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The Antiarrhythmic Effect of n-3 Polyunsaturated Fatty Acids: Modulation of Cardiac Ion Channels as a Potential Mechanism

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Abstract. Sudden cardiac death remains one of the most serious medical challenges in Western countries. Increasing evidence in recent years has demonstrated that the n-3 polyunsaturated fatty acids (PUFAs) can prevent fatal ventricular arrhythmias in experimental animals and probably in humans. Dietary supplement of fish oils or intravenous infusion of the n-3 PUFAs prevents ventricular fibrillation caused by ischemia/reperfusion. Similar antiarrhythmic effects of these fatty acids are also observed in cultured mammalian cardiomyocytes. Based on clinical observations and experimental studies in vitro and in vivo, several mechanisms have been postulated for the antiarrhythmic effect of the n-3 PUFAs. The data from our laboratory and others have shown that the n-3 PUFAs are able to affect the activities of cardiac ion channels. The modulation of channel activities, especially voltage-gated Na^+ and L-type Ca^{2+} channels, by the n-3 fatty acids may explain, at least partially, the antiarrhythmic action. It is not clear, however, whether one or more than one mechanism involves the beneficial effect of the n-3 PUFAs on the heart. This article summarizes our recent studies on the specific effects of the n-3 PUFAs on cardiac ion channels. In addition, the effect of the n-3 PUFAs on the human hyperpolarization-activated cyclic-nucleotide-modulated channel is presented.

Key words: Cardiac arrhythmia — Eicosapentaenoic acid — Docosahexaenoic acid — Hyperpolarization-activated cyclic-nucleotide-modulated channel — Mutation

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Introduction

Evidence from either clinical observations or from animal studies suggests that the n-3 polyunsaturated fatty acids (PUFAs) can prevent fatal arrhythmias [1, 29, 36]. The dietary n-3 PUFAs are α-linolenic acid (LA, C18:3n-3), largely derived from plants, and eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), which are primarily derived from seafood (Fig. 1). The early observations showed that the Greenland Inuit Eskimos, who had a diet rich in the n-3 PUFAs, had a low mortality from coronary heart disease (CHD) [6, 17]. In another early epidemiological study on the regional differences in incidence of CHD, the lowest incidence of CHD (0.6%) was in the fishermen compared to 19% in the population who lived inland [13]. A survey on 45,722 men suggests that the n-3 PUFAs from both seafood and plant sources may reduce the risk of CHD. Plant-based n-3 PUFAs may particularly reduce CHD risk when seafood-based n-3 PUFA intake is low, which has implications for populations with low consumption or availability of fatty fish [40]. The n-3 PUFAs not only seem to lower the incidence of CHD, but apparently also have direct antiarrhythmic effects as shown in preliminary human trials [40]. In a recent clinical study, ten patients with implanted defibrillators and a history of ventricular tachycardia were infused with n-3 fatty acids and underwent electrically induced ventricular tachycardia [49]. At the beginning of the study, the arrhythmia was induced in seven out of the 10 participants. After the infusion of fish oil, only in two of the ten patients were arrhythmias successfully induced [49]. Also, the PUFA administration substantially reduced the incidence of postoperative atrial fibrillation by 54.4% and shortened the hospital stay in patients undergoing coronary artery bypass graft surgery (10). Several other human interventional trials have also

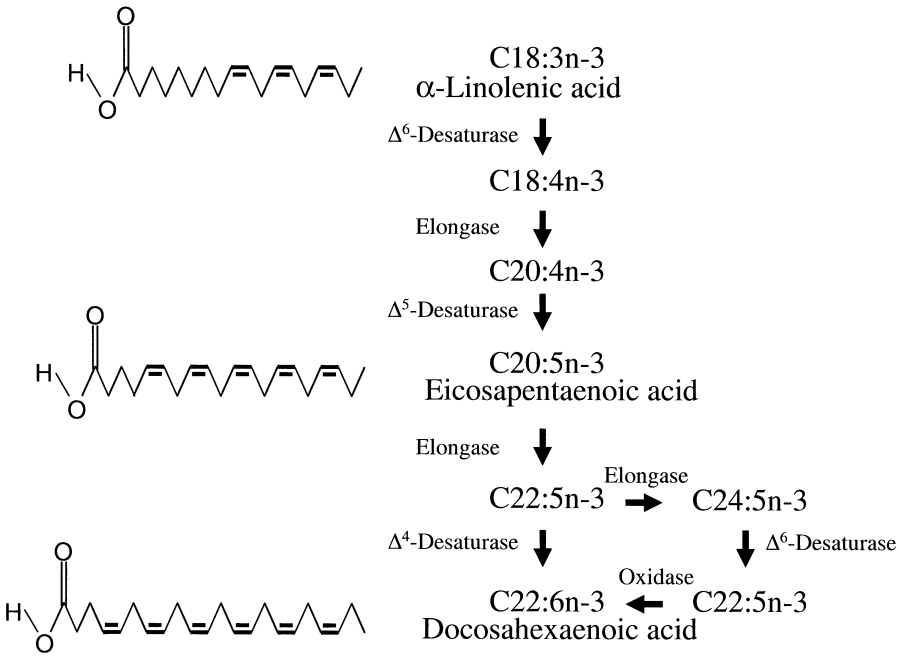

Fig. 1. Schematic diagram of the metabolic pathway of the n-3 polyunsaturated fatty acids.

shown that diets rich in long-chain n-3 fatty acids significantly reduce the incidence of sudden death from CHD [2, 16, 20, 50] and even among men without evidence of prior cardiovascular disease [2].

Animal data support the clinical observations that a diet high in fish oil, in contrast to saturated fat or monounsaturated olive oil, prevented ventricular fibrillation induced by coronary artery ligation in rats and increased the electrical ventricular fibrillation thresholds in marmosets [37, 38]. The cardioprotective effect of the n-3 PUFAs was further confirmed by Billman and colleagues in a dog model of sudden ventricular fibrillation [7]. In this model, intravenous delivery of an emulsion of fish oil [8] or of one of the two major n-3 fatty acids EPA and DHA just prior to occluding the left circumflex coronary artery prevented fatal ventricular fibrillation. Dietary flaxseed, which is the richest plant source of one of the n-3 PUFA, α-linolenic acid, was associated with reduced ventricular fibrillation during ischemia-reperfusion in normal and hypercholesterolemic rabbits, possibly via shortening of the action potential duration [4]. To further understand the physiological, biochemical, and biophysical bases of the antiarrhythmic action in vivo by the n-3 PUFAs, Kang and colleagues used cultured neonatal rat cardiac cells as an in vitro model of arrhythmogenesis [24, 25]. Perfusion of a number of agents known to produce lethal arrhythmias in humans, e.g., cardiac glycosides, elevated extracellular Ca^{2+}, isoproterenol, thromboxane, lysophosphatidylcholine, etc., to the cultured myocytes accelerated their beating rates, developed contractures and fibrillations. Addition of EPA or DHA before adding the arrhythmogenic agents prevented fibrillation [24, 25]. If an arrhythmia was induced by one of the toxic agents and EPA or DHA was then added to the perfusate, the arrhythmia stopped and the cells beat regularly again. If the fatty acids were removed from the cardiac cells with delipidated bovine serum albumin in the presence of the arrhythmogenic agents, the arrhythmia resumed [24, 25]. These results suggest that the n-3 PUFAs acted directly on the heart cells without formation of covalent bonds because if they had, the delipidated serum albumin would not have been able to wash away the fatty acids from the myocytes [24, 25].

To understand how the n-3 PUFAs protect hearts in vivo or cardiomyocytes in vitro from arrhythmias, the effects of the fatty acids on membrane excitability have been examined in isolated rodent cardiomyocytes. Electrophysiological data indicate that the n-3 PUFAs, but not monounsaturated and saturated fatty acids, reduced membrane electrical excitability by significantly increasing the threshold of depolarizing current that was required to initiate an action potential and by prolonging the refractory period following an action potential [26]. These two major effects of the PUFAs on cardiomyocytes are important for their antiarrhythmic actions. Current-clamp analysis showed that these effects on cardiac action potentials were due to a hyperpolarization of the membrane resting potential and an increase in the threshold potential (Table 1). Further studies have shown that the PUFAs significantly suppressed Na^+ currents and shifted the steady-state inactivation of the Na^+ channel to more hyperpolarized potentials [55]. PUFAs also inhibit voltage-gated Ca^{2+} [56] and K^+ currents in mammalian heart cells [57]. These

Table 1. Significant changes of the parameters of cardiac action potentials in the presence of eicosapentaenoic acid

	RMP mV	APT mV	APD_{75} ms	Current PA	CL ms
Control	−52	−52	204	18	525
EPA	−57*	−43*	163**	27*	1225*

The data in each column represent the means of 8 cells in the absence or presence of 10 μM EPA. *RMP*, resting membrane potential; *APT*, action potential threshold; APD_{75} action potential duration measured at 75% repolarization; *Current*, currents required to initiate the first action potential; *CL*, cycle length of an excitable response between two consecutive stimuli. *, $P < 0.05$;**, $P < 0.01$; versus the control.

Table 2. Effects of eicosapentaenoic acid on cardiac Na^+ currents

		Human cardiac Na^+ channel					
	Rat neonatal	$hH1_\alpha$	$hH1_{\alpha\beta}$	N406K	F1760K	Y1767K	L409C/A410W
IC_{50} (μM)	4.8	0.51	3.9	18.0	10.5	6.7	3.9 ($I_{Na,peak}$) 0.9($I_{Na,late}$)
$\Delta V_{1/2}$ (mV)	−19	−28	−22	−7	−18	−26	−19 ($I_{Na,peak}$) ? ($I_{Na,late}$)

The data in each column represent the means of multiple cells ($n \geq 7$) in the absence or presence of EPA. *Rat neonatal*, cultured neonatal rat cardiac myocytes. IC_{50}, concentration to produce 50% inhibition of cardiac Na^+ currents. $\Delta V_{1/2}$, difference of the values of the steady-state inactivation of Na^+ channels at the point of 50% inactivation in the absence and presence of 5 μM EPA. Human cardiac Na^+ channels, the wild-type or its mutated channels, were transfected into HEK293t cells. As $I_{Na,late}$ of the double mutants of $hH1_\alpha$ was completely inhibited in the presence of 5 μM EPA, the value of the $\Delta V_{1/2}$ was not available.

electrophysiological effects of the n-3 PUFAs on ion channels may be important for their antiarrhythrnic actions in vivo. This article focuses on reviewing the current evidence of the modulation of cardiac ion channels by the n-3 PUFAs.

Modulatory Effects of the n-3 PUFAs on Cardiac Ion Channels

Na^+ Channels

The voltage-gated Na^+ current (I_{Na}) is critical for excitation of heart cells. In the heart, activation of Na^+ channels leads to a rapid influx of sodium ions and subsequently initiates action potentials. The effect of the n-3 PUFAs on Na^+ channels may, therefore, be closely related to their antiarrhythmic action. In cultured neonatal rat ventricular myocytes, we used the whole-cell patch-clamp technique to assess the effects of these fatty acids on $I_{Na,rat}$ [55]. Extracellular application of free EPA produced a concentration-dependent suppression of $I_{Na,rat}$ with an IC_{50} of 4.8 μM (Table 2). Likewise, DHA also significantly inhibited the cardiac $I_{Na,rat}$. In contrast, monounsaturated or saturated fatty acids did not cause significant inhibition of $I_{Na,rat}$. The EPA-induced inhibition of I_{Na} was voltage-dependent and significantly shifted the steady-state inactivation curve to the hyperpolarizing direction (Table 2).

To extend understanding of the possible actions of the n-3 PUFAs in human hearts, we investigated their effects on the α-subunit of the human cardiac Na^+ channel ($hH1_\alpha$). The $hH1_\alpha$ cDNA was transiently transfected into cultured human embryonic kidney (HEK293t) cells. EPA significantly reduced the $hH1_\alpha$ Na^+ current ($I_{Na,\alpha}$) with an IC_{50} of 0.51 μM [58]. EPA shifted the $V_{1/2}$ of the steady-state inactivation by −28 mV (Table 2). In addition, EPA inhibited $I_{Na,\alpha}$ with a higher "binding affinity" to $hH1_\alpha$ channels in the inactivated state than in the resting state. EPA markedly accelerated the transition from the resting state to the inactivated state and slowed the recovery from the inactivated state to the resting, available for activation state [58]. We are surprised by the result that besides PUFAs, saturated or monounsaturated fatty acids also significantly inhibited $I_{Na,\alpha}$ in transfected HEK293t cells [58]. These data indicate that free fatty acids suppress human $I_{Na,\alpha}$ with high "binding affinity" to inactivated channels and prolong the duration of inactivation of $hH1_\alpha$ channels.

The human cardiac Na^+ channel consists of one large α-subunit that alone creates a functional membrane channel. In addition, there is also a small β_1-subunit [12]. The β_1-subunit modulates the voltage-dependent Na^+ channel [3, 35, 47]. As described above, the fatty acid specificity was lost in the α-subunit alone expressed in HEK293t cells, in which monounsaturated and even saturated fatty acid had some suppressing effects on human $I_{Na,\alpha}$ [58], whereas only PUFAs suppressed $I_{Na,rat}$ in isolated rat cardiac myocytes [55]. To evaluate how the complete human myocardial Na^+ channel ($hH1_{\alpha\beta}$) would be affected by the antiarrhythmic PUFAs and to determine whether the addition of the β_1-subunit

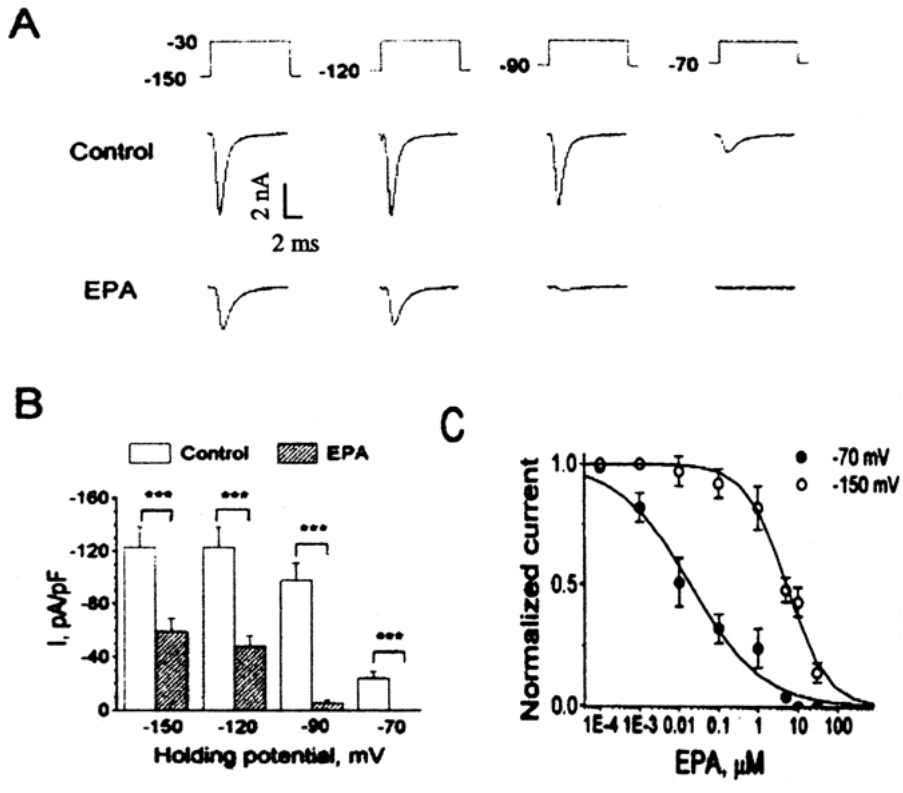

Fig. 2. Voltage-dependent suppression of hHl $I_{Na,\alpha\beta}$ by extracellular application of eicosapentaenoic acid (EPA). (*A*) Original current traces of $I_{Na,\alpha\beta}$ in the absence (*Control*) and presence (*EPA*) of 5 μM EPA were evoked by depolarizing pulses from −150, −120, −90, and −70 mV to −30 mV (*see* voltage step protocols at top). (*B*) Extracellular application of 5 μM EPA significantly reduced peak $I_{Na,\alpha\beta}$ densities ($n = 15$). ***$P <$ 0.001. (*C*) Suppression of resting (○) and inactivated (●) $hH1_{\alpha\beta}$ channels by EPA was concentration-dependent. Data were fitted by a logistical equation. Each value represents 6–15 cells (mean ± SE). Normalized current was calculated as $I_{Na,\alpha\beta(EPA)}/I_{Na,\alpha\beta(control)}$ from the same corresponding cell.

to $hH1_\alpha$ would reestablish the specificity by only PUFAs for modulating the fast voltage-dependent Na^+ current in the rat, we assessed the effects of coexpressing the β_1-subunit on the kinetics of the α-subunit of hH1 in the absence or presence of the n-3 PUFAs in HEK293t cells. Compared to the expression of $hH1_\alpha$ alone, coexpressing hH1 α- and β_1-subunits ($I_{Na,\alpha\beta}$) significantly increased current density in transfected HEK293t cells [59]. Compared with $I_{Na,\alpha}$ the voltage-dependent steady-state inactivation of $I_{Na,\alpha\beta}$ significantly shifted in the depolarizing direction. EPA significantly inhibited $I_{Na,\alpha\beta}$ in a voltage- and concentration-dependent manner (Fig. 2). EPA shifted the $V_{1/2}$ of the steady-state inactivation by −22 mV (Table 2). EPA also significantly accelerated channel transition from the resting state to the inactivated state and prolonged the recovery time from inactivation [59]. Other PUFAs also significantly inhibited $I_{Na,\alpha\beta}$. In contrast, saturated and monounsaturated fatty acids had no effects on $I_{Na,\alpha\beta}$. This finding differs from the results for $I_{Na,\alpha}$, which was significantly inhibited by both saturated and unsaturated fatty acids. Our data demonstrate that functional association of the β-subunit with $hH1_\alpha$ modifies the kinetics and fatty acid block of the Na^+ channel.

To assess how the PUFAs modulate the activity of cardiac Na^+ channels, we introduced specific site-directed point mutations of single amino acids in $hH1_\alpha$ to determine whether a single amino-acid mutation would cause a loss or significant reduction of the expected action of the PUFAs on the channel conductance. Three mutated single amino-acid substitutions with lysine were made in the $hH1_\alpha$ Na^+ channel at domain 4-segment 6 (D4-S6) for F1760, Y1767 and at D1-S6 for N406, sites which are in the putative regions for binding of local anesthetics and batrachotoxin, respectively. EPA or DHA significantly reduced Na^+ currents in the HEK293t cells expressing the wildtype, Y1767K, or F1760K of $hH1_\alpha$ [60, 61]. The inhibition was voltage- and concentration-dependent with a significant hyperpolarizing shift of the steady-state inactivation (Table 2). In contrast, the mutant N406K was much less sensitive to the inhibitory effect of EPA. Coexpression of N406K with the β_1 subunit in HEK293t cells further decreased the inhibitory effect of EPA with a significantly smaller hyperpolarizing shift of the $V_{1/2}$ of the steady-state inactivation of the Na^+ current. These results demonstrate that substitution of asparagine with lysine at site 406 in D1-S6 significantly reduced the inhibitory effect of the PUFAs on the Na^+ current, and coexpression with β_1 decreased this effect even more. Therefore, asparagine at the 406 site in $hH1_\alpha$ appears critical for the inhibition by the PUFAs of cardiac Na^+ currents, which may play a significant role in the antiarrhythmic action of the n-3 fatty acids. These findings suggest that the $hH1_\alpha$ protein may have a specific binding site for the n-3 PUFAs.

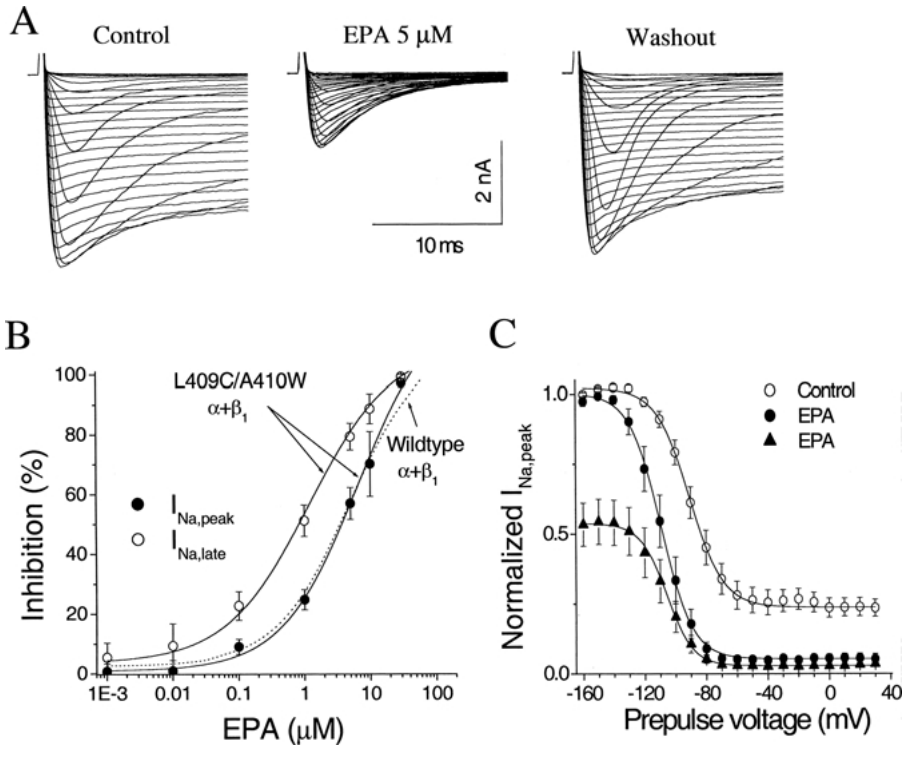

Fig. 3. Effects of EPA on persistent Na^+ currents in HEK293t cells transfected with the mutant L409C/A410W of $hH1_\alpha$ plus β_1 subumt. (*A*) Superimposed Na^+ current traces were recorded in the absence (*Control* and *Washout*) and presence (*EPA 5* μM) of EPA. Following 400 ms hyperpolarizmg pulses to −160 mV, currents were elicited by 200 ms pulses from −90 mV to 50 mV with 5 mV increments. The membrane potential was held at −90 mV and the pulse rate was 0.2 Hz. Compared to the control, both $I_{Na,peak}$ and $I_{Na,late}$ were inhibited by 5 μM EPA, but $I_{Na,late}$ was almost completely inhibited. The current recovered after washout of EPA. (*B*) Concentration-dependent suppression of persistent Na^+ currents by EPA in HEK293t cells expressing inactivation-deficient $hH1_\alpha$ Na^+ channels. The inhibition on $I_{Na,peak}$ (●) and $I_{Na,late}$ (○) was proportional to the concentrations of the fatty acid ($n \geq 6$). The dotted line is the concentration-dependent inhibition of Na^+ currents in HEK293t cells transfected with the wildtype hHI_α Na^+ channel plus β_1 subunit [59]. (*C*) The steady-state inactivation of $I_{Na,peak}$ in the absence (○) or presence (●, $n = 16$) of 5 μM EPA is shown. $I_{Na,peak}$ was inhibited by 50% with prepulses from −160 mV to −130 mV and almost completely suppressed with prepulses more positive than −70 mV (▲, relative inhibition). The data were fitted with a Boltzmann equation and show a significant shift in the hyperpolarizing direction in the presence of EPA. A portion (~25%) of $I_{Na,peak}$ in the absence of EPA was not inactivated even with highly depolarizing prepulses, but EPA at 5 μM almost completely abolished the non-inactivated portion. Currents were elicited by 200 ms test pulses to −30 mV following 500 ms conditional prepulses varying from −160 mV to 30 mV with 10 mV increments.

Along with the inhibition of cardiac I_{Na}, the n-3 PUFAs significantly enhance the transition of cardiac Na^+ channels into the inactivation state and markedly shift the steady-state inactivation curve to the hyperpolarizing direction [55]. Aside from these specific effects, we explored whether the PUFAs would have any effect on inactivation-deficient cardiac Na^+ channels. We investigated the effect of the n-3 PUFAs on persistent Na^+ currents in HEK293t cells transfected with the Na^+ inactivation-deficient mutant L409C/A410W of $hH1_\alpha$ Na^+ channels [62]. The double mutants at L409/A410 comprise part of the receptor site for the docking of the inactivation particle so that the inactivation particle is unable to close the Na^+ channel [54]. Extracellular application of EPA or DHA significantly and reversibly inhibited the Na^+ current of the mutant, but the late portion (measured near the end of each pulse) of I_{Na} was almost completely suppressed (Fig. 3). The inhibitory effect of EPA on I_{Na} was concentration-dependent with IC_{50} values of 4.0 μM for the peak and 0.9 μM for the late portion of I_{Na} (Table 2). EPA shifted the steady-state inactivation of I_{Na} by −19 mV to the hyperpolarizing direction (Fig. 3, Table 2). Our data demonstrate that the double mutations at sites 409 and 410 in the D1-S6 region of $hH1_\alpha$ induce inactivation-deficient Na^+ channels and that the n-3 PUFAs are able to inhibit the Na^+ current and to restore the inactivation of the inactivation-impaired channel.

It is widely known that local anesthetics, such as lidocaine and procaine, have antiarrhythmic properties. However, other local anesthetics, such as cocaine [18, 39] and bupivacaine [15] are cardiotoxic, associated with arrhythmias, which may be fatal. To compare the effects of lidocaine and cocaine with the n-3 fatty acids, EPA and DHA, on Na^+ channels and to learn how lidocaine and n-3 fatty acids are

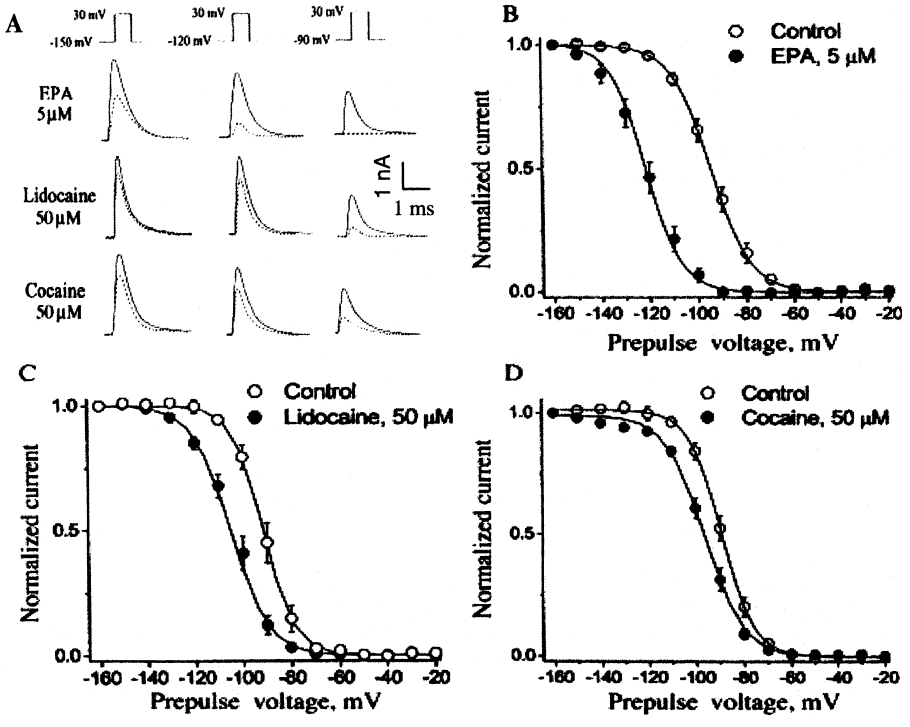

Fig. 4. Effects of EPA, lidocaine, and cocaine on $I_{Na,\alpha}$ recorded in HEK293t cells expressing hH1$_\alpha$ Na^+channels. (*A*) Single step depolarizations from −150, −100, and −90 to +30 mV each in the absence (*solid lines*) and presence (*dotted lines*) of EPA (5 μM), lidocaine (50 μM), or cocaine (50 μM). Normalized steady-state inactivation was fitted by the Boltzmann equation in the absence and presence of 5 μM EPA (*B*), 50 μM lidocaine. (*C*), and 50 μM cocaine (*D*). Currents were elicited by 10-ms test pulses to 30 mV following 500-ms conditional prepulses varying from −160 to −20 mV with 10-mV increments. The membrane potential was held at −150 mV and the pulse rate was 0.1 Hz. The values of the $\Delta V_{1/2}$ shifts were −27.1, −13.6, and −6.8 mV for EPA (5 μM), lidocaine (50 μM), and cocaine (50 μM), respectively.

cardioprotective, whereas cocaine can be cardiotoxic, we examined their electrophysiologic effects on human and rat cardiac I_{Na}. Our data show that the n-3 fish oil fatty acids and lidocaine share three actions on I_{Na}: a potent inhibition; a strong voltage-dependent inhibition; and a significant shift of the steady-state inactivation in the hyperpolarized direction [63]. In contrast, cocaine shows only the potent inhibition of I_{Na}. The voltage-dependent inhibition of I_{Na} by cocaine is much decreased with only a very small leftward shift of the steady-state inactivation (Fig. 4). Further we observed that in cultured neonatal rat cardiomyocytes n-3 fish oil fatty acids terminate the tachycardia induced by the α_1 adrenergic agonist, phenylephrine, whereas cocaine accelerates the tachycardia [63]. Our data suggest that the large leftward shift (towards more negative potential) of the steady-state inactivation is very critical to the prevention of fatal arrhythmias by the n-3 fatty acids or lidocaine. It is likely that the small leftward shift of the steady-state inactivation, not a lesser inhibition of I_{Na}, by cocaine, impairs its ability to prevent cardiac arrhythmias. In addition, the sympathomimetic action of cocaine via inhibition of presynaptic reuptake of norepinephrine and via the local anesthetic properties of Na^+ channel blocker described above might synergistically cause cardiac arrhythmias.

Ca^{2+} Channels

The voltage-gated L-type Ca^{2+} current ($I_{Ca,L}$) is responsible for the Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum into the cytosol of heart cells. Increase in cytoplasmic free calcium concentration is critical for electro-mechanical coupling and contraction of the heart. Intracellular Ca^{2+} overload, however, can cause cardiac arrhythmias. The inhibitory effects of the n-3 PUFAs on $I_{Ca,L}$ may thus partially be responsible for their antiarrhythmic properties. One study using isolated cultured neonatal rat cardiac myocytes showed that the n-3 PUFAs, but not monounsaturated or saturated fatty acids, were antiarrhythmic during Ca^{2+} overload [24]. The antiarrhythmic effect occurred quickly, but only with the free fatty acid form of the PUFAs, while the ethyl ester or triglyceride forms were not promptly antiarrhythmic [24]. The antiarrhythmic effect is quickly reversed when the free PUFAs are extracted from the cells by adding delipidated bovine serum albumin to the bathing solution. This observation suggests that the PUFAs are neither fully incorporated into membrane phospholipids nor covalently bound to any constituents of the myocyte to produce the antiarrhythmic effect [24].

We further examined the role of $I_{Ca,L}$ and sarcoplasmic reticulum (SR) Ca^{2+} release in the action of the PUFAs on cardiomyocytes [56]. We observed clear reductions in $I_{Ca,L}$ and SR Ca^{2+} release, both important in Ca^{2+} overload arrhythmias. We observed that the direct action of PUFAs responsible for the reduced SR Ca^{2+} release was on $I_{Ca,L}$. The concentration of EPA to produce 50% inhibition of $I_{Ca,L}$ was 0.8 μM in neonatal rat heart cells and 2.1 μM in adult rat ventricular myocytes [56]. While EPA suppressed $I_{Ca,L}$, it produced a small, but significant, shift of the steady-state inactivation curve in

the hyperpolarizing direction (Fig. 5). Other PUFAs, unlike saturated or monounsaturated fatty acids, had similar effects on $I_{Ca,L}$ as EPA. EPA did not change the temporal or spatial character of the Ca^{2+}-sparks, nor did it alter the ability of $I_{Ca,L}$ to trigger Ca^{2+}-sparks [56]. Our data indicate that the PUFAs may act as antiarrhythmic agents in vivo in normal and Ca^{2+}-overloaded cells principally because they reduce Ca^{2+} entry by blocking $I_{Ca,L}$. The finding that only PUFAs with a free carboxyl group blocked cardiac Ca^{+} channels suggests that these carboxyl groups might be interacting with the positively charged amino acids of the α-subunit of cardiac Ca^{2+} channels. We have also shown that the action of EPA to reduce $I_{Ca,L}$ produces a reduction of the $[Ca^{2+}]_i$ transient [56]. While there is a reduction of the probability that Ca^{2+}-sparks are triggered by a depolarization, the relationship between $I_{Ca,L}$ and Ca^{2+} -sparks is unchanged. Similarly, there is no change in the kinetics of the Ca^{2+} -spark, the elementary unit of SR Ca^{2+} release. These results demonstrate that there is no direct action of EPA on the SR Ca^{2+}-release process or on SR Ca^{2+} re-uptake [21]. One study, however, demonstrated that the PUFAs have two specific effects on the SR: they reduce the availability of calcium for uptake and they inhibit the release mechanism [42]. The n-3 PUFAs may also modify the action of dihydropyridine agonists and antagonists on the L-type Ca^{2+} channels [45]. The finding of an inhibition of $I_{Ca,L}$ in our patch-clamp study [56] is consistent with the earlier reports that the n-3 PUFAs were able to displace [^{3}H]nitrendipine from its binding site at the extracellular pole of the calcium channel [22].

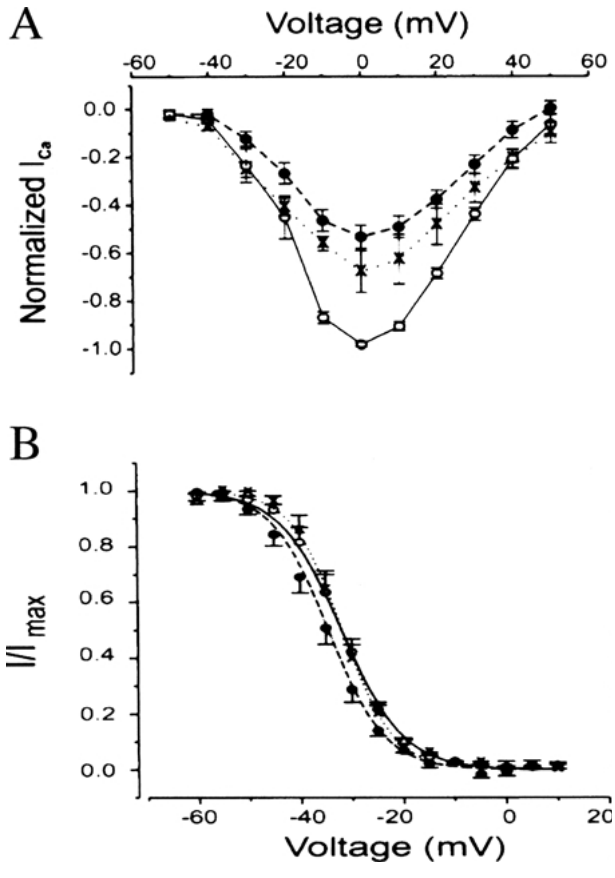

Fig. 5. Inhibition of the voltage-gated L-type Ca^{2+} current in adult rat ventricular myocytes. (*A*) Normalized current-voltage relationships are shown for control (*empty circles*, $n = 11$), 1.5 μM EPA (*filled circles*, $n = 11$) and washout (*exes*, $n = 5$). The current density values were normalized to the maximum level observed under control conditions for each experiment [56]. (*B*) Normalized steady-state inactivation of peak I_{Ca} is shown for control (*empty circles*) with fitting parameters of $V_{1/2} = -31.97 \pm 1.1$ mV and $K = 5.2 \pm 0.26$ ($n = 11$), 10 μM EPA (*filled circles*) with fitting parameters of $V_{1/2} = -34.53 \pm 1.1$ mV and $K = 5.11 \pm 0.15$ ($n = 11$) and washout (*X-symbols*) with fitting parameters of $V_{1/2} = -31.86 \pm 0.9$ mV and $K = 4.9 \pm 0.13$ ($n = 5$). Control and EPA curves are significantly different ($P < 0.05$).

K^+ Channel

I_{to}, I_K, and I_{K1}, are the three major potassium currents across cardiac cell membrane. After analyzing the effects of the n-3 PUFAs on cardiac Na^+ and Ca^{2+} channels, we further examined the effect of these fatty acids on the voltage-activated K^+ currents in isolated adult mammalian cardiomyocytes [57]. We found that the two outward K^+ currents, the transient outward K^+ current (I_{to}) and the delayed rectifier K^+ current (I_K), were both inhibited by the n-3 PUFAs, while the inwardly rectifying K^+ current (I_{K1}) was unaffected by the n-3 PUFAs. The lack of an effect of the n-3 PUFAs on I_{K1} may result from differences of channel structures among I_K, I_{to}, and I_{K1} [11]. DHA produced a concentration-dependent suppression of I_{to} and I_K in adult ferret cardiomyocytes with an IC_{50} of 7.5 and 20 μM, respectively; but had no effect on I_{K1}. The inhibition of I_{to} by the PUFAs was also reported by a different laboratory [9]. Honore and colleagues showed that DHA and the n-6 polyunsaturated fatty acid arachidonic acid (C20:4n-6, AA) blocked the major cardiac delayed rectifier K^+ channel (Kv1.5) expressed in a Chinese hamster ovarian cell line and I_K in cultured mouse and rat cardiac myocytes [23]. Their results demonstrate that the inhibition occurred when DHA was applied extracellularly and not when included in the patch electrode [23].

EPA showed effects on I_{to}, I_K, and I_{K1} similar to DHA. However, AA had a biphasic action on I_K: initially inhibition and then activation. The late activation of I_K by AA was prevented by pretreatment with indomethacin, a cyclooxygenase inhibitor. This late activation of I_K by AA was thus not due to the free AA but to cyclooxygenase metabolites [57]. Monounsaturated and saturated fatty acids, which are not antiarrhythmic, lack any significant effects on

the major K^+ currents. Our results demonstrate that the n-3 PUFAs inhibited cardiac I_{to} and I_K with higher concentrations (Table 2). Inhibition of the outward K^+ currents, I_{to} and I_K, by the n-3 PUFAs would prolong the duration of the action potential rather than shorten it [4, 26]. Therefore, the inhibitory effects of the PUFAs on the $I_{Ca,L}$ and I_{Na} would have to dominate over the weaker inhibition of the outward K^+ currents to explain the observed small, but significant, reduction in the action potential duration of cultured cardiomyocytes [26] and the electrocardiographic QT interval in dogs (30).

I_{K1} is essential for maintaining different resting membrane potential in pacemaker or non-pacemaker cardiomyocytes [43] and in other types of cells [14]. During myocardial ischemia intracellular K^+ leaks out due to membrane damage, which can cause membrane depolarization and arrhythmias. A lower resting potential in ischemic (−70 mV) than in non-ischemic (−78 mV) human ventricular myocytes has been reported [41]. In addition, intravenous injection of anesthetic thiopental has been shown to increase the incidence of ventricular arrhythmias via depression of I_{K1} and depolarization of the resting membrane [44]. Our studies have shown that the n-3 PUFAs inhibit I_K and I_{to}, but not I_{K1} [57]. These effects result in a decrease in the efflux of K^+ without a depolarization of cardiac resting membrane potential, which is consistent with our previous finding of slight hyperpolarization of resting cardiomyocytes in the presence of the PUFAs [26]. Therefore, the effects of the n-3 PUFAs on Ca^{2+} and Na^+ channels without inhibition of I_{K1} may protect the heart from arrhythmias during myocardial ischemia.

Pacemaker Channels

The normal excitatory stimuli of the heart are generated in the sinoatrial (SA) node. The "pacemaker" channel may play a critical role in automaticity of the SA node. This channel is commonly referred to as the hyperpolarization-activated and cyclic nucleotide-modulated (HCN) channel. HCN channels in the heart modulate cardiac automaticity via the hyperpolarization-activated cation current (I_f, "funny" current). Hyperpolarization activates the channel and cyclic nucleotides modulate its activity. Recent studies have unveiled the molecular identity of HCN (HCN 1-4) channels [32, 48]. The SA node shows the highest level of HCN4 in the heart and exhibits both the largest and most positively activating pacemaker currents [64]. In order to evaluate the effects of n-3 PUFAs on HCN, we transfected HEK293 cells with human cardiac HCN4 cDNA. In non-transfected HEK293 cells, there were no detectable HCN currents (*data not shown*). However, robust HCN4 currents were elicited by different voltages in HCN4-transfected HEK293 cells (Fig. 6).

Activation of HCN4 channels is quite slow and requires several seconds to reach a steady state. The amplitude of the tail currents at a fixed holding potential is usually analyzed for a voltage-dependent activation curve of HCN currents. Figure 6 shows that 10 μM DHA had a very minor effect on HCN4 currents in the HEK293 cells transfected with the human HCN4 cDNA vector. DHA at 10 μM did not significantly change the activation of HCN4 channels and had no significant effect on the activation time constant (Fig. 7). The reversal potential of HCN4 currents was not altered by DHA in the HCN4-transfected HEK293 cells (Fig. 8). These results indicate that the minor effect of DHA on HCN4 channels may not influence cardiac automaticity and heart rate in vivo, while these fatty acids have anti-arrhythmic actions during myocardial ischemia.

Other Ion Channels

To further examine the effects of the n-3 PUFAs on other cardiac ion channels, we studied the acetylcholine-activated K^+ channel ($I_{K,ACh}$), cardiac Na^+-Ca^{2+} exchanger (NCX1), and cAMP-activated cardiac chloride channel. EPA significantly inhibited cardiac $I_{K,ACh}$ and cAMP-activated Cl^- currents [31]. The significance of the inhibition of $I_{K,ACh}$ or Cl^- currents is currently not clear. EPA or DHA, but not the saturated fatty acid, stearic acid (C18:0), significantly inhibited cardiac Na^+-Ca^+ exchanger currents in HEK293t cells transfected with the canine NCX1 cDNA [65]. The suppression was concentration-dependent with an IC_{50} of 0.82 μM of EPA.

It has been shown that the n-6 fatty acid AA can modulate T-type Ca^{2+} channels [52]. AA inhibited α_{1G} currents in HEK-293 cells heterologously expressing the T-type Ca^{2+} channel [52]. The inhibition occurred within a few minutes after perfusion of AA, regardless of preceding exposure to inhibitors of AA metabolism (ETYA and 17-ODYA). Single-channel recordings of cell-free inside-out patches showed that current inhibition was due to a decrease of the open probability without changes in the size of unitary currents. AA shifted the inactivation curve to more negative potentials, increased the speed of macroscopic inactivation, and decreased the extent of recovery from inactivation [52]. During AA perfusion, a tonic current inhibition was observed regardless of whether the channels were held in resting or inactivated states. This result suggests a state-independent interaction with the channel. Compared with the wildtype, the α_{1G} mutants with slow inactivation showed an increased affinity for AA, indicating that the structural determinants of fast inactivation are involved in the AA-channel interaction. Model simulations indicate that AA inhibits T-type currents by switching the channels into a nonavailable conformation and by

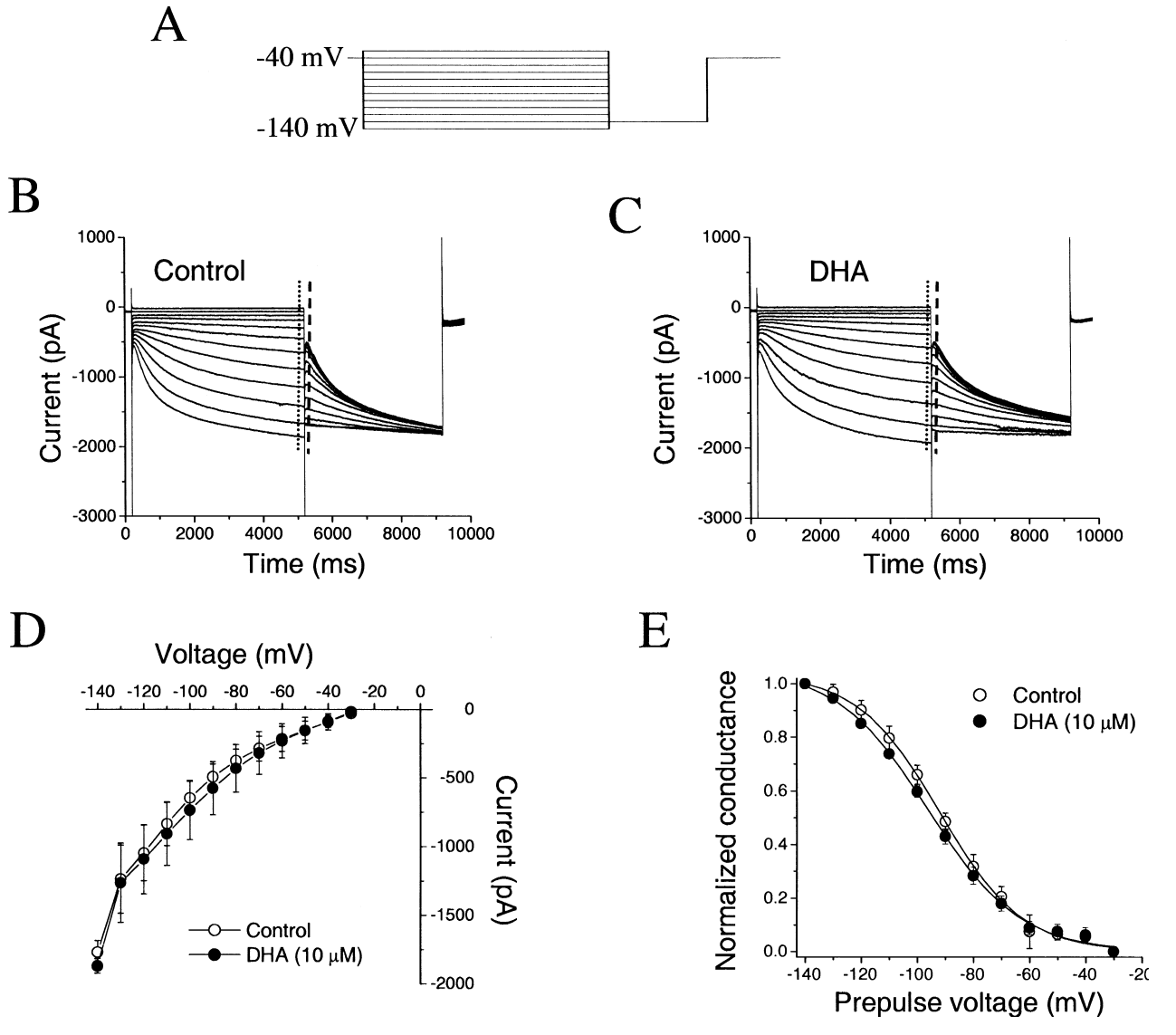

Fig. 6. Effects of DHA on HCN4 currents in HEK293 cells transfected with human HCN4 cDNA. Panel *A* shows the voltage-clamp protocol. The durations of the pulses are corresponding to the *X*-axes of panels *B* and *C*. The holding potential was −40 mV and the pulse rate was every 30 s. Test pulses from −40 mV to −140 mV in 10 mV increments were applied. Panel *B* and *C* are the superimposed I_f traces recorded from an HEK293 cell in the absence (*Control*) and presence (*DHA*) of 10 μM DHA. (*D*) Mean current-voltage relationships ($n = 5$) of I_f plotted according to the values measured at the dotted vertical lines for control (○) and DHA (●) in panels *B* and *C*. (*E*) Mean normalized activation curves of I_f ($n = 5$) were drawn from the values measured at the dashed vertical line for control (○) and DHA (●) of the tail currents in panels *B* and *C*. The data points were fitted by a Boltzmann equation. DHA at 10 μM significantly altered neither the amplitudes of the currents nor the activation parameters.

affecting transitions between inactivated states, which results in the negative shift of the inactivation curve [52].

Potential Sites for the Action of the n-3 PUFAs

Membrane phospholipid is one of the possible sites of the action of the n-3 PUFAs on ion channels. Klausner and colleagues showed that these same unsaturated fatty acids, to the extent they were tested, could alter the "fluidity" of membrane phospholipids [28]. This has been thought 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 the n-3 PUFAs that inhibited cardiac ion channels in our experiments. At these low concentrations, the molar ratio of the n-3 PUFAs to phospholipds in red cell ghost is less than 1:100 [46], which is too low to have

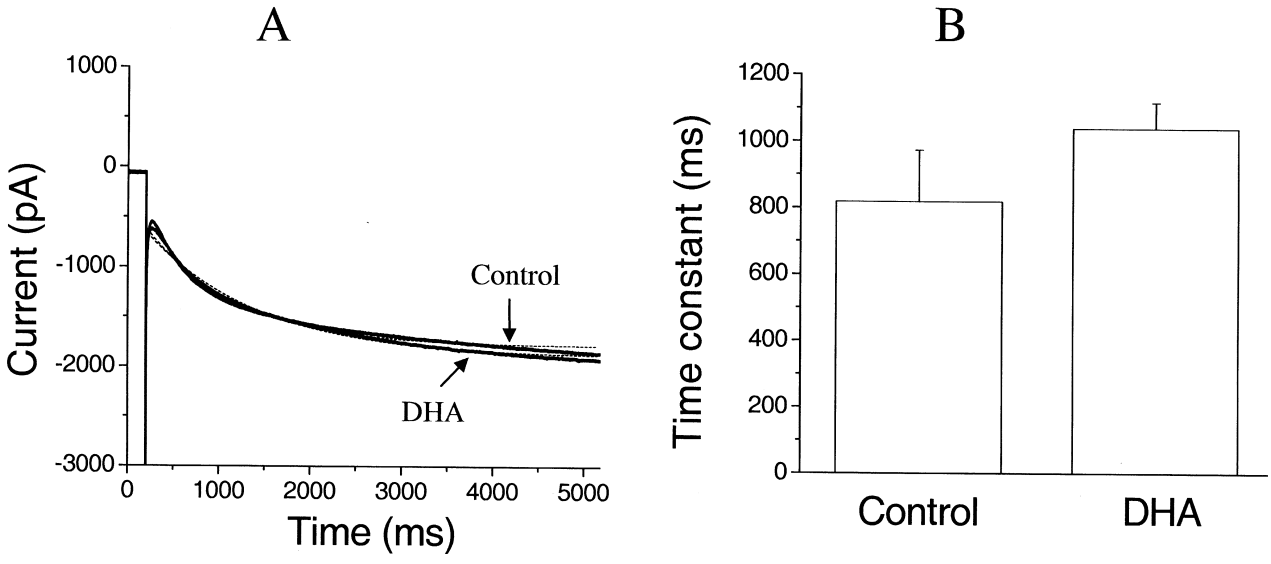

Fig. 7. Effect of DHA on time constant of activation of I_f in HEK293 cells transfected with human HCN4 cDNA. (*A*) Original current traces (*solid lines*) and the lines (*dotted lines*) fitted by the single-exponential equation in the absence (*Control*) and presence (*DHA*) of 10 μM DHA. The holding potential was −40 mV. The currents were elicited by test pulses from −40 to −140 mV. (*B*) Mean time constants ($n = 5$) of I_f recorded in the absence (*Control*) and presence (*DHA*, $P > 0.05$) of 10 μM DHA.

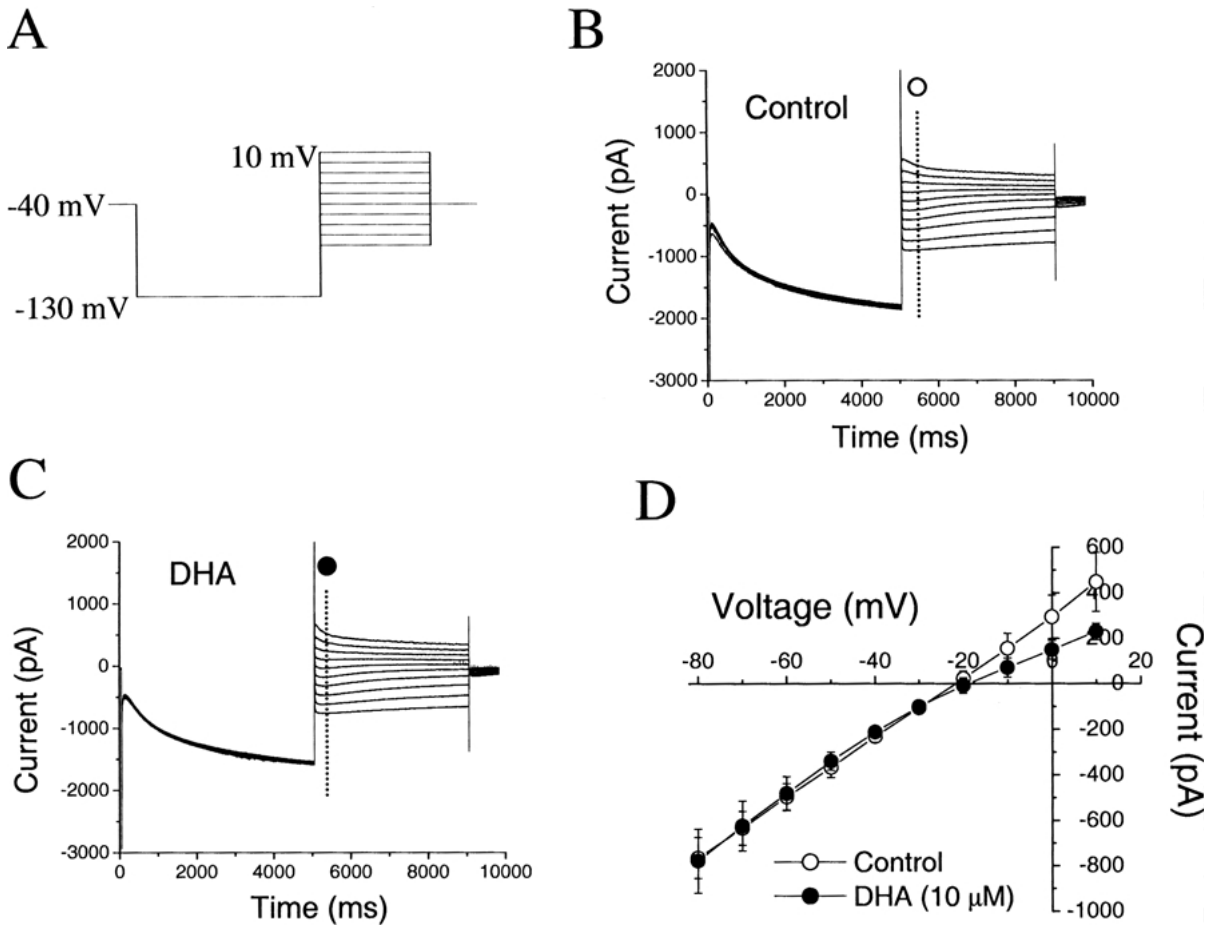

Fig. 8. Effects of DHA on the reversal potential of I_f in HEK293 cells transfected with human HCN4 cDNA. The mean reversal potential curves were plotted from the I_f tail currents ($n = 5$) measured at the dotted vertical lines for control (*B*, ○) and 10 μM DHA (*C*, ●) against the test voltages of the tail pulses (shown in *A*). DHA at 10 μM had no significant effect on the I_f reversal potential (*D*).

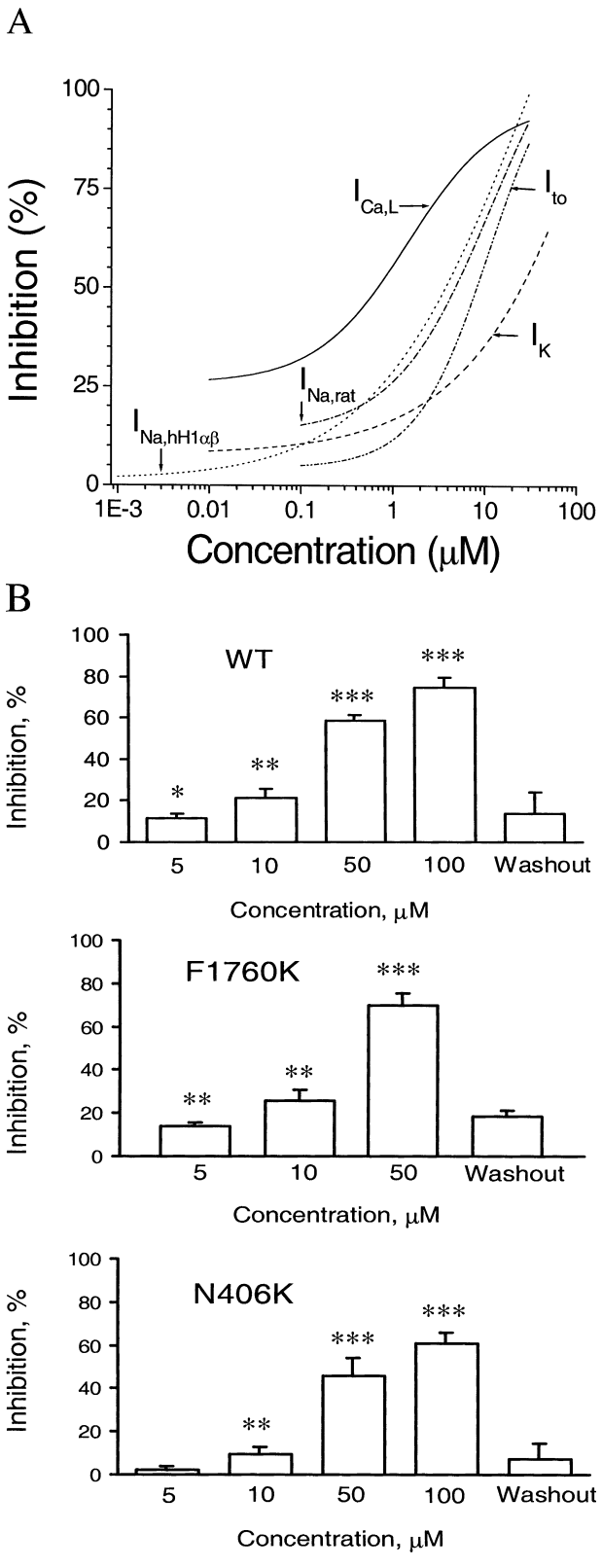

Fig. 9. The curves in (*A*) are the fitting lines based on the data of the concentration-dependent inhibition of $I_{Na,rat}$, $I_{Na,hH1\alpha,\beta}$, $I_{Ca,L}$, I_K, and I_{to} by EPA or DHA. (*B*) Effects of Triton X100 on human hHl_α Na^+ currents in the HEK293 cells transfected with the wildtype (WT) and the mutants, F1760K and N406K. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus their corresponding controls.

a general effect of increasing the "fluidity" of the membrane phospholipids.

During the past decade 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 [5, 19, 33, 34]. They have tested their hypothesis on the cationic-permeable short gramicidin channel with two nonionic detergents, Triton X100 and β-octyl glucoside. They reported that these two compounds with no chemical similarity to the PUFAs also affect the Na^+ and Ca^{2+} currents of mammalian channels [5, 33]. They explained their results by alteration of stresses between channel and membrane when the hydrophobic length of the transmembrane channel protein does not match the hydrophobic thickness of the resting membrane phospholipid bilayers (*see* Fig. 5 in reference 31). This tension exerts locally on the channel protein, which affects the conformational state and conductance of the ion channel. Compounds that can incorporate into the phospholipid membrane close to its junction with a channel protein may change the tension of the channel protein. This would change the conformational state of the transmembrane segment of the channel protein and affect its conductance.

Our recent observations showed that Triton X100 and β-octyl glucoside significantly inhibited Na^+ currents of human cardiac Na^+ channels transiently expressed in HEK293 cells and shifted the steady-state inactivation to more hyperpolarized potentials [55]. The detergents probably act on the phospholipid cell membrane, which abuts the transmembrane channel protein. Thus, other ion channels, such as Ca^{2+} channels, were also affected [56]. These effects are similar to those of the n-3 PUFAs. The n-3 PUFAs affect many types of ion channels as we have observed [31]. This low selectivity thus suggests that the modulation of ion channels by the n-3 PUFAs is possible via local action on membrane phospholipids, as the two nonionic detergents do.

However, the above hypothesis of PUFAs' action on membrane phospholipids does not give a satisfactory explanation to several of our findings. For example, I_{K1}, HCN4, as well as mutated N406K hH1 Na^+ channels were not sensitive to the n-3 PUFAs. In addition, the effects and the concentrations to cause 50% inhibition differ among different types of cardiac ion channels (Fig. 9*A*). It is, thus, quite possible that the n-3 fatty acids directly bind to the ion channel proteins and modulate channel activities. This possibility is supported by our observation that a single point mutation at 406 of D1-S6 of $hH1_\alpha$ Na^+ channels significantly decreased the sensitivity to the PUFAs [61], but did not significantly alter the effective inhibition of the mutant by Triton X100

(Fig. 9*B*). This result suggests that the n-3 fatty acids may directly act on the Na^+ channel protein via binding to a specific location (probably the D1-S6 region), as most local anesthetics are able to bind to D4-S6. It is well known that free PUFAs bind to plasma proteins and are transported to other tissues by the blood. Our data clearly demonstrate that extracellular perfusion of delipidated bovine serum albumins washed away the inhibitory effects of the PUFAs, which indicates that bovine serum albumins bind to PUFAs and remove them away from their active sites. In addition, the data from two receptor-binding studies strongly support the hypothesis that the modulatory effects of the n-3 PUFAs on ion channels result from direct action on channel proteins [22, 27]. Specific binding of [^{3}H]nitrendipine to intact cardiomyocytes was noncompetitively inhibited by EPA or DHA via reduction of high- and low-affinity binding sites [22]. In neonatal rat cardiac myocytes EPA reversibly inhibited the binding of the radioligand, [benzoyl-2,5-(3)H] batrachotoxin, to voltage-gated Na^+ channels [27]. The purported receptor for batrachotoxin locates at the D1-S6 region of cardiac Na^+ channels. This could explain why the mutant N406K of D1-S6 significantly reduces the sensitivity to the n-3 fatty acids. It has been reported that batrachotoxin can bind inside the pore [53]. The binding affinity of the n-3 PUFAs to an ion channel is probably dependent on its sequence and structural conformation. Different affinities may explain why the responses to the n-3 fatty acids are different among different types of cardiac ion channels (Fig. 9) and why DHA or EPA has no significant effects on I_{K1} and HCN4 channels.

The antiarrhythmic action of these fatty acids may result from their significant inhibition of cardiac Na^+ and Ca^{2+} channels. For example, ischemic cardiomyocytes can be partially depolarized (e.g., perhaps −70 mV rather than −90 mV) and more vulnerable. A small depolarizing stimulus (e.g., current of injury) can activate Na^+ channels to initiate action potentials and arrhythmias. Fortunately, the n–3 PUFAs can prevent their occurrence by voltage-dependent inhibition and by shifting the steady-state inactivation to more negative potentials [55–65]. Furthermore, in partially depolarized heart cells, the n-3 PUFAs enhance the transition of Na^+ channels directly into an inactive state in milliseconds without eliciting an action potential [59]. The result of these events is that the partially depolarized cells are promptly and completely inactivated by the presence of the n-3 fatty acids. The PUFAs have no significant effects on I_{K1} and HCN channels, which may be important for maintaining normal cardiac function because I_{K1} channels are critical for maintaining resting membrane potential in most cardiomyocytes and HCN channels are critical for pacemaker cells in the heart.

In summary, our studies and others demonstrate that the n-3 PUFAs can modulate the electrophysiological kinetics of several ion channels. As the effects of these fatty acids on ion channels are fully reversible after extraction with albumin, the PUFAs may act either on channel proteins directly or via non-covalent incorporation into membrane phospholipids. It is also possible that the n-3 PUFAs are able to bind to a specific site on channel proteins and also to non-specifically incorporate into lipid cell membrane. More studies are needed to further dissect the mechanism(s) by which n-3 PUFAs modulate ion channels and protect the heart from arrhythmias.

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