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The Modulation of Ionic Currents in Excitable Tissues by n-3 Polyunsaturated Fatty Acids

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My (AL) first awareness of Professor Hans H. Ussing's work was in 1953 and still is a vivid memory because of the impact it has had on my career. I was then an aspiring young nephrologist, or 'salt and water' investigator, as we called ourselves —there was yet no recognized clinical specialty of nephrology. I was trying to learn how the body fluid volumes and concentration were regulated in health and disease, when I chanced on an issue of a Report of the Josiah Macy Foundation in which Ussing presented his brilliant demonstration of active transport of Na^+ across the frog skin [23]. I was fascinated but very frustrated. I understood the individual words in his essay, but I had not a clue what he was saying. I did realize that he was describing the single most important function of the kidney, the reabsorption of Na^+ from the glomerular filtrate. If I could not understand that phenomenon, I had better find another field in which to work. So I timidly approached my Professor and Chief of Medicine, Dr. Walter Bauer, to request a year's leave of absence to spend 6 months studying biophysics with Ussing in Denmark and 6 months biochemistry with Hans Krebs in England. My plans got modified by the intervention of Fritz Lipmann, who convinced Dr. Bauer that I should forego the biophysics and spend two years seriously learning biochemistry with Krebs. But Krebs had just been appointed Professor of Biochemistry at Oxford and would be moving his laboratory form Sheffield to Oxford, so I would likely be serving as a stevedore were I to go directly to Krebs. So I wrote a letter thanking Krebs for the privilege of working in his Laboratory, but since he would be very busy moving to Oxford, I would be in Copenhagen with Ussing and would come to him as soon as he had settled into his labs in Oxford. That gave me four wonderful months with Ussing, which were perhaps the most pleasant and intense learning experience I've had. Not only did I have the opportunity to learn the principles involved in Ussing's description of active transport of ions and of diffusive and osmotic movement of water across epithelia, but also to repeat his classic transport experiments. At Ussing's suggestion I found that the transparently thin urinary bladder of the toad, *Bufo marinus*, proved an excellent tissue with which to study active reabsorption of Na^+ and water and their control by hormones, e.g., vasopressin and aldosterone. This provided me and others a model tissue in which to study fundamental aspects of kidney function for many years following my return to Boston.

The lessons I learned from Ussing have still stood me in good stead, after my major medical interests shifted from Medicine and nephrology to Preventive Medicine and preventive cardiology around 1980. By that time I had concluded that Medicine was doing little to relieve the burden of the chronic diseases of middle age despite the escalating costs of the many palliative therapies being introduced into practice. I had seen in my travels enough to know that coronary heart disease, the major killer in Western industrialized countries, was rare then in some three-quarters of the world, where diets were quite different from ours. This was just at a time when interest was starting in the possibility that dietary long chain, polyunsaturated fatty acids of the n-3 class (also popularly called $\omega 3$ fatty acids) might be beneficial in the prevention of coronary heart disease. These are the fatty acids that are present in our diets largely today from fish.

Two Australian investigators, McLennan and Charnock, showed that these fish oils also prevented ischemia-induced fatal ventricular arrhythmias in rats [18, 19] and marmosets [17] when their animals were fed diets for months in which the major source of fats

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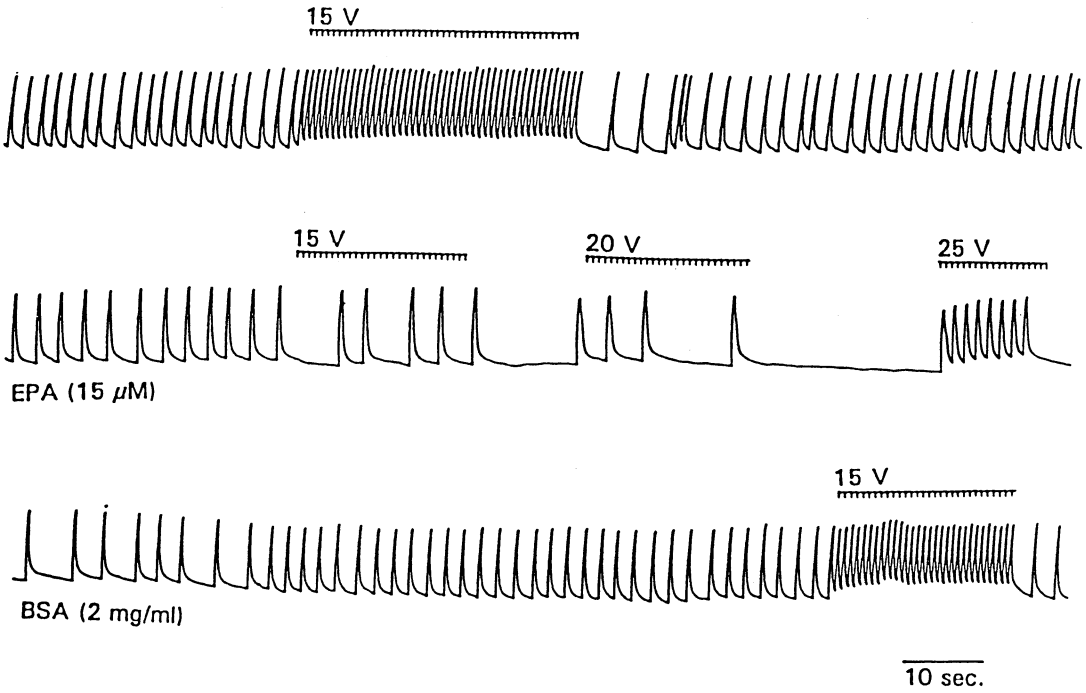

Fig. 1. The effect of EPA on the response of the cultured neonatal rat cardiomyocytes to electrical stimuli delivered from an external applied electrical voltage source [9]. The three strips are continuous tracings of the contraction rate and amplitude of a single myocyte within a clump of myocytes. The spontaneous beating rate and amplitude of contraction is apparent in the top tracing. An external electrical field of 15 V delivered stimuli at a rate that readily doubled the beating rate. The second tracing shows, that with EPA (15 μM) added to the superfusate, the beating rate began to slow, but when an external electrical field of 15 V was applied, the cells paid no attention to the stimuli, nor did they at 20 V. At 25 V they responded, but only to every other stimulus. Upon addition of delipidated bovine serum albumin to the superfusate the free EPA was extracted from the cardiomyocytes, the contractions returned to the control rate, and now the cells doubled their beating rate in response to stimuli delivered at 15 V, just as they had initially.

were the fish oil fatty acids. It seemed then very strange that a dietary factor could provide such protection, but it seemed worth testing to see if the findings of these investigators could be confirmed. To do this, I collaborated with Prof. George E. Billman, Ph.D., of the Department of Physiology, The Ohio State University School of Medicine, Columbus, Ohio, who had developed a highly reproducible dog model of sudden cardiac arrhythmic death. The dogs are prepared by a surgical ligation of the anterior descending coronary artery to produce a large anterior wall infarct and a hydraulic cuff is placed around the left circumflex coronary artery so that later a new area of ischemia may be produced at will. The dogs are then taught to run on a treadmill. Under these conditions, when the left circumflex artery is occluded, the dogs we studied invariably developed fatal ventricular arrhythmias within two minutes, but could be defibrillated successfully. In this dog model we found that infusing an emulsion of fish oil intravenously just prior to occluding the left circumflex coronary artery prevented the fatal ventricular fibrillation from occurring with high probability ($P < 0.005$) [2, 3]. Since fish oil contains many ingredients, we tested the two major n-3 fatty acids EPA (eicosapentaenoic acid. C20:5n-3) and DHA (docosahexaenoic acid, C22:6n-3) in fish oils as the pure, free fatty acids individually and found them each to be potent antiarrhythmic agents in our dog study [4].

With these encouraging, but still surprising findings, we wanted to learn what physiologic, biochemical or biophysical effects of these n-3 polyunsaturated fatty acids (PUFAs) might explain their antiarrhythmic action. We chose to study cultured neonatal rat cardiac cells. These cells can be cultured on microscope cover slips to which they adhere and grow. By the second day in culture these cells have grown into monolayer clumps and each clump is beating spontaneously, rhythmically and simultaneously. Placing the coverslip with the adherent myocytes in a perfusion chamber on an inverted microscope, we can observe the effects on the function of the myocytes of adding test substances to the perfusate bathing the cells while we record with a video camera and a monitor the effect of the agents on the beating rate and amplitude of contractions of a single cell in a clump [7]. When we added to the perfusate a number of agents known to produce lethal arrythmias in humans, e.g., cardiac glycosides, elevated extracellular Ca^{2+}, isoproterenol, thromboxane, lysophosphatidylcholine, etc., the myocytes promptly accelerated their beating rate, developed

contractures and fibrillated. Addition to the superfusate of 1.0 to 10 μM concentrations of EPA or DHA before adding the arrhythmogenic agent prevented the expected arrhythmia. If we first induced an arrhythmia with a toxic agent and then added the EPA or DHA to the perfusate, the arrhythmia was stopped and the cells resumed a regular beating rate. If we then removed the fatty acids from the cardiac cells with delipidated bovine serum albumin in the continued presence of the toxic agents, the arrhythmia resumed. From this we learned that the PUFAs acted directly on the heart cells and needed only to partition into the hospitable hydrophobic environment provided by the acyl chains of the fatty acids in the membrane phospholipids to exert their antiarrhythmic action. They did not from covalent bonds with any constituent in the cells in order to be antiarrhythmic. If they had, we would not have been able to withdraw the fatty acids from the myocytes with the delipidated serum albumin. Saturated and monounsaturated fatty acids possessed none of these antiarrhytmic actions [7].

With these functional studies we realized that the PUFAs must be affecting the electrical processes in these excitable, contractile cells. With Dr. Jing X. Kang we quickly found [8] that the presence of EPA or DHA produced two physiologic effects that we think are important. Their presence requires a 50% stronger depolarizing electrical stimulus just to elicit an action potential. Without an action potential there is no contraction of the myocytes. Also the PUFAs cause a 2- to 3-fold prolongation of the refractory period following an action potential. These two effects of the PUFAs on every contractile cell in the heart in our cultured, perfused myocytes stabilize the

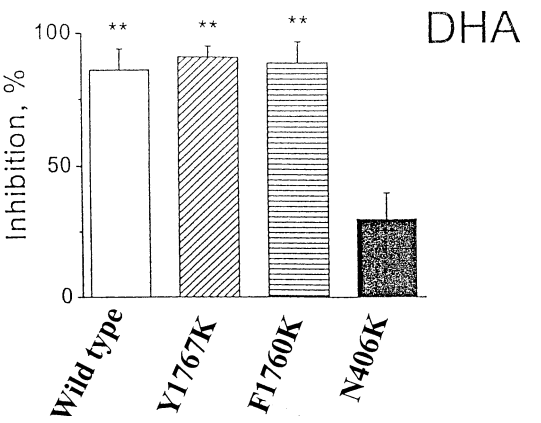

Fig. 2. Inhibition of I_{Na} in the 'wild type' and in point amino-acid mutants of the human Na^+ ion α-subunit channel transiently expressed in HEK293 cells. The mutant Y1760K was tested because it prevents the expected inhibitory action of local anesthetics, e.g., lidocaine or procaine, on the I_{Na}. The mutant F1767K was also tested as a close neighbor, but inactive mutant, on the inhibitory action of local anesthetics. Our interests in local anesthetics is because they are inhibitors of the I_{Na}, acting in many ways very similar to the actions of the n-3 fatty acids on the Na^+ channel. We had actually expected that the n-3 fatty acids would share the same "binding site" on the Na^+ channel as has been identified as the purported specific binding site for local anesthetics in D4,S6 of the Na^+ channel protein. N406K was chosen because it is part of the purported receptor for the potent cardiac and neural toxin batrachotoxin on D1, S6 of the Na^+ channel protein, which n-3 fatty acids specifically displace from this receptor (binding site) [9]. Na^+ currents were evoked by single-step voltages from a holding potential of −120 to 30 mV. The expected I_{Na} inhibition by 5 μM DHA (C22:6 n-3) was not blocked in the wild type, the Y1767K or the F1760K mutant Na^+ channel proteins, but strongly blocked in the N406K mutant. This would be interpreted to indicate that the n-3 PUFAs bind to a specific receptor at the D1, S6 of the Na^+ channel protein at a site shared with batrachotoxin.

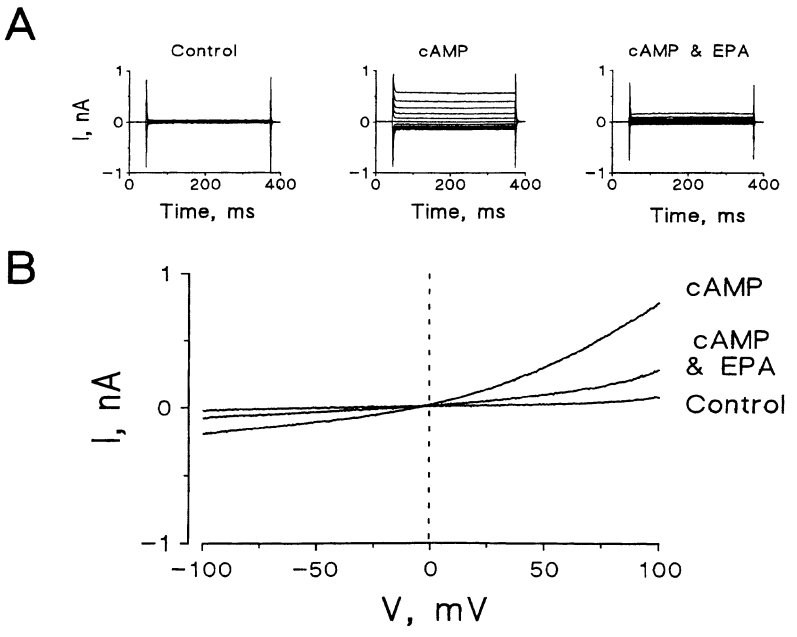

Fig. 3. The inhibitory action of EPA (5 μM) on the ligand-activated cAMP Cl^- current of a rat cardiomyocyte. (*A*) Protocol for this experiment. (*B*) The current-voltage response of the myocyte in the control, the cAMP- and the AMP &EPA-treated myocyte. The inward Cl^- current activated by cAMP-dependent protein kinase A (PKA) was determined in cultured neonatal rat cardiomyocytes according to the published procedure [6].

cells electrically and are important to the antiarrhythmic actions of the PUFAs [8], as the Fig. 1 [10] illustrates.

When one considers that this electrical stabilizing effect of the n-3 PUFAs is an action on every contractile myocyte in the heart in the absence of neural or hormonal controls, one can sense what a potent antiarrhythmic action these fatty acids may exert.

This electrical stabilizing effect of the PUFAs in turn result from their ability to modulate the conductances of ion channels in the cardiomyocyte plasma membranes. Of these actions we think that the inhibitory action they exert on the voltage-gated fast Na^+ current [25, 27, 29], which initiates the action potential, and the L-type Ca^{2+} channel current [26], which initiates the calcium-induced calcium-release from the sarcoplasmic reticulum into the cytosol of heart cells, which result in electro-mechanical coupling and contraction in myocytes, are the two ion channels affected by the PUFAs, which are most important to their antiarrhythmic effects.

We have pursued the mechanism of action of the n-3 PUFAs further by trying to determine what the primary site of action of these antiarrhythmic fatty acids is that results in the effects described. It is generally accepted evidence today that the primary site of a drug on ion channel conductance has been found if an amino-acid point mutation of the ion channel protein abolishes the effect of the drug on the ion channel conductance. If that effect of an amino-acid point mutation occurs, then it is accepted that the original amino acid, which had been replaced, must be part of a specific binding site for the drug on the ion channel protein. For an example, this method has been used to identify the primary binding site for local anesthetics to be on the Domain 4, Segment 6 (D4,S6) of the α-subunit of the rat and human myocardial sodium channel [22, 24]. Recently we have adopted this approach and found EPA or DHA, the two important n-3 fatty acids in fish oils, lose their blocking effect on the human myocardial Na^+ channel α-subunit transiently expressed in HEK293 cells, when the asparagine in the 406 position in D1, S6 was substituted by a lysine [28]. According to the accepted criteria for establishing a primary site of action for an agent or drug that affects the conductance of ion channels, this would mean a specific binding site for the n-3 fatty acids exists to which the fatty acids bind and that this action results in the modulatory effects of the fatty acids on the ion channel currents. This result is shown in Fig. 2 [28].

This to us does not seem a satisfactory explanation of the primary site of action of the n-3 PUFAs. The main reason for our dissatisfaction with this explanation is that we have found that, beside the voltage-gated cation channel currents i.e., Na^+, Ca^{2+}, and the two repolarizing K^+ currents, which share considerable amino-acid homology, other cardiac currents, e.g., the ligand-gated cAMP-activated Cl^- and the acetylcholine-activated K^+ currents, are also inhibited by the n-3 PUFAs (unpublished results) – *see* Figs. 3 & 4. These ligand-gated channels lack amino-acid homology with the voltage-gated cation channel proteins. It seems quite unlikely that they

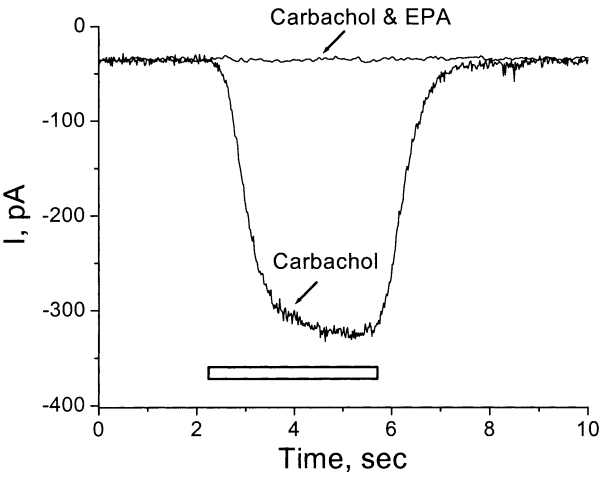

Fig. 4. The inhibitory effect of EPA (5 μM) on the ligand-gated acetylcholine-dependent K^+ current of a rat cardiomyocyte. The current-voltage response to carbachol was eliminated by the EPA (5 μM). The method used to measure this current was as published by Xiao [30].

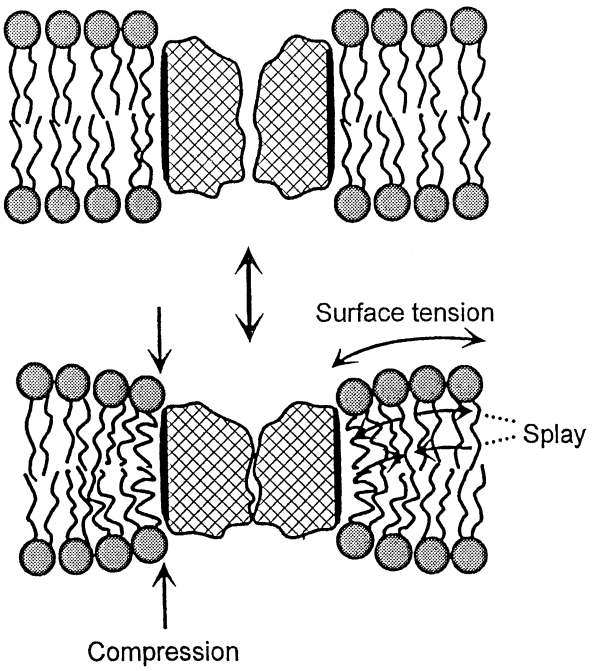

Fig. 5. Schematic representation of how hydrophobic coupling mismatch between the transmembrane channel protein and the bilayer acyl chains might cause ion channel protein conformational changes (Reproduced with permission from [14].)

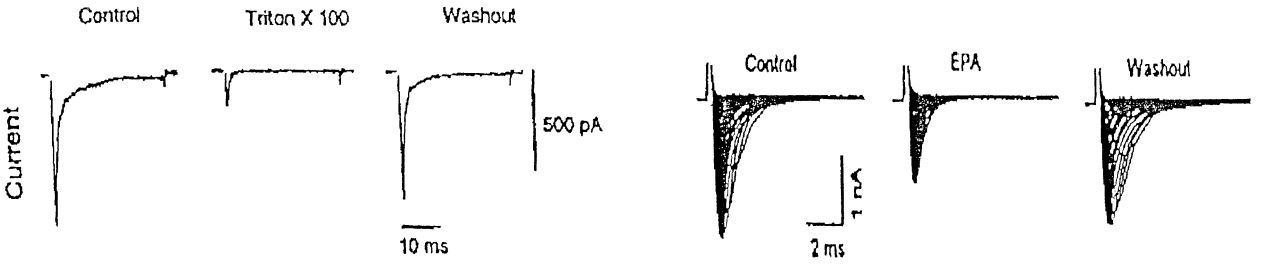

Fig. 6. Comparison of the effects of Triton X-100 (100 μM) on rat muscle μl sodium channel α-subunit expressed in HEK293 cells (reproduced with permission from [1]) on the left with the inhibitory effects of n-3 EPA (5 μM) on the whole-cell voltage-clamp traces from $I_{Na\alpha}$ of human myocardial channel also expressed in HEK293 cells, on the right [25]. For the figure on the right, the traces of $I_{Na\alpha}$ are superimposed. They were elicited by 10-sec test pulses from −90 to 55 mV in increments of 10 mV at 0.2 Hz for control, 5 μM EP and washout. The cell was held at −80 mV and hyperpolarized to −160 mV for 200 msec before a test pulse. The same cardiac myocyte was measured in the control, after EPA and following washout, so that each of the superimposed currents, respectively, for each of the three conditions, are comparable.

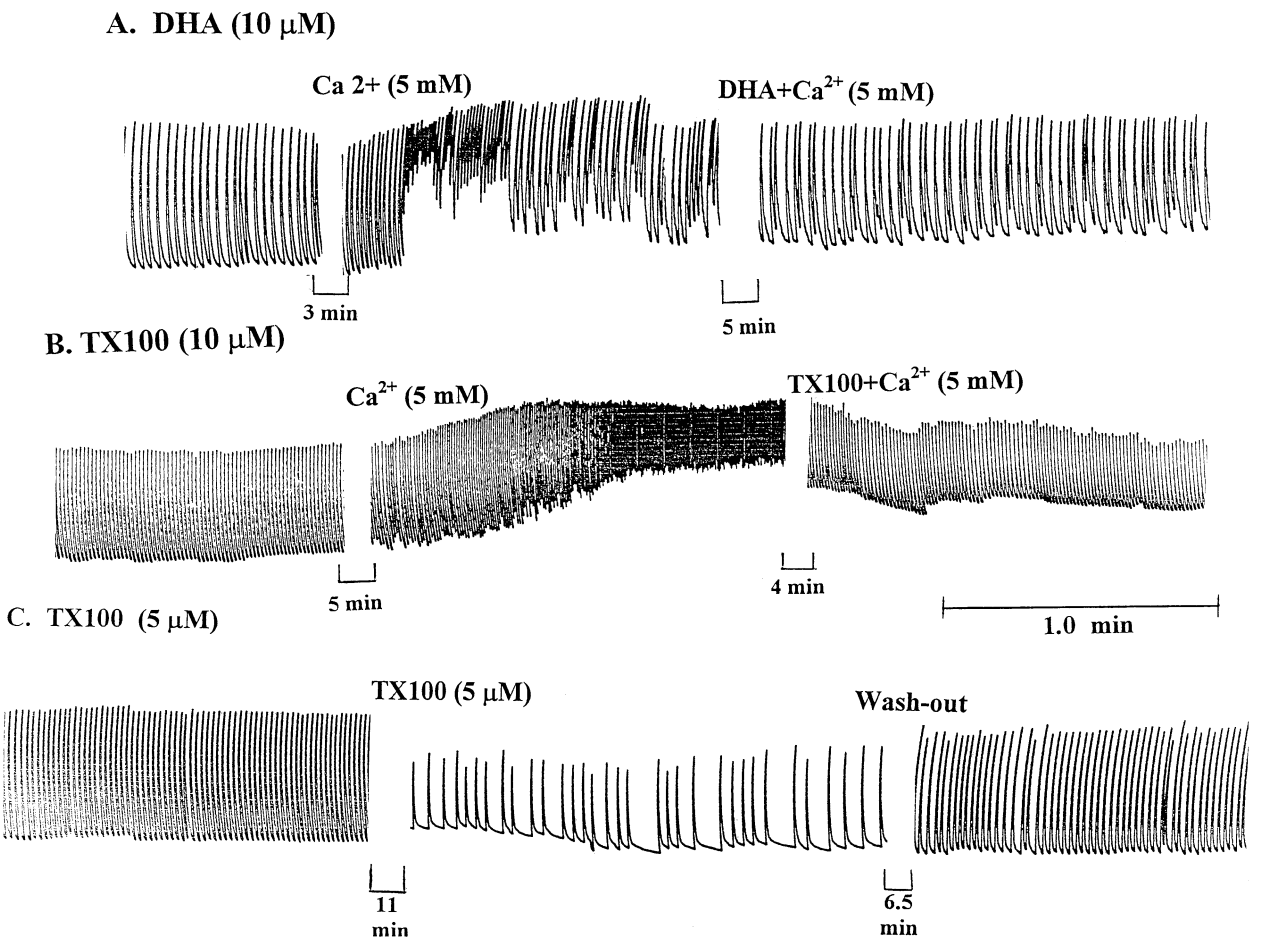

Fig. 7. Effects of DHA (*A*) and of Triton X-100 (*B*) (each 10 μM) on arrhythmias by high Ca^{2+} (5 μM) in the medium bathing cultured neonatal rat cardiomyocytes. (*C*) Triton X-100 (5 μM) causes the reversible slowing of the spontaneous beating rate of cultured neonatal rat cardiomyocytes. This slowing effect is an invariable action of the n-3 polyunsaturated fatty acids [7].

would have similar specific receptors to accommodate the binding of the n-3 PUFAs as do the voltage-gated cation channels. There seems to be a need for a more general conceptual framework or paradigm to explain the primary site of action of these n-3 PUFAs.

An Alternative Paradigm – Preliminary Supportive Data

The phospholipids of cardiac myocyte plasma membranes may be a more general locus for the primary site of action of the n-3 PUFAs. Ever since it was

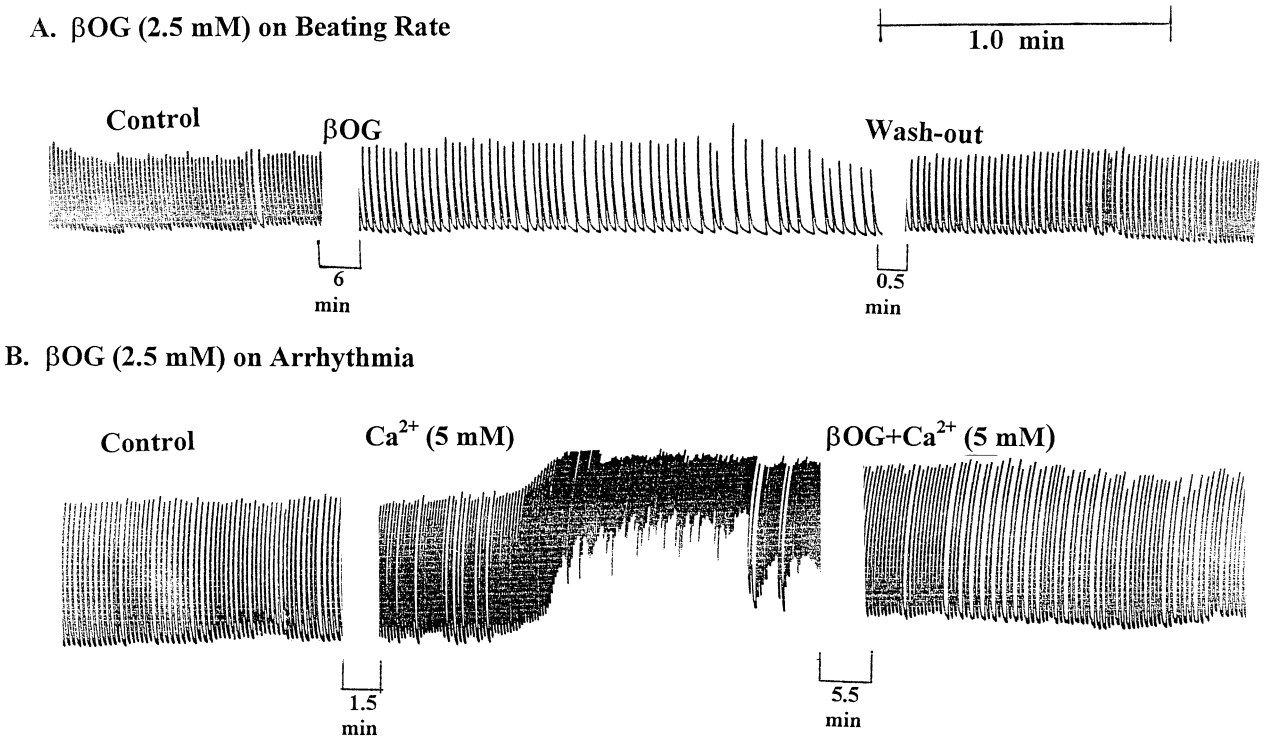

Fig. 8. Effects of β-octyl-glucoside (βOG, 2.5 mM) on (*A*) slowing of the spontaneous beating rate of cultured neonatal rat cardiomyocytes, and (*B*) arrhythmias induced by high Ca^{2+} (5 mM) in the medium bathing the neonatal cardiomyocytes.

reported by Klausner et al. [11] that these same unsaturated fatty acids, to the extent they were tested, could alter the "fluidity" of membrane phospholipids, this has been regarded as a possible means by which addition of free fatty acids to cells might alter the actions of membrane proteins, i.e., channels, carriers and enzymes. However, the concentrations of fatty acids used in their study was some 10-fold or greater than the nM to low μM concentrations of n-3 PUFAs we find will inhibit the ion channels. We recently reported [21] that the molar ratio of PUFAs to phospholipid in red cell ghost is less than 1:100 at these low concentrations we use. This molar ratio is too low for the n-3 PUFAs to have a general effect to increase the "fluidity" (packing) of the membrane phospholipids, but not too low to affect the micro domains of the cell membrane through which the ion channels penetrate. During the past decade O.S. Andersen and colleagues have been rigorously developing and testing a hypothesis that fatty acids and other agents could be affecting ion channels by a primary effect on the cell membrane in the immediate vicinity of the channel protein rather than by a direct action on the channel protein [1, 5, 12–16, 20, a partial list]. We have now made preliminary tests of this hypothesis.

They have hypothesized that if the hydrophobic length of the transmembrane channel protein does not match the hydrophobic thickness of the resting membrane phospholipid bilayer, such a mismatch would create stresses between channel and membrane. Frequently the hydrophobic length of the channel protein is slightly less than the hydrophobic thickness of the acyl chains of the phospholipid bilayer in its "resting" state. As a result this requires that the thickness of the cell membrane be decreased at its contact with the transmembrane segment of the channel protein in order that their hydrophobic regions match. The resulting compression of the acyl chains of the membrane phospholipid fatty acids occupying the Sn-1 and Sn-2 positions in the phospholipids is opposed by a spring-like tension. This tension the membrane exerts locally on the channel protein, which affects the conformational state and conductance of the ion channel. They further reason that if agents exists, which would incorporate into the phospholipid membrane close to its junction with the channel protein and reduce the curvature of the phospholipid and the compression of its acyl chains, the spring-like tension on the channel protein would be altered. This would affect the conformational state of the transmembrane segment of the channel protein and its conductance would change. They have illustrated their hypothesis schematically[14] - *see* Fig. 5.

They have tested their hypothesis on the cationic-permeable short gramicidin channel with two non-ionic detergents, Triton X-100 and β-octyl glucoside. They have also reported that these compounds, which form micelles, bear no chemical similarity to the PUFAs, also affect the Na^+ and Ca^{2+} currents of

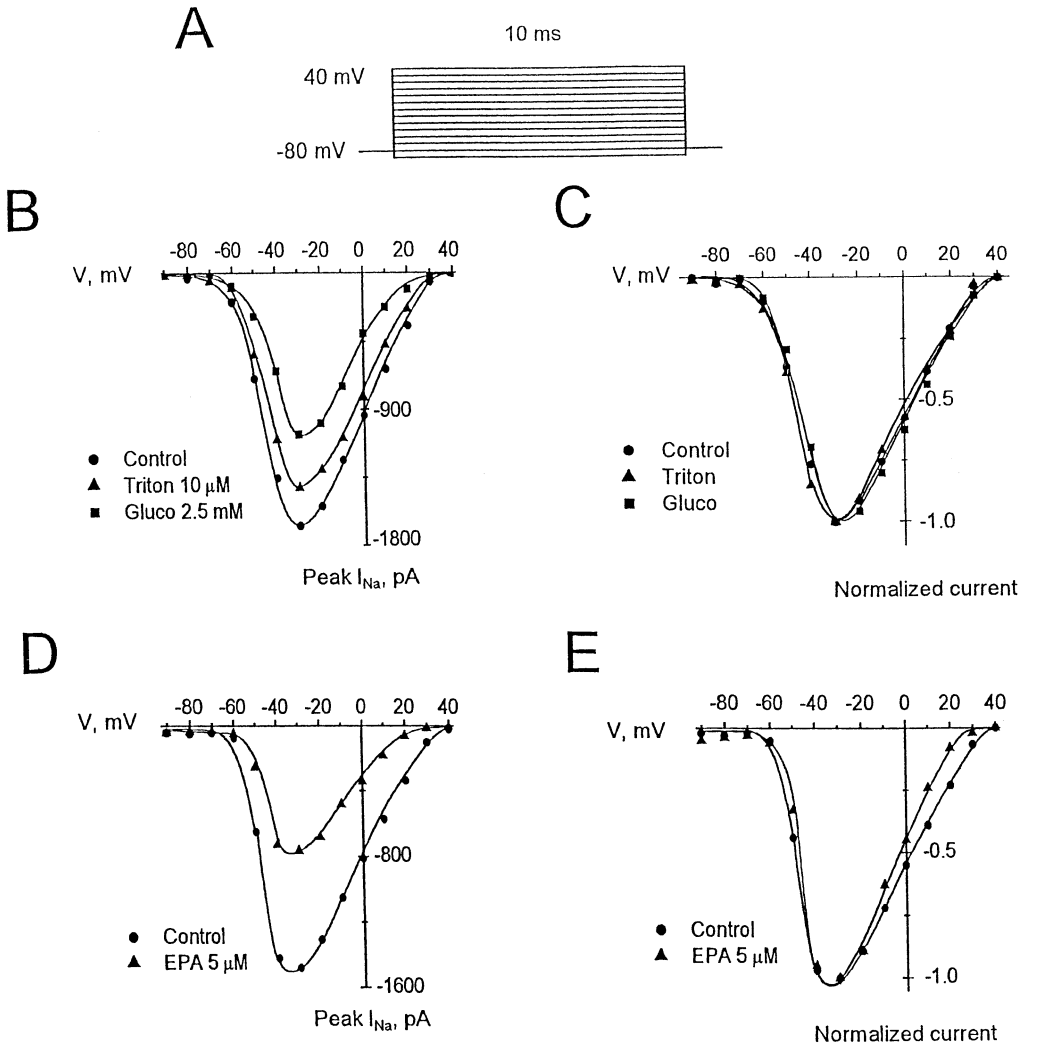

Fig. 9. Effects of Triton X-100, β-octyl glucoside, and n−3 EPA on the current-voltage relationship. (*A*) The experimental protocol is shown. Currents were elicited by 10 ms voltage pulses from −90 to 40 mV with 10 mV increments every 4 sec. The membrane potential was held at −80 mV. (*B*) The amplitude of I_{Na} was inhibited in the presence of 10 μM Triton X-100 (Triton) and more so by 2.5 mM β-octyl glucoside (Gluco). (*C*) Normalized current-voltage relationships are shown in the absence (Control) and presence of the 10 μM Triton X-100 and 2.5 mM β-octyl glucoside. (*D*) I_{Na} was inhibited by 5 μM n-3 EPA. (*E*) Normalized current-voltage relationships are shown in the absence (Control) and presence of 5 μM EPA (unpublished results).

mammalian channels [1, 15]. Figure 6 shows their reported finding on the Na^+ channel current [1], which is similar to what we have reported for the n-3 fatty acid, EPA [25, 27, 29].

The n-3 PUFAs modulate ion currents in cardiac myocytes, which stabilize the cells electrically [10], and prevent or terminate induced arrhythmias and reversibly slow the spontaneous beating rate of neonatal rat cardiac myocytes [7]. Therefore, we compared the effects of n-3 DHA and Triton X-100 (each 10 μM) on tachyarrhythmias induced in neonatal rat cardiomyocytes by 5mM $[Ca^{2+}]_e$. This is shown in Fig. 7 (unpublished results).

The results with β-octyl glucoside (βOG) are shown in Fig.8 (unpublished results). Much higher concentrations of βOG (2.5 mM) were required than of DHA or Triton X-100 (10 μM), consistent with the greater water solubility of βOG than of DHA or of Triton X-100.

As we have reported for neonatal rat cardiomyocytes [27] and for human myocardial hH1 α and α + β1-subunits of the Na^+ channel transiently expressed in HEK293 cells [25, 29], the n-3 fatty acids reversibly inhibit in a dose-dependent manner the Na^+ currents with a shift of the steady-state inactivation potential to more hyperpolarized potentials. Therefore, preliminary observations have been made on the ability of the two nonionic detergents, Triton X-100 and β-octylglucoside, to do the same. These preliminary whole-cell patch-clamp studies are shown

in Fig. 9 (unpublished results). Not shown is our unpublished preliminary finding that the detergents also shift the steady-state inactivation potential to hyperpolarized potentials, as do the n-3 PUFAs [25, 29].

Our preliminary observations indicate that so far these two nonionic detergents mimic both the electrophysiologic and functional effects we have observed with the fish oil fatty acids on cardiac myocytes. This would be consistent with the hypothesis of Andersen and colleagues. This paradigm is that the fatty acids are acting primarily on the phospholipid cell membrane, which abuts the transmembrane channel protein. Thus other ion channels, as we have observed, may similarly be affected, as are the cation-permeable voltage-gated channels, even though the other channels may share no amino-acid homology with the Na^+ or Ca^{2+} channel proteins to provide binding sites for the PUFAs on the respective ion channels. Further studies to test this hypothesis are in progress.

My hope is that Professor Ussing, whose research repeatedly challenged established dogmas, would at least look favorably on the direction our thinking and research is currently headed.

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