing similar PUFA-mediated inhibition of the human cardiac Na^+ channel when it was expressed in isolation in a laboratory cell line (23). This supports the notion that many of the results obtained in the rat myocytes may also be valid in their human counterparts.

2.3 Conclusions

The aforementioned studies illustrate the power of a reductionist approach in elucidating the basis for the n-3 FAs' physiological effects *in vivo*. This line of inquiry began with epidemiological evidence in humans, and subsequently progressed to experiments at the level of whole animals, cardiac cells, isolated ion channels, and finally individual amino acid residues within those ion channels. Although the studies discussed above clearly show that n-3 FAs have anti-arrhythmic effects on cardiac cells *in vitro* and on whole hearts in experimental animals, even Leaf himself conceded in a recent review that there is presently insufficient evidence to state conclusively that these effects actually prevent fatal cardiac arrhythmias *in vivo* in humans (31). There is still a need for more randomized, double-blind, placebo-controlled studies to

solidify these findings. At the same time, recent years have seen the publication of several studies in humans that further support this anti-arrhythmic role for n-3 FAs. Two recent studies (32, 33) found that compared to placebo, the consumption of purified DHA produced a significant decrease in heart rate in human subjects. Others noted a beneficial effect on heart rate variability (known to be a predictor of arrhythmic events and sudden cardiac death) from the consumption of n-3 FAs, both in survivors of a first myocardial infarction (34) and in healthy men and women (35).

The link between CVD and n-3 intake remains controversial because human epidemiological and population studies have mostly - but not always - shown an inverse relationship between n-3 FA consumption and risk of CVD mortality (37-42). However, variability in the types of human populations used in these studies may account for some of the inconsistent findings in the literature. For instance, in a recent meta-analysis of human studies linking fish consumption and CVD mortality (43), the authors concluded that increasing fish consumption only produces a large reduction in risk of CVD mortality for populations that are already at high-risk. Alternatively, differences in the quantities of n-3 FA examined in various studies might help explain some of the inconsistent results in this field. For instance, compared with no fish intake, a moderate intake of 1-2 fatty fish meals per week significantly reduces CVD mortality, but there is little evidence indicating that mortality can be further reduced by even larger intakes (44). Finally, differences in the types of disease outcomes examined might also play a role. For instance, while many studies have documented the inverse relationship between n-3 FA intake and sudden cardiac death, there is little evidence indicating a significant reduction in the risk of non-fatal cardiac events (39-41).

✓

+
low
ω 3

Nonetheless, the fact remains that the beneficial effects of n-3 FAs on CVD have been documented in hundreds of studies, including tissue culture experiments, animal studies, and well-controlled clinical trials in humans. Taken together, the current body of literature on this subject is convincing. As mentioned previously, there are a number of potential mechanisms thought to underlie the cardiovascular benefits of n-3 FAs, over and above the one discussed here (making the heart less vulnerable to ventricular fibrillation), including inhibiting atherosclerosis, modulating eicosanoid synthesis, inhibiting cytokine synthesis, reducing blood pressure, decreasing platelet aggregation, etc. Based on the available evidence (as perceived by the authors of this report), it seems appropriate to recommend the moderate consumption of n-3 FAs for the maintenance of optimal human health, and for the prevention of CVD in particular. Although achieving the same dietary intake of n-3 FAs as our hunter-gatherer ancestors in the Paleolithic era is unrealistic, a modest increase in our present intake of this class of PUFA would likely confer significant health benefits.

4. Figures

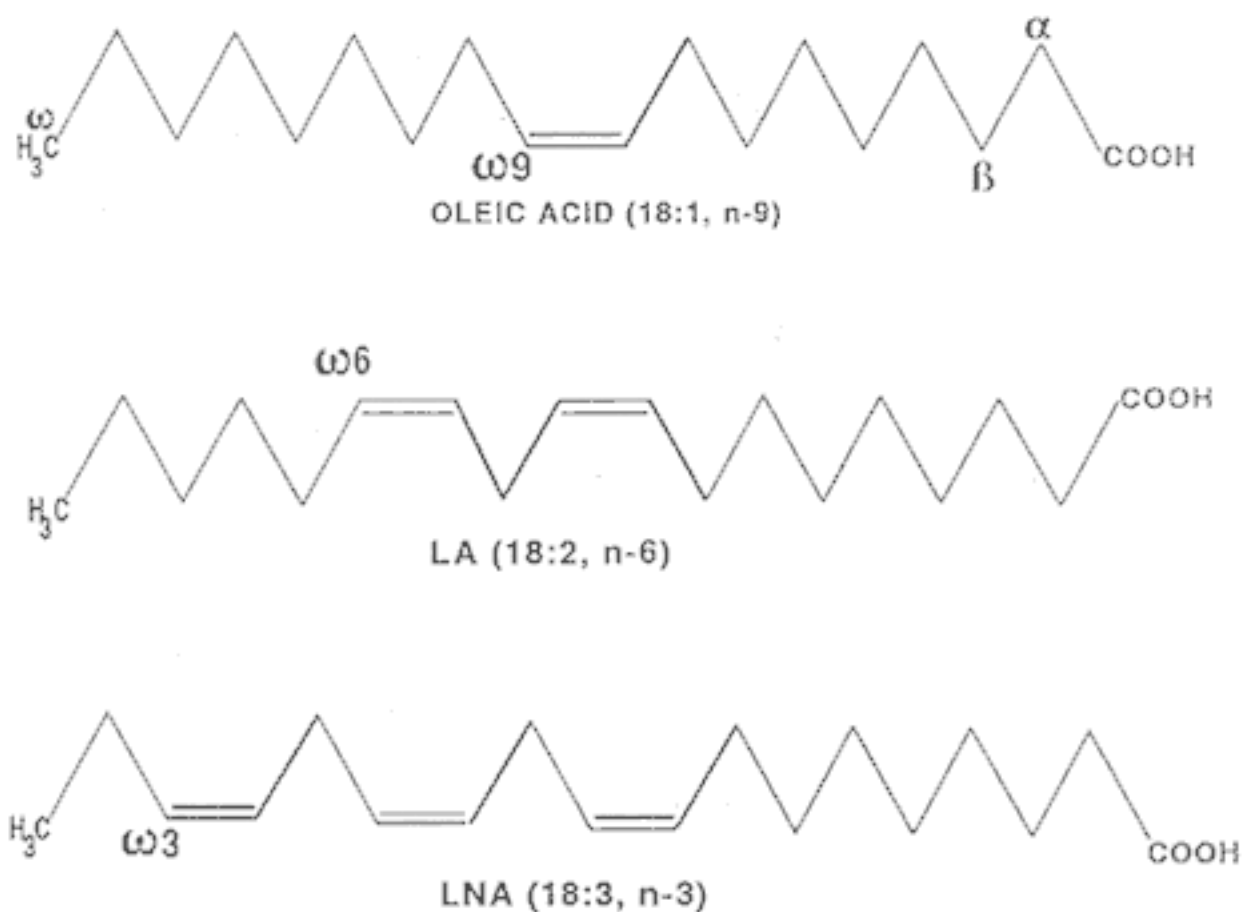
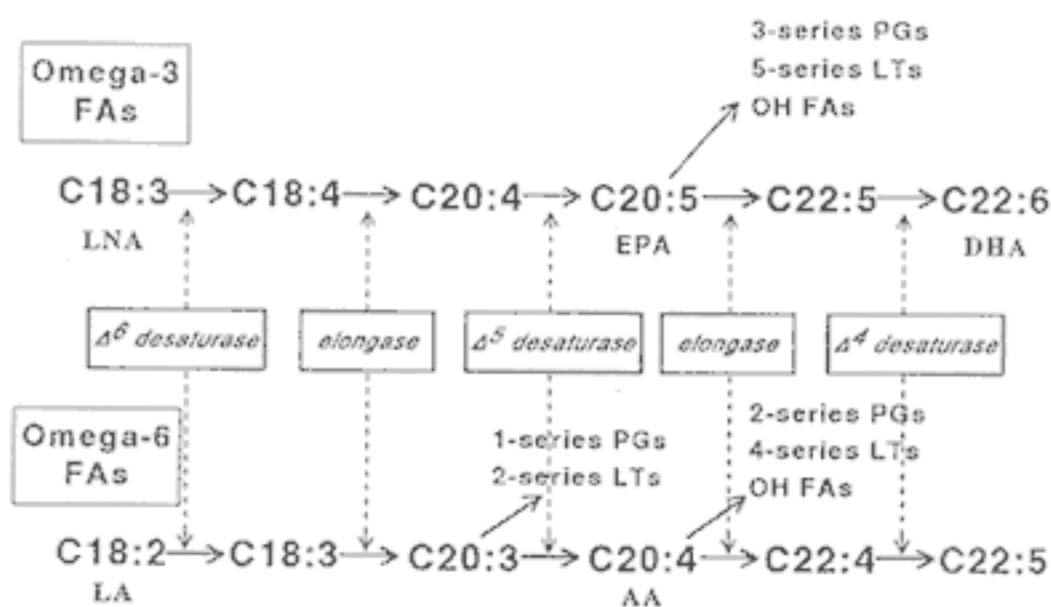


Figure 1. Structures of representative n-9, n-6 and n-3 fatty acids. An alternative nomenclature accepted in the nutritional sciences is to number a fatty acid's carbons from the methyl end of the molecule, using the ω- or n- system. Unsaturated fatty acids are subdivided into 3 families depending on the site of the first double bond in the molecule: either n-3, n-6 or n-9. Shown are the structures of oleic acid (18:1, n-9), linoleic acid (LA; 18:2, n-6), and α-linolenic acid (LNA; 18:3, n-3). [from ref 1]



excellent

Figure 2. Metabolism of n-3 and n-6 fatty acids by the same set of elongases and desaturases. LA and LNA are metabolized to longer-chain PUFAs by the addition of more 2-carbon units and the insertion of additional double bonds between n-9 and the carboxy terminus of the molecule. LNA (18:3, n-3) is converted to eicosapentaenoic acid (EPA; 20:5, n-3) and docosahexaenoic acid (DHA; 22:6, n-3), while LA (18:2, n-6) is converted to γ -linolenic acid (20:3, n-6) and arachidonic acid (AA; 20:4, n-6). Note that γ -linolenic acid is the starting material for synthesis of the 1-series prostaglandins and 2-series leukotrienes, AA is the starting material for synthesis of the 2-series prostaglandins and 4-series leukotrienes, and EPA is the starting material for synthesis of the 3-series prostaglandins and 5-series leukotrienes. [from ref 1]

DIETARY LINOLEIC (AND ARACHIDONIC) ACID

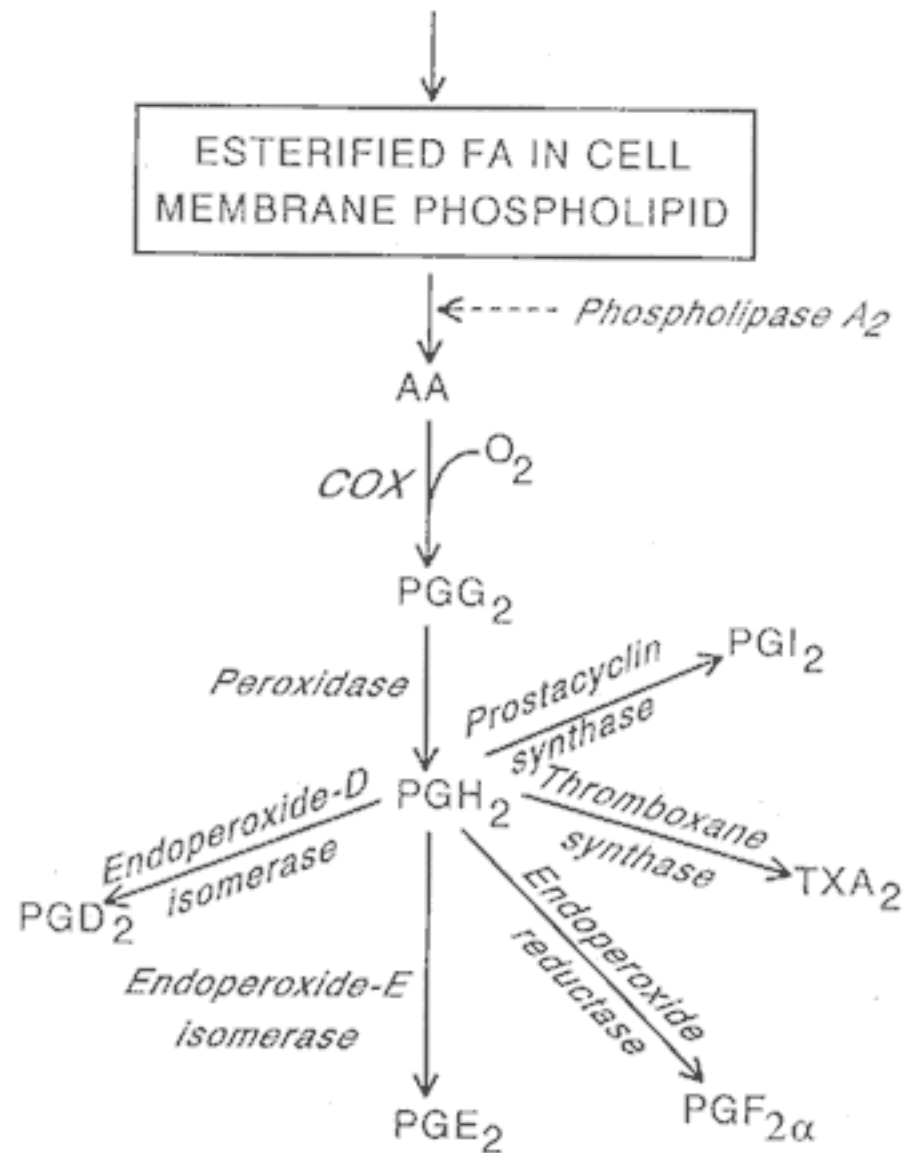


Figure 3. Metabolism of arachidonic acid to the prostanooid family of eicosanoids. The prostanooid subfamily of the eicosanoids consists of the prostaglandins (PGs), prostacyclin (PGI₂), and thromboxanes (TXs). The first step in their synthesis is the cleavage of arachidonic acid (AA) from phospholipids in the cell membrane by phospholipase A₂. The cyclooxygenase activity of the enzyme PG synthase then converts AA to PGG₂, which is subsequently reduced to PGH₂ by the enzyme's peroxidase activity. Further derivatives are synthesized from other enzymes acting on PGH₂. [from ref 1]

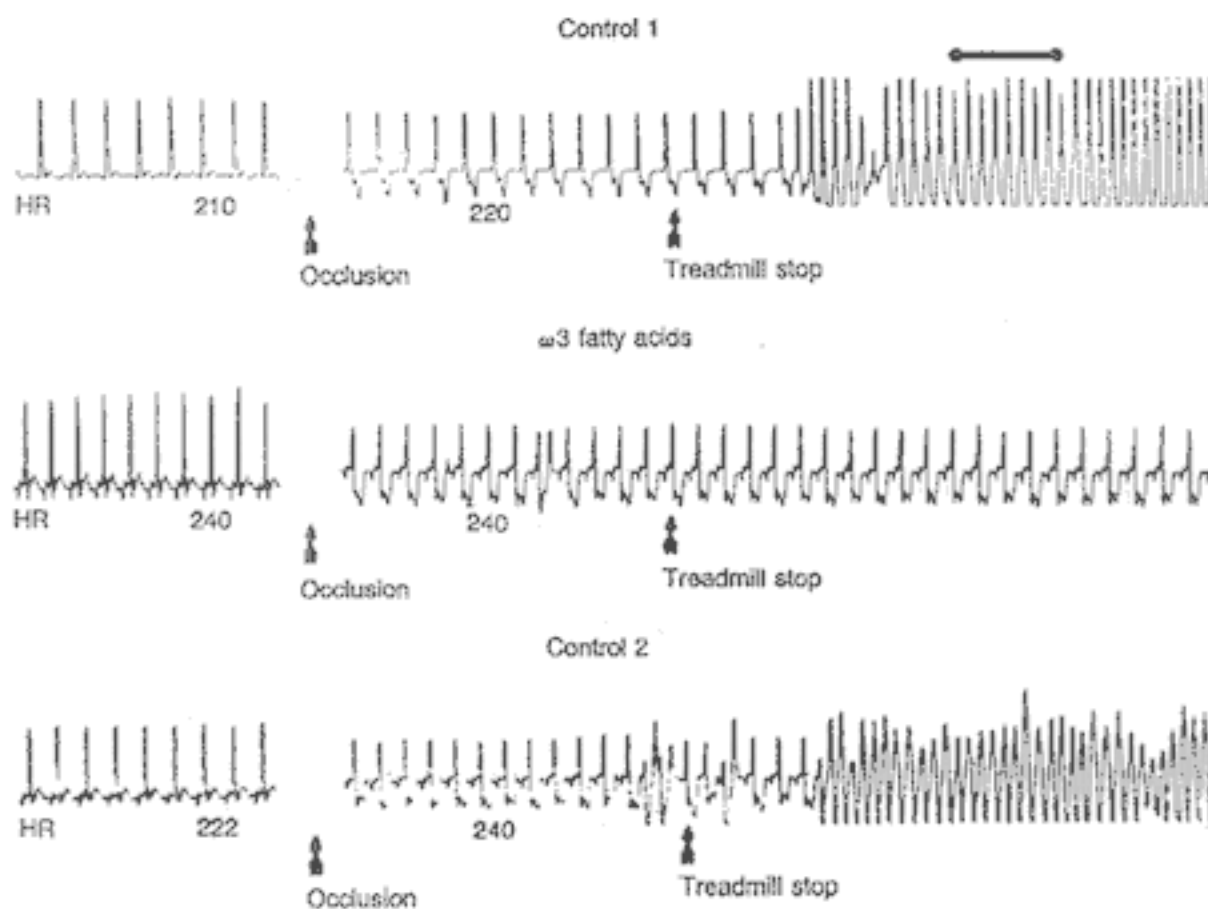
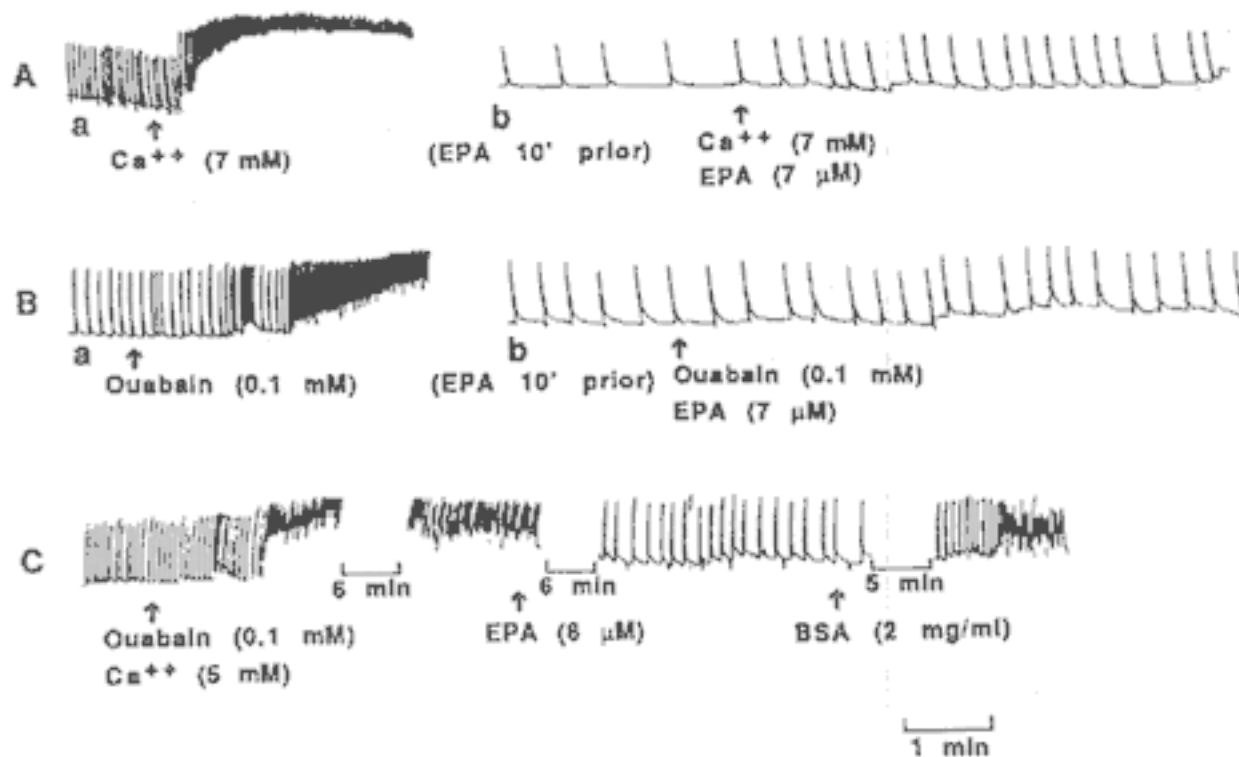


Figure 4. Electrocardiograms from a surgically-prepared dog during an exercise/ischemia test, with and without intravenous infusion with n-3 fatty acids. In control 1 (top panel), the dog received an infusion of saline solution. Ventricular fibrillation (VF) occurred shortly after occlusion of the animal's left circumflex coronary artery. With administration of an emulsion of ω 3 fatty acids (middle panel), the VF was averted. In control 2 (lower panel), the dog received a soybean oil emulsion lacking DHA and EPA. Again, VF occurred shortly after occlusion of the animal's left circumflex coronary artery. HR = heart rate in beats/min. Dark bar in upper right of figure represents a time of 1 s. [from ref 17]



*different
time
scale?
a vs b*

Figure 5. Prevention and termination of Ca²⁺- and/or ouabain-induced arrhythmias in neonatal rat myocytes by addition of eicosapentaenoic acid (EPA; 20:5, n-3) to the medium. (A) (a) The addition of 7 mM Ca²⁺ to the medium induced violent arrhythmias in the myocytes. (b) If 7 μM EPA was present in the medium, addition of 7 mM Ca²⁺ was not able to induce the arrhythmias. (B) (a) Similarly, the addition of 0.1 mM ouabain to the medium induced violent arrhythmias in the myocytes. (b) Again, if 7 μM EPA was present in the medium, addition of 0.1 mM ouabain was not able to induce the arrhythmias. (C) Arrhythmias caused by 0.1 mM ouabain plus 5 mM Ca²⁺ could be terminated by addition of 8 μM EPA. Subsequent removal of the EPA by addition of delipidated BSA caused the arrhythmias to recur. [from ref 18]

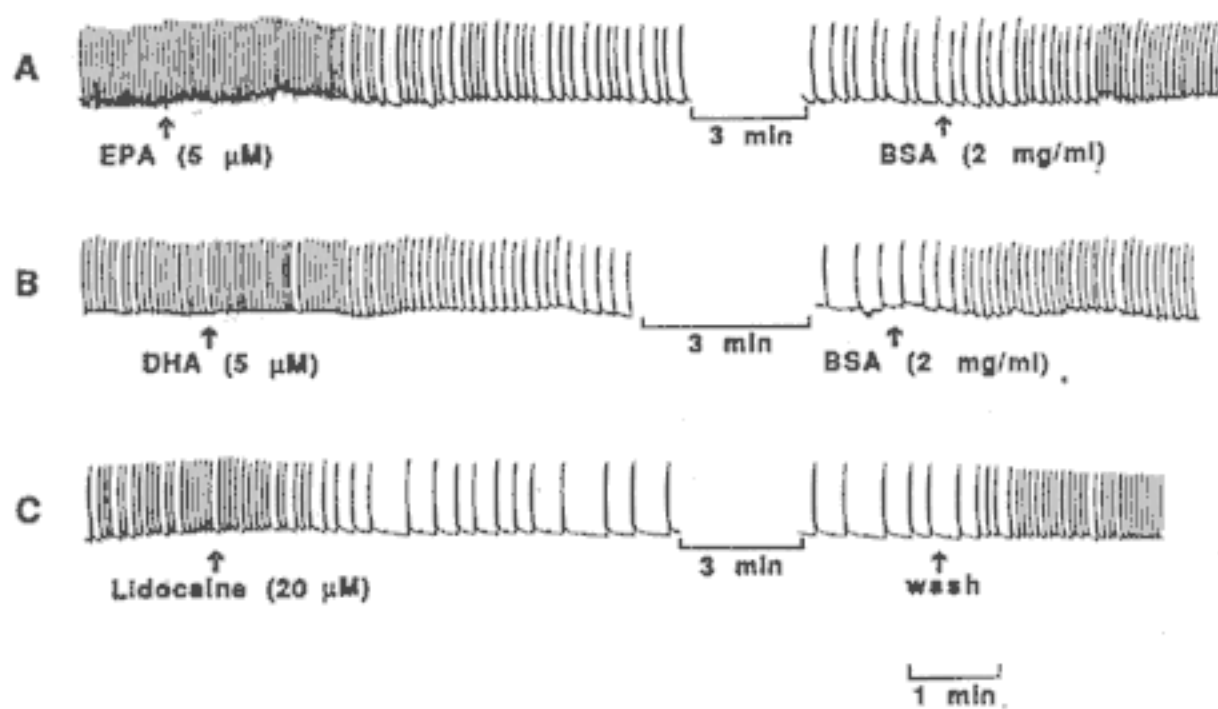


Figure 6. Effects of eicosapentaenoic acid (EPA; 20:5, n-3) and docosahexaenoic acid (DHA; 22:6, n-3) on the normal contraction of neonatal rat myocytes. Addition of 5 μ M EPA (A) or DHA (B) resulted in a significant decrease (approximately 50%) in the normal contraction rate of the myocytes. Both effects could be reversed by removal of the n-3 fatty acid with delipidated BSA. The effects were similar to those of the anti-arrhythmic drug lidocaine at 20 μ M (C).

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