Single point mutations affect fatty acid block of human myocardial sodium channel α subunit Na^+ channels

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Suppression of cardiac voltage-gated Na^+ currents is probably one of the important factors for the cardioprotective effects of the n-3 polyunsaturated fatty acids (PUFAs) against lethal arrhythmias. The α subunit of the human cardiac Na^+ channel ($hH1_\alpha$) and its mutants were expressed in human embryonic kidney (HEK293t) cells. The effects of single amino acid point mutations on fatty acid-induced inhibition of the $hH1_\alpha$ Na^+ current (I_{Na}) were assessed. Eicosapentaenoic acid (EPA, C20:5n-3) significantly reduced I_{Na} in HEK293t cells expressing the wild type, Y1767K, and F1760K of $hH1_\alpha$ Na^+ channels. The inhibition was voltage and concentration-dependent with a significant hyperpolarizing shift of the steady state of I_{Na}. In contrast, the mutant N406K was significantly less sensitive to the inhibitory effect of EPA. The values of the shift at 1, 5, and 10 μM EPA were significantly smaller for N406K than for the wild type. Coexpression of the β_1 subunit and N406K further decreased the inhibitory effects of EPA on I_{Na} in HEK293t cells. In addition, EPA produced a smaller hyperpolarizing shift of the $V_{1/2}$ of the steady-state inactivation in HEK293t cells coexpressing the β_1 subunit and N406K. These results demonstrate that substitution of asparagine with lysine at the site of 406 in the domain-1-segment-6 region (D1-S6) significantly decreased the inhibitory effect of PUFAs on I_{Na}, and coexpression with β_1 decreased this effect even more. Therefore, asparagine at the 406 site in $hH1_\alpha$ may be important for the inhibition by the PUFAs of cardiac voltage-gated Na^+ currents, which play a significant role in the antiarrhythmic actions of PUFAs.

human cardiac Na^+ channel | β_1 subunit | polyunsaturated fatty acids

Dietary polyunsaturated fatty acids, especially of the n-3 class (PUFAs), have been shown to be potent cardiac antiarrhythmic agents in animals, and probably in humans (reviewed in ref. 1). Much has been learned about the mechanism(s) by which these antiarrhythmic effects operate by directly and rapidly modulating the conduction of ion channels in the sarcolemma, so as to make every cardiomyocyte resistant to arrhythmias (1). Major uncertainties, however, still remain as to how the PUFAs accomplish these modulatory actions. The current dominant view is that antiarrhythmic agents affect channel conductances by directly interacting (binding) with the protein of the ion channel as it penetrates the sarcolemmal phospholipid bilayer. The technique used to establish such an interacting site of the ligand with the ion channel protein is to introduce specific site-directed point mutations of single amino acids in the wild-type ion channel protein to determine whether a single or cluster of juxtaposed amino acids will cause a loss or significant reduction of the expected action of the ligand on the channel conductance. If such a point mutation is experimentally discovered, then the amino acid that has been replaced in the mutated channel protein is presumed to be in the binding site for the ligand to the channel protein.

The present study reports that a point amino acid mutation expressed in the human cardiac sodium channel, $hH1_\alpha$, transiently expressed in a human embryonic kidney cell line, HEK293t, seems to fulfill this criterion for an interactive or binding site for PUFAs.

Methods

Cell Culture and Transient Transfection of Na^+ Channels. Oligonucleotide-directed point mutations of $hH1_\alpha$ were performed by using the transformer site-directed mutagenesis kit (CLONTECH), modified to increase the *in vitro* yield DNA synthesis. The detailed method has been described (2, 3). HEK293t cells were cultured with the method described elsewhere (3). Cells transfected with the wild type or its mutants of $hH1_\alpha$ Na^+ channels or plus the rat brain β_1 subunits were seeded with an appropriate density in 35-mm tissue culture dishes (which also served as recording chambers). Transfected cells were used within 5 days.

Electrophysiologic Recordings. In an experiment, HEK293t cells transfected with the wild type or its mutants of $hH1_\alpha$ or plus β_1 were continuously superfused (1–2 ml/min) with Tyrode's solution containing 137 mM NaCl/5 mM KCl/1 mM $MgCl_2$/1.8 mM $CaCl_2$/10 mM Hepes/10 mM glucose, pH 7.4. The pipette solution contained 100 mM NaF/30 mM NaCl/10 mM EGTA/10 mM Hepes (titrated with cesium hydroxide to pH 7.3). The bath solution containing 65 mM NaCl/85 mM choline chloride/2 mM $CaCl_2$/10 mM Hepes (titrated with tetramethyl ammonium hydroxide to pH 7.4) was rapidly exchanged by a modified puffer-pipette system (4). After forming the whole-cell configuration, cells were dialyzed for 20 min before data collection. Various concentrations of fatty acids were applied by the puffer-pipette system. Concentrations of ethanol used for dissolving fatty acids were negligible and had no effect on Na^+ currents in HEK293t cells. Currents were acquired and analyzed as before (3, 5). Experiments were conducted at 22–23°C.

Data Analysis. Data are presented as mean ± SEM. Inactivation and voltage-block data were fit by the Boltzmann equation, $1/[1 + \exp(V_{1/2} - V)/k]$, where $V_{1/2}$ is the midpoint voltage of the function and k is the slope factor (in mV/*e*-fold change in current). The data of concentration-dependent inhibition of

Abbreviations: PUFAs, n-3 polyunsaturated fatty acids; EPA, eicosapentaenoic acid (C20:5n-3); $hH1_\alpha$, human myocardial sodium channel α subunit; HEK293, transformed human embryonic kidney cell line; LAs, local anesthetics; BTX, batrachotoxin; I_{Na}, Na^+ current.

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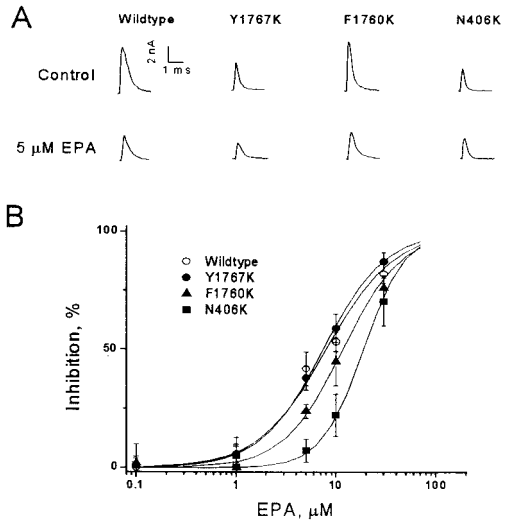

Fig. 1. Concentration-dependent suppression of human cardiac Na^+ currents by EPA. (*A*) Original current traces of I_{Na} in the absence (Control) and presence (EPA) of 5 μM EPA were evoked by depolarizing pulses from a holding potential of −150 mV to 30 mV for the wild type and three mutants. The membrane holding potential was −150 mV and the rate of pulses was 0.1 Hz. (*B*) The amplitude of peak Na^+ currents shown as in *A* was calculated in the absence and presence of various concentrations of EPA. The EPA-induced inhibition of I_{Na} is concentration-dependent. Each data point represents the average value of at least seven individual cells.

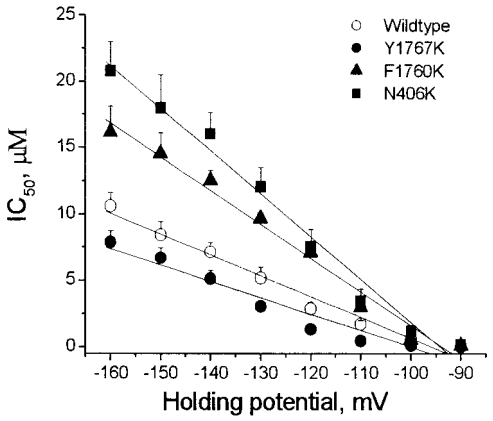

Fig. 2. Effects of different holding potentials on the IC_{50} of EPA. I_{Na} was elicited by 10-ms test pulses from different holding potentials to 30 mV every 10 s. Concentration-dependent inhibitions of I_{Na} by EPA were fit with a logistical equation at different holding potentials, and IC_{50} of EPA was calculated by the equation. The data of IC_{50} were fit with the equation of linear regression for the wild type (○), Y1767K (●), F1760K (▲), and N406K (■) of $hH1_\alpha$ Na^+ channels. The regression coefficients were similar for the wild type (−0.157 ± 0.008) and Y1767K (−0.124 ± 0.015). However, the regression coefficients were significantly different ($P < 0.01$) between the wild type and N406K (−0.322 ± 0.018), as well as between the wild type and F1760K (−0.254 ± 0.014).

peak I_{Na} were fit with the logistical equation, $(A_1 - A_2)/[1 + (x/x_0)^P + A_2]$, where x_0 is the center, p is power; A_1 is the initial Y value, and A_2 is the final Y value. The concentration of fatty acids that produced 50% inhibition of cardiac Na^+ currents is the IC_{50}. The data of IC_{50} at different holding potentials are fit with the equation of linear regression, $y = A + Bx$, where A is the intercept and B is the slope. The unpaired Student's t test was used to determine statistical differences between two experimental groups. Data derived from three or more experimental groups were examined by variance analysis (ANOVA). The level of $P < 0.05$ was considered as statistical significance.

Results

Inhibition of I_{Na} by EPA. The PUFAs not only inhibit voltage-gated Na^+ currents in rat cardiomyocytes (6) but also inhibit Na^+ currents in HEK293t cells expressing $hH1_\alpha$ alone (5) or $hH1_\alpha$ plus the β_1 subunit (3). To determine whether single-point mutations of the hH1 α subunit altered the PUFAs block of the Na^+ channel, the effects of EPA on Na^+ currents were examined in HEK293t cells expressing either the wild type ($hH1_\alpha$) or one of the three mutants (Y1767K, F1760K, or N406K) of $hH1_\alpha$. Fig. 1*A* shows that 5 μM EPA significantly inhibited I_{Na} in HEK293t cells transfected with the wild type, Y1767K, and F1760K of $hH1_\alpha$. In contrast, substitution of asparagine with lysine at the site of 406 (N406K) in the D1-S6 region did not significantly reduce the EPA-induced inhibition of I_{Na}. The average inhibition of I_{Na} by 5 μM EPA was 42 ± 7%, 38 ± 5%, 24 ± 3%, and 7 ± 5% for the wild type ($n = 8$, $P < 0.001$), Y1767K ($n = 7$, $P < 0.01$), F1760K ($n = 9$, $P < 0.01$), and N406K ($n = 8$, $P > 0.05$), respectively. The inhibition of I_{Na} commenced within 20 s and peaked within 3 min after application of 5 μM EPA. I_{Na} returned toward the pretreatment level after washout of EPA with 0.2% BSA solution. Fig. 1*B* shows that the inhibitory effects of EPA on I_{Na} for the wild type (○), Y1767K (●), F1760K (▲), and N406K (■) were concentration-dependent. Again, N406K shows a greatly reduced sensitivity to EPA over the range of concentrations tested. When currents were elicited by single-step pulses from −150 mV to 30 mV, the values of IC_{50} of EPA were 8.5 ± 1.0 μM, 6.7 ± 0.8, 14.5 ± 1.6, and 18.0 ± 2.5 μM for the wild type, Y1767K, F1760K, and N406K, respectively. These data indicate that single point mutations of $hH1_\alpha$ alter the inhibitory effects of EPA on I_{Na}. Especially, N406K reduced the inhibitory effect of EPA on Na^+ currents by 2-fold based on the values of IC_{50} for the wild type and the mutant of $hH1_\alpha$.

Voltage-Dependent Inhibition of I_{Na} by EPA. We had demonstrated that PUFAs produced a voltage-dependent inhibition of cardiac Na^+ currents in cardiomyocytes and in HEK293t cells expressing $hH1_\alpha$ or $hH1_\alpha$ plus β_1 (3, 5, 6). To learn whether the effects of the PUFAs on these mutated channels would follow the same pattern of inhibition, we assessed whether single point mutations modified the EPA-induced voltage-dependent inhibition. I_{Na} for the wild type and three mutants was evoked by single-step pulses from the holding potential of −160, −150, −140, −130, −120, −110, −100, or −90 mV to 30 mV in the presence of various concentrations of EPA (Fig. 2). The reduction of I_{Na} was concentration-dependent for the wild type and three mutants with different holding potentials. Fig. 2 shows the IC_{50} of EPA with different holding potentials for the wild type (○), Y1767K (●), F1760K (▲), and N406K (■). However, the inhibition of I_{Na} by EPA was more profound when test pulses were stepped from more positive holding potentials. For example, at the holding potential of −100 mV, the IC_{50} was 0.55 ± 0.21, 0.48 ± 0.04, 0.55 ± 0.20, and 1.22 ± 0.44 μM for the wild type, Y1767K, F1760K, and N406K, respectively. In contrast, at the holding potential of −160 mV, the IC_{50} was 10.6 ± 1.0, 7.9 ± 0.9, 16.1 ± 2.0, and 20.8 ± 0.2.2 μM for the wild type, Y1767K, F1760K, and N406K, respectively.

Depolarization of the cell membrane potential causes inactivation of cardiac voltage-gated Na^+ channels. To determine whether single point mutations affected the EPA-induced voltage-dependent block of $hH1_\alpha$ Na^+ channels, we used HEK293t cells transfected with the wild type and its three mutants. The voltage protocol consisted of a 10-s conditioning pulse ranging from −180 mV to −50 mV with 10-mV decrements followed by a 100-ms (Fig. 3*A*, *Inset*) or 5-ms (Fig. 3*B*,

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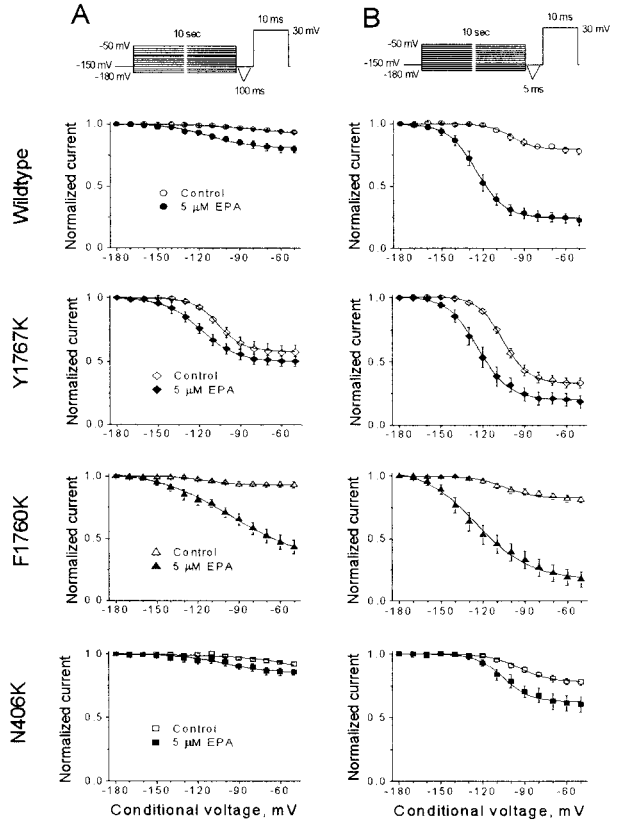

Fig. 3. Voltage-dependent inhibition of I_{Na} by EPA in HEK293t cells expressing the wild type or three mutants of $hH1_\alpha$ Na^+ channels. (*A*) Whole-cell currents were normalized by I_{Na} recorded with the conditioning voltage of −180 mV for their corresponding controls. The experimental protocol is shown in the *Inset*. Currents were elicited by 10-ms test pulses to 30 mV following a 10-s conditioning pulse varying from −180 mV to −50 mV with 10-mV increments. A 100-ms interval was inserted between the conditioning pulse and the test pulse. The membrane potential was held at −150 mV, and the pulse rate was 0.1 Hz. Except for the mutant F1760K, EPA at 5 μM did not show profound voltage-dependent suppression of I_{Na} for the wild type, Y1767K, and N406K with the protocol of a 100-ms recovery interval. (*B*) Voltage-dependent suppression of I_{Na} in the presence of 5 μM EPA is shown. The *Inset* is the voltage protocol with a recovery interval of 5 ms. EPA at 5 μM produced a profound voltage-dependent suppression of I_{Na} for the wild type, Y1767K, and F1760K, but the suppression for N406K is relatively less profound with the protocol of a 5-ms recovery interval. The data were fit with a Boltzmann equation.

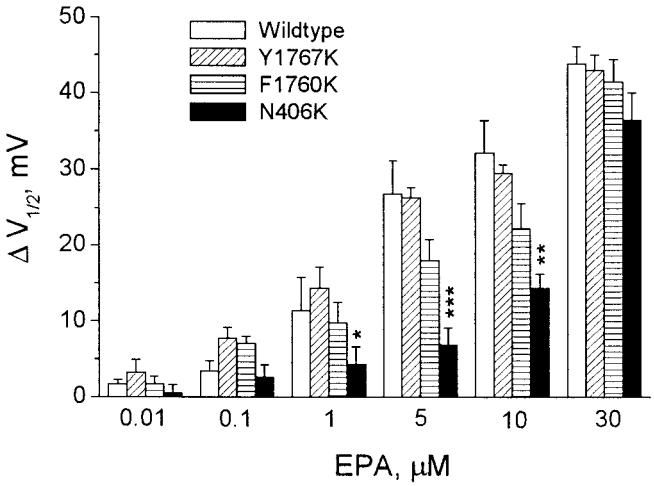

Fig. 4. Effects of EPA on the shift of the steady-state inactivation of I_{Na}. Currents were elicited by 10-ms test pulses to 30 mV following 500-ms conditional prepulses varying from −160 mV to −20 mV with 10-mV increments. The experimental protocol was the same as shown for the *Inset* of Fig. 6*B*. The membrane potential was held at −150 mV, and the pulse rate was 0.1 Hz. Normalized steady-state inactivation was averaged and fit with a Boltzmann equation. The values of $V_{1/2}$ voltages were calculated in the absence and presence of EPA. Delta changes of hyperpolarizing shift of $V_{1/2}$ caused by various concentrations of EPA were plotted for the wild type, Y1767K, F1760K, and N406K of $hH1_\alpha$ Na^+ channels. Each bar represents the mean and SE of at least eight individual cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; N406K versus the wild type or the other two mutants, Y1767K and F1760K.

Inset) interval at −150 mV and a subsequent 10-ms test pulse to 30 mV. Fig. 3*A* shows that with the 100-ms recovery interval, I_{Na} only of the mutant, Y1767K, was significantly reduced when the voltages of conditioning pulses were depolarized above −120 mV; the I_{Na} of the wild type and the other two mutants was not affected. Also, in the presence of 5 μM EPA, only the mutant F1760K showed profound voltage-dependent suppression of I_{Na} by the 100-ms protocol. However, with the 5-ms protocol, the EPA-induced voltage-dependent block of I_{Na} occurred in HEK293t cells expressing the wild type or the mutants of Y1767K and F1760K. The mutant Y1767K had greater voltage-dependent reduction of I_{Na} by the 5-ms protocol even in the absence of EPA (Fig. 3*B*). This makes it difficult to interpret the effect of the fatty acid on the further inhibition of the F1767K. However, clearly the N406K had much larger and more consistent effects in diminishing the action of the PUFAs on the Na^+ channel α subunit. These results indicate that the single point mutation at the site of 406 in the D1-S6 region of $hH1_\alpha$ reduces the EPA-induced voltage-dependent block of the mutated Na^+ channel.

Reduction of EPA-Induced Hyperpolarizing Shift of I_{Na} of the Mutant N406K. Recent data demonstrated that PUFAs significantly shifted the steady-state inactivation of cardiac Na^+ currents to more hyperpolarized values (3, 5, 6). To test whether single point mutations altered the EPA-induced shift of the steady-state inactivation, we investigated the effects of different concentrations of EPA on inactivation of the wild type and its three mutants of $hH1_\alpha$ Na^+ channels. The experimental protocol was the same as shown for the *Inset* of Fig. 6*B*. The membrane potential was held at −150 mV, and the pulse rate was 0.1 Hz. Currents were elicited by 10-ms test pulses to 30 mV following 500-ms conditional prepulses varying from −160 mV to −20 mV with 10-mV increments. Normalized steady-state inactivation was averaged and fit with a Boltzmann equation. The values of $V_{1/2}$ voltages were calculated in the absence and presence of EPA. Changes of the hyperpolarizing shift of $V_{1/2}$ were plotted against various concentrations of EPA (Fig. 4). The shift of $V_{1/2}$ was concentration-dependent for the wild type and the three mutants. However, compared with the wild type, the mutant N406K significantly reduced the shifts of $V_{1/2}$ at 1 μM ($P < 0.05$), 5 μM ($P < 0.001$), and 10 μM ($P < 0.01$) of EPA. In contrast, the shifts of $V_{1/2}$ for Y1767K and F1760K were similar to those of the wild type at all of the EPA concentrations tested. These results indicate that the point mutation at the site of 406 in the D1-S6 region reduces the EPA-induced shift of the steady-state inactivation of I_{Na}.

Inhibition of I_{Na} by Other Fatty Acids. Other fatty acids, including monounsaturated and saturated fatty acids, inhibited cardiac Na^+ currents in HEK293t cells expressing $hH1_\alpha$ Na^+ channels (5), but not with the β1 subunit also present. To assess whether single point mutations of the $hH1_\alpha$ Na^+ channel altered the inhibitory effects of other fatty acids on cardiac Na^+ currents,

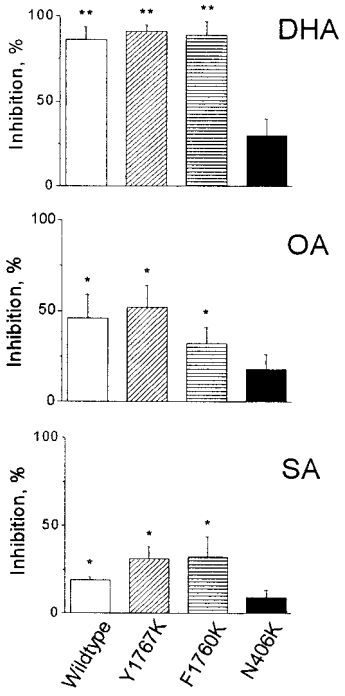

Fig. 5. Inhibition of I_{Na} by other fatty acids. Na^+ currents were evoked by single-step voltage pulses from a holding potential of −120 to 30 mV. I_{Na} of the wild type, Y1767K, and F1760K was significantly inhibited by 5 μM docosahexaenoic acid (DHA, C22:6n-3), oleic acid (OA, C18:1n-9), or stearic acid (SA, C18:0). In contrast, I_{Na} of N406K was not significantly inhibited by the fatty acids. Each bar represents the average value of at least four individual cells. *, $P < 0.05$; **, $P < 0.01$; I_{Na} in the presence of 5 μM fatty acids versus the corresponding control for the wild type or their mutants.

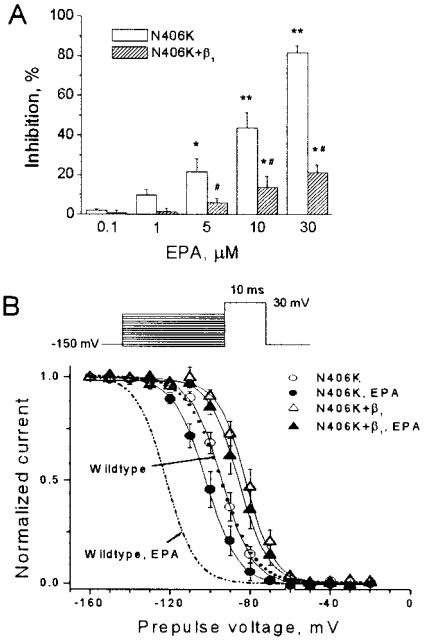

Fig. 6. Effects of coexpressing β_1 subunits on the steady-state inactivation and EPA-induced inhibition of Na^+ currents in HEK293t cells expressing N406K or plus the β_1 subunit. (*A*) Concentration-dependent inhibition of Na^+ currents by EPA in HEK293t cells transfected with N406K alone (N406K) or cotransfected with N406K and β_1 subunits (N406K + β_1). Each bar represents the average value of at least seven individual cells. *, $P < 0.05$; **, $P < 0.01$; versus control. #, $P < 0.05$; N406K plus β_1 versus N406K. (*B*) The effects of coexpressing β_1 subunits on the steady-state inactivation of I_{Na} in the absence or presence of 5 μM EPA for N406K and N406K plus β_1. Currents were elicited by 10-ms test pulses to 30 mV following 500-ms conditional prepulses varying from −160 mV to −20 mV with 10-mV increments. The membrane holding potential was −150 mV, and the pulse rate was 0.1 Hz. The dotted line and dashed-dotted line correspondingly represent in the absence and presence of 5 μM EPA for the steady-state inactivation of the wild type of $hH1_\alpha$ Na^+ channels. The data were fit with a Boltzmann equation.

docosahexaenoic acid (C22:6n-3), oleic acid (C18:1n-9), and stearic acid (C18:0) at 5 μM were applied to the bath solution. Na^+ currents were evoked by single-step voltage pulses from a holding potential of −120 mV to 30 mV. Fig. 5 shows that 5 μM docosahexaenoic acid significantly inhibited I_{Na} by 86 ± 8%, 91 ± 4%, and 89 ± 8% for the wild type ($P < 0.01$, $n = 6$), Y1767K ($P < 0.01, n = 6$), and F1760K ($P < 0.01, n = 5$) of $hH1_\alpha$ Na^+ channels, respectively. In contrast, the inhibition of Na^+ currents was not significant in HEK293t cells expressing the mutant N406K (30 ± 10%, $P > 0.05$, $n = 6$). Fig. 5 also shows that 5 μM oleic acid significantly suppressed I_{Na} by 46 ± 13%, 52 ± 12%, and 32 ± 9% for the wild type ($P < 0.05$, $n = 7$), Y1767K ($P < 0.05, n = 4$), and F1760K ($P < 0.05, n = 4$) of $hH1_\alpha$ Na^+ channels, respectively. In addition, 5 μM stearic acid inhibited I_{Na} of the wild type, Y1767K, and F1760K by 19 ± 2% ($P < 0.05$, $n = 7$), 31 ± 7% ($P < 0.05$, $n = 5$), and 32 ± 12% ($P < 0.05$, $n = 7$), respectively. Again, I_{Na} of N406K was not significantly inhibited by 5 μM oleic acid ($18 \pm 8\%, P > 0.05, n = 6$) or by stearic acid (9 ± 4%, $P > 0.05$, $n = 7$). These results indicate that the single point mutation at the 406 site reduces the sensitivity of the mutant of $hH1_\alpha$ Na^+ channels to PUFAs and monounsaturated and saturated fatty acids.

Modification of the EPA-Induced Suppression of I_{Na} by Coexpression with the β_1 Subunit. Coexpression of $hH1_\alpha$ and β_1 reduced the affinity of resting channels to lidocaine by 2-fold in oocytes (7) and elicited a positive shift in state-dependent cocaine block of the Na^+ channel (8). Our recent experiments demonstrated that coexpression of $hH1_\alpha$ and β_1 significantly reduced the EPA-induced inhibition of I_{Na} (3). In this study, we find that the mutant N406K significantly decreased the suppression of I_{Na} by EPA. To determine whether coexpression of N406K and β_1 had a similar effect on EPA block of $hH1_\alpha$ channels as observed in our previous work (3), experiments were designed to assess the effects of EPA on I_{Na} in HEK293t cells expressing N406K and β_1. The results were compared with those of the wild type plus β_1. Fig. 6*A* shows a concentration-dependent inhibition of I_{Na} by extracellular application of EPA in HEK293t cells transfected with N406K or N406K plus β_1. I_{Na} was evoked by single-step pulses from −150 mV to 30 mV. Whereas I_{Na} of N406K plus β_1 was significantly inhibited by EPA at 10 and 30 μM, the reduction of I_{Na} caused by 5, 10, and 30 μM EPA is significantly less (Fig. 6*A*, $P < 0.05$) for N406K plus β_1 than for N406K alone. These results demonstrate that functional association of β_1 with N406K further reduces the apparent affinity of the channel to EPA and decreases the degree of block of I_{Na} by EPA.

The data in Fig. 6*A* suggest that reduction of channel sensitivity to EPA after coexpression of N406K and β_1 may be related to the β_1-induced shift of the steady-state inactivation to more depolarized potentials, as we reported previously (3). Therefore, the effects of coexpressing β_1 subunits on the steady-state inactivation of Na^+ currents were examined in the absence and presence of EPA in HEK293t cells expressing N406K alone or N406K plus the β_1 subunit. Currents were elicited by 10-ms test pulses to 30 mV following 500-ms conditional prepulses varying from −160 mV to −20 mV with 10-mV decrements. The membrane holding potential was −150 mV, and the pulse rate was 0.1 Hz. EPA at 5 μM shifted the steady-state inactivation of the mutant N406K to the negative direction (Fig. 6*B*). The $V_{1/2}$

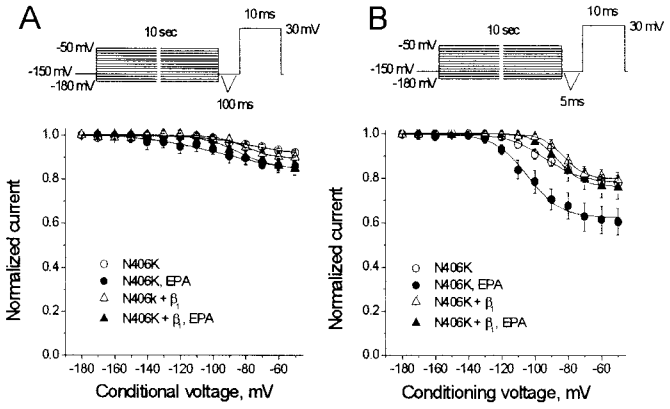

Fig. 7. Voltage-dependent suppression of I_{Na} by EPA in HEK293t cells coexpressing N406K and the β_1 subunit of hH1$_\alpha$ Na^+ channels. (*A*) Whole-cell currents were normalized by I_{Na} recorded with the conditioning voltage of −180 mV for their corresponding controls. The experimental protocol is shown in the *Inset*. Currents were elicited by 10-ms test pulses to 30 mV following a 10-s conditioning pulse varying from −180 mV to −50 mV with 10-mV increments. A 100-ms interval was inserted between the conditioning pulse and the test pulse. The membrane potential was held at −150 mV, and the pulse rate was 0.1 Hz. EPA at 5 μM did not show a significantly voltage-dependent suppression of I_{Na} for N406K alone or N406K plus β_1 with the protocol of a 100-ms recovery interval. (*B*) Voltage-dependent suppression of I_{Na} in the presence of 5 μM EPA is shown. The *Inset* is the voltage protocol with a recovery interval of 5 ms. With this protocol, 5 μM EPA did not produce a significant voltage-dependent suppression of I_{Na} in HEK293t cells coexpressing N406K and β_1. The data were fit with a Boltzmann equation. Each data point represents the average value of at least eight individual cells.

was shifted by -7.7 ± 0.3 mV with the $V_{1/2}$ and k values of -94.0 ± 0.97 mV and 6.6 ± 0.56 mV, respectively, in the absence of EPA ($n = 18$), and of -101.7 ± 1.07 mV and 8.0 ± 0.44 mV, respectively, in the presence of EPA. Coexpressing N406K and β_1 ($n = 11$) shifted the $V_{1/2}$ by 12.5 ± 1.2 mV to the depolarizing direction, from -94.0 ± 0.97 mV for N406K to -81.5 ± 0.42 mV (Fig. 6*B*). In the presence of 5 μM EPA, the $V_{1/2}$ of the steady-state inactivation of N406K plus β_1 was shifted to hyperpolarizing direction by -3.9 ± 0.78 mV, from -81.5 ± 0.42 mV with a k value 6.9 ± 0.82 mV, respectively, in the absence of EPA, to -85.4 ± 0.26 mV with a k value 7.5 ± 0.61 mV, respectively, in the presence of EPA ($n = 11$). In contrast, the EPA-induced shift of $V_{1/2}$ for the wild type was much greater, from -94.3 ± 0.43 mV with a k value of 8.5 ± 0.11 mV (dotted line, $n = 33$) for the control to -121.8 ± 1.18 mV with a k value of 7.4 ± 0.82 mV (dashed-dotted line) for 5 μM EPA. These results indicate that functional association of β_1 with N406K caused a depolarizing shift of $V_{1/2}$ by 12.5 mV, which was much less than a 22-mV shift of hH1$_\alpha$ plus β_1 reported before (3). In addition, in the presence of 5 μM EPA, the hyperpolarizing shifts of the $V_{1/2}$ of the steady-state inactivation were significantly greater for the wild type alone (−27 mV) and the wild type plus β_1 (−23 mV) than for N406K (−8 mV) and N406K plus β_1 (−4 mV), respectively.

To investigate whether coexpression of N406K and β_1 altered the voltage-dependent block of the Na^+ channel, the effects of EPA on voltage-dependent inhibition of I_{Na} by 5 μM EPA were examined in HEK293t cells transfected with N406K alone or N406k plus β_1. Fig. 7 shows that whole-cell currents were normalized by I_{Na} recorded with the conditioning voltage of −180 mV for their corresponding controls. Currents were elicited by 10-ms test pulses to 30 mV following a 10-s conditioning pulse varying from −180 mV to −50 mV with 10-mV increments. A 100-ms or 5-ms interval was inserted between the conditioning pulse and the test pulse. The membrane potential was held at −150 mV, and the pulse rate was 0.1 Hz. EPA at 5 μM did not show a significant voltage-dependent suppression of I_{Na} for N406K alone or N406K plus β_1 with the 100-ms protocol (Fig. 7*A*). However, coexpressing β_1 subunits significantly reduced voltage-dependent inhibition of I_{Na} by 5 μM EPA in HEK293t cells with the protocol of a 5-ms recovery interval (Fig. 7*B*). The data indicate that functional association of the β_1 subunit reduces the EPA-induced voltage-dependent inhibition of the mutant N406K Na^+ channel.

Discussion

The aim of this study was to further understanding of how dietary polyunsaturated fatty acids prevented fatal ventricular arrhythmias. Having found PUFAs to be potent inhibitors of the voltage-dependent fast Na^+ channels, which initiate action potentials in excitable tissues, we needed to know how the PUFAs interacted with the ion channel or membrane to affect conductance through the ion channel. We have excluded an effect of the PUFAs on the general packing of the membrane phospholipids (membrane fluidity) at the low concentrations of the PUFAs that modulate Na^+ channel conductance (9) in cardiac myocytes. Our experiments did not, however, exclude a possible effect of the PUFAs on the microdomains of the sarcolemma through which the ion channels penetrate. The generally accepted evidence by electrophysiologists that a ligand is acting on the protein of an ion channel is to make point amino acid mutations in the channel protein. If one or a few amino acids within a restricted cluster of amino acids is changed and results in the ligand no longer affecting its modulating effect on the channel conductance, it is considered that the amino acid(s) that has been replaced is the site of interaction or binding of the ligand to the channel protein. This is the logic we have applied in this study. With some 1,800 to 2,000 amino acids comprising the structure of the α subunit of the Na^+ channel (10), we chose to start with point amino acid mutations that have been shown to block the action of local anesthetics (LA) on the Na^+ channel. This choice was based on several striking similarities between the effects of the two agents: (*i*) the fact that LAs have long been used clinically to prevent or treat ischemia-induced malignant arrhythmias, and the PUFAs have now been shown to be antiarrhythmic in animals and probably humans (reviewed in ref. 1); (*ii*) both inhibit the voltage-dependent fast Na^+ current (1, 11); (*iii*) they do so by shifting the steady-state inactivation to hyperpolarized levels (6, 12, 13), and this makes the inhibition of I_{Na} highly voltage-dependent; (*iv*) both prolong the inactivated state of the Na^+ channel (3, 11); (*v*) both noncompetitively displace bound batrachotoxin from its known binding site on the Na^+ channel (14–16): and (*vi*) both reversibly reduce the spontaneous beating rate of cultured neonatal rat cardiomyocytes (1). Thus, we hypothesized that the PUFAs would interact with the same sites within the α subunit of the Na^+ channel as has been demonstrated for LAs.

The putative site of binding or interaction of LAs with the Na^+ channel has been localized to the middle of domain IV-segment 6 of the α subunit of the Na^+ channel of rat brain RBIIA isoforms (17, 18) We have studied the homologous residues hH1-F1760 and hH1-Y1767 of human heart Na^+ channels involved in LA binding (2). Point mutations at these sites have affected the action of LA on the Na^+ channel. We have also tested Asn-406 because DI-S6 has been shown to contain the putative binding site for batrachotoxin (BTX) (16), an alkaloid neurotoxin. It has also been shown that batrachotoxin can be displaced from its binding site by LAs (15, 19, 20). Wang and Wang (20) demonstrated that three lysine point mutations in this region make the rat skeletal muscle Na^+ channel α subunit completely resistant to BTX. They concluded that the putative BTX receptor is probably located near the middle of the DI-S6 segment, which includes the residues of μ1 Ile-433, Asn-434, and

Leu-437 of the rat skeletal muscle Na^+ channel α subunit. In this study, we have used the Asn-406 residue in the human cardiac Na^+ channel α subunit, which is homologous with Asn-434 residue in the rat μ1 skeletal muscle Na^+ channel α subunit.

With the α subunit in its folded channel configuration, however, these DI-S6 and D4-S6 sites are apparently juxtaposed and thought to be within the pore lining region of the internal vestibule of Na^+ channels. The close functional association, as well as the close positional association, has further been demonstrated by the findings that point mutations in D4-S6, which inhibit the action of LAs on conductance of Na^+ channels, also block the binding of BTX to the Na^+ channel (21, 22). Likewise, mutations in D1-S6, which block the binding of BTX, also block the inhibiting action of local anesthetics on the Na^+ channel (23).

Indeed, in this study, we report that point mutations within the putative site of the interaction or binding of LAs in D4-S6, namely L1760K and Y1767K, did have some modulating effects on I_{Na}. However, clearly N406K had much larger and more consistent effects in blocking the actions of the PUFAs on the Na^+ channel α subunit, and the effects persisted and were even greater when the β subunit was transiently coexpressed with the hH1 α subunit in the HEK293t cell line to produce a more complete human myocardial Na^+ channel. We had reported that 5 μM EPA inhibited I_{Na} in the $hH1_\alpha$ coexpressed with the β1 subunit by 48.3% (3). Whereas with the N406 mutant coexpressed with the β1 subunit, 5 μM EPA inhibited I_{Na} by only 5.8% (Fig. 6*A*). Thus. in the presence of the β1 subunit, the N406K results in some 8.3-fold loss of the inhibitory action of EPA on I_{Na}.

Thus, we conclude from this limited probing in the D4-S6 and the D1-S6 regions of the human Na^+ channel that the PUFAs do mimic the LAs in their sites of interaction with the protein of the α subunit of the Na^+ channel, but their major site of interaction seems within the D1-S6, rather than in D4-S6, where the LAs seem primarily to interact.

We believe this interpretation of our findings is consistent with the current paradigm generally accepted by most electrophysiologists. However, Andersen and associates (24, 25) recently carefully examined experiments and clearly reproposed an alternative paradigm that ligands may act by primarily affecting the phospholipid bilayer surrounding the transmembrane ion channels in a way that secondarily affects stresses on the ion channels causing changes in their conformation, which then alter the conductance of the channels. It seems that this alternative paradigm might equally be consistent with our results.

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