Biochemical and Biophysical Research Communications **281,** 45–52 (2001)
doi:10.1006/bbrc.2001.4309, available online at http://www.idealibrary.com on IDEAL®

Point Mutations in α-Subunit of Human Cardiac Na^+ Channels Alter Na^+ Current Kinetics

Yong-Fu Xiao,*,†,‡ Qingen Ke,*,† Sho-Ya Wang,§ Yinke Yang,*,† Ging Kuo Wang,*,¶ James P. Morgan,*,† and Alexander Leaf*,‡,[1]

†*Charles A. Dana Research Institute and Harvard–Thorndike Laboratory, Cardiovascular Division, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, Massachusetts;* ‡*Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts;* ¶*Department of Anesthesia, Brigham and Women's Hospital, Boston, Massachusetts;* **Harvard Medical School, Boston, Massachusetts 02215; and* §*Department of Biology, State University of New York at Albany, Albany, New York*

Received January 9, 2001

Dietary polyunsaturated fatty acids (PUFAs) prevent ischemia-induced fatal cardiac arrhythmias in animals and probably in humans. This action results from inhibition of ion currents for Na^+, Ca^{2+}, and possibly other ions. To extend understanding of this protection we are seeking a possible binding site for the PUFAs on the α-subunit of the human cardiac Na^+ channel, $hH1_\alpha$, transiently expressed in HEK293t cells. Three mutated single amino acid substitutions with lysine were made in the α-subunit at Domain 4-Segment 6 (D4-S6) for F1760, Y1767 and at D1-S6 for N406. These are in the putative sites of binding of local anesthetics and batrachotoxin, respectively. The mutants F1760K, Y1767K, and N406K, separately and to different extents, affected the current density, the steady-state inactivation potential, accelerated inactivation, delayed recovery from inactivation, and affected voltage-dependent block, but did not affect activation of the $hH1_\alpha$. It is essential to learn that single point mutations in D1-S6 and D4-S6 alone significantly modify the kinetics of human cardiac $hH1_\alpha$ Na^+ currents. The effects of PUFAs on these mutant channels will be the subject of subsequent reports. © 2001 Academic Press

Key Words: **human cardiac Na^+ channel; α-subunit; point mutation; activation; inactivation.**

Dietary long chain polyunsaturated fatty acids (PUFAs), especially of the n-3 class, have been shown to prevent ischemia-induced fatal cardiac ventricular arrhythmias in animals and probably also in humans (1). This protective action has been shown to result from modulation of ion currents for Na^+, Ca^{2+}, K^+, and other ions (1), of which the Na^+ and Ca^{2+} currents seem now to us most important. These effects on ion channels appear to be direct, fast effects, which do not require covalent binding of the PUFAs with any constituent of the plasma membranes and have been demonstrated on single isolated cardiac myocytes in the absence of neural or humoral influences (1–3).

In seeking further the mechanism(s) by which these PUFAs prevent malignant arrhythmias, we have been searching for the primary site of their interaction with the cardiac myocytes. A primary action on the general physical state (packing) of phospholipid cardiac sarcolemma, which might allosterically alter the ion channel conductance has been excluded, though an action on micro-domains of phospholipids through which ion channels penetrate, has not been excluded (4). Another site of possible primary action on sarcolemma is an interaction or binding directly to the protein of transmembrane ion channels. Suggestive evidence for such effects have been shown for the voltage-dependent Na^+ channel, by the ability of the antiarrhythmic PUFAs to specifically displace bound ^{3}H-batrachotoxin (BTX) from its binding site in domain 1, transmembrane segment 6 (D1-S6) of the α-subunit of the Na^+ channel (5). But this effect resulted from noncompetitive inhibition, indicating that the site of binding differed from that of the BTX but could allosterically displace the bound BTX. Similarly it had been shown that the PUFAs displaced the dihydropyridine L-type Ca^{2+} channel blocker, nitrendipine, from its specific binding site in the vestibule of the Ca^{2+} channel. But again this effect resulted from noncompetitive inhibition of the nitrendipine binding to the Ca^{2+} channel (6). So again we were left with a suggestive binding of the PUFAs to the

[1] To whom correspondence should be addressed at Massachusetts General Hospital East, 149 13th Street, Charlestown, MA 02129. Fax: (617)726-6144. E-mail: aleaf@partners.org.

0006-291X/01 $35.00
Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

ion channel proteins, but not definitive evidence. This study takes a different approach to this problem by testing whether point amino acid mutations in the 1800– to 2000-amino-acid sequence of the human Na^+ channel α-subunit (7) could affect the ion channel conductance. A definite effect of inhibition of current by a specific agonist or antagonist of the wildtype ion channel conductance has constituted evidence that the agent was interacting or binding to the ion channel at the site of the altered amino acid. To conduct such a test of binding to the ion channel protein we chose sites for mutated point amino acid substitutions which have now been shown to prevent the characteristic effects of local anesthetics to inhibit the Na^+ channel α-subunits, namely amino acid residues in the putative binding site Domain 4-Segment 6 for local anesthetics and in D1-S6, the putative binding site for BTX. The decision to begin with these local anesthetic sites was based on what seems like a remarkable concurrence of the effects of local anesthetics and of PUFAs on functions of the heart, which will be discussed later. The present paper deals with the quite considerable effects on the Na^+ conductance of the human wildtype α-subunit ($hH1_\alpha$) from the effects of the three mutated amino acids alone on the Na^+ currents of the $hH1_\alpha$ transiently expressed in HEK293t cells. The effects of the PUFAs and the further possible interactions of PUFAs in comparison with local anesthetics are the subjects of subsequent publications.

MATERIALS AND METHODS

Mutagenesis and transient transfection of Na^+ channels. Oligonucleotide-directed point mutations of $hH1_\alpha$ were performed by using the Transformer Site-Directed Mutagenesis Kit (Clontech Lab., Palo Alto, CA), with some modifications to increase the yield of *in vitro* DNA synthesis. Mutations were identified by restriction mapping and further confirmed by DNA sequencing of regions containing the mutation. The detailed method has been described in a previous report (8). The methods for the culture of HEK293t cells and the transfection of the wildtype and its mutants of $hH1_\alpha$ Na^+ channels, as well as for the cotransfection with the rat brain β_1 subunit were described in detail previously (2, 8).

Electrophysiological recordings. During an experiment HEK293t cells cultured in a culture dish for 2 to 5 days after transfection were continuously superfused (1–2 ml/min) with the Tyrode's solution, which contained (in mM): NaCl 137, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, Hepes 10, glucose 10, pH 7.4. Recording glass electrodes with a resistance of 1–3 MΩ were filled with the pipette solution which contained (in mM): NaF 100, NaCl 30, EGTA 10, and Hepes 10 (titrated with cesium hydroxide to pH 7.3). The recording method of Na^+ currents was as described (2, 9). The average membrane capacitance of the HEK293t cells patched during experiments was 32 ± 0.8 pF (n = 69). The bath solution during voltage-clamp measurements contained (in mM): NaCl 65, choline chloride 85, $CaCl_2$ 2, and Hepes 10, was rapidly exchanged (10). The 65 mM Na^+ concentration in the bathing solution was to reduce peak Na^+ currents to manageable amplitudes. Experiments were conducted at 22–23°C.

Statistics. Data are presented as means ± SEM. Whole-cell activation conductance (g_m) at a given voltage step was calculated from the equation $g_m = I_{Na}/(E_m - E_{Na})$, where I_{Na} is the current, E_m is the amplitude of voltage step, and E_{Na} is the reversal potential of the Na^+ current. Activation conductance, 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 results of development of inactivation were fit by the least-squares fitting ($y = A_0 + A_1\exp^{-t/\tau 1}$...Origin 6.0, Microcal Software, Inc., Northampton, MA) with a single exponential function. The recovery from inactivation was fit with a logistical equation, $Y = \{(A_1 - A_2)/[1 + (\chi/\chi_0)^p] + A_2\}$; where χ_0 is center, p is power, A_1 is initial Y value, and A_2 is final Y value. 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.

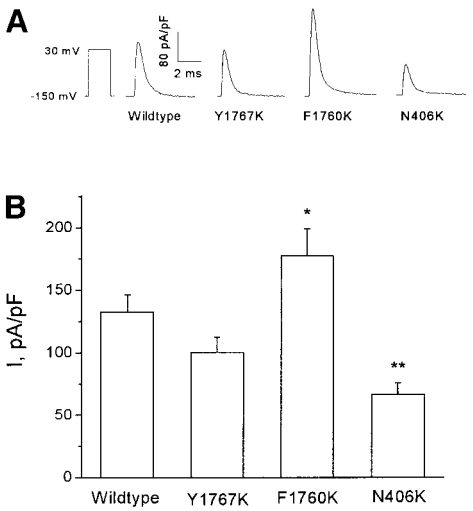

FIG. 1. Current densities of voltage-activated human cardiac Na^+ channels. (A) Current traces were evoked by depolarizing test pulses from a holding potential of −150 to 30 mV (see the protocol of inset) for the wildtype (wildtype) and the mutants (Y1767K, F1760K, and N406K) of $hH1_\alpha$ Na^+ channels expressed in HEK293t cells. (B) Peak current densities evoked by test pulses were averaged for the wildtype (n = 69), Y1767K (n = 43), F1760K (n = 44), and N406K (n = 32). *$P < 0.05$; **$P < 0.01$; vs wildtype.

RESULTS

Current Densities in HEK293t Cells Expressing the Wildtype and Three $hH1_\alpha$ Mutants

Voltage-activated Na^+ currents with fast activation and fast inactivation kinetics were evoked by depolarizing pulses in HEK293t cells 2 to 5 days in culture after transient transfection with either the wildtype (Fig. 1A, Wildtype) or one of three mutants (Fig. 1A, Y1767K, F1760K and N406K) of $hH1_\alpha$. Substitution of phenylalanine at the site of 1760 with lysine significantly enhanced the peak current densities in HEK293t cells transfected with the mutant F1760K. The average current density (elicited by voltage pulses from −150 to 30 mV) was 132 ± 14 pA/pF for the wildtype (n = 69) and 177 ± 22 pA/pF for F1760K (n =

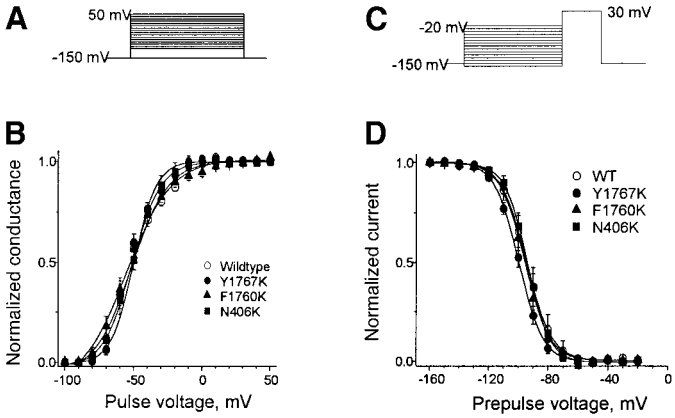

FIG. 2. Effects of single point mutations on the activation and inactivation of the Na^+ current in HEK293t cells. (A) The experimental protocol is shown. Na^+ currents were elicited by 10-ms test pulses from −90 to 50 mV with 10-mV increments every 5 s. The membrane potential was held at −150 mV. (B) Relative whole-cell activation conductances for the wildtype (○, $n = 32$), Y1767K (●, $n = 28$), F1760K (▲, $n = 29$), and N406K (■, $n = 22$) of $hH1_\alpha$ Na^+ channels. (C) The voltage protocol for recording current traces of the steady-state inactivation is shown. Currents were elicited by 10-ms test pulses to 30 mV following 500 ms conditional prepulses varying from −150 to −20 mV with 10-mV increments. The membrane potential was held at −150 mV and the pulse rate was 0.1 Hz. (D) Normalized steady-state inactivation was averaged for the wildtype (○, $n = 32$), Y1767K (●, $n = 28$), F1760K (▲, $n = 29$), and N406K (■, $n = 22$) of $hH1_\alpha$ Na^+ channels. The data were fit with a Boltzmann equation.

44, $P < 0.05$; Fig. 1B), respectively. In contrast, substitution of asparagine at the site of 406 with lysine significantly reduced the peak current densities in HEK293t cells transfected with the mutant N406K. The average current density was 73 ± 9 pA/pF for N406K ($n = 32$, $P < 0.001$, vs the wildtype; Fig. 1B). Compared to the wildtype, the current density in HEK293t cells expressing the mutant Y1767K (substitution of tyrosine at the site of 1767 with lysine) was not significantly changed (101 ± 12 pA/pF, $n = 43$, $P > 0.05$, vs the wildtype; Fig. 1B).

Hyperpolarizing Shift of Inactivation of the Mutant Y1767K

Na^+ currents were elicited by various voltage pulses (see the protocol, Fig. 2A). The normalized whole-cell activation conductances of the wildtype and three mutants of $hH1_\alpha$ were calculated from peak Na^+ currents (Fig. 2B) with an equation (see Materials and Methods). Na^+ currents were activated at around −70 mV and reached a maximal conductance at −20 mV for the wildtype and the mutants (Fig. 2B). Compared to the wildtype, the values of the $V_{1/2}$ and k (slope) of activation were not significantly altered after point mutations at D1-S6 (N406K) and D4-S6 (F1760K and Y1767K). The average $V_{1/2}$ and k values fitted with a Boltzmann equation were −53.8 ± 1.64 mV and 13.9 ± 1.26 mV, respectively, for the wildtype ($n = 32$); −50.1 ± 0.81 mV and 8.2 ± 0.47 mV, respectively, for Y1767K ($n = 28$); −55.3 ± 2.20 mV and 16.0 ± 1.30 mV, respectively, for F1760K ($n = 29$); and −51.5 ± 1.62 mV and 10.4 ± 1.37 mV, respectively, for N406K ($n = 22$). These results indicate that the point mutations at the regions of D1-S6 and D4-S6 did not modify the activation process of $hH1_\alpha$ Na^+ channels.

The effects of the point mutations on the steady-state inactivation were examined by measuring the amplitude of peak currents evoked by a two-pulse protocol. From a holding potential of −150 mV, we delivered 500-ms prepulses ranging from −150 mV to −20 mV (in 10 mV increments) and then measured the available current elicited by a 10-ms test pulse to 30 mV (Fig. 2C). The average $V_{1/2}$ of the steady-state inactivation curve for the wildtype was −94.3 ± 0.43 mV with a k value of 8.5 ± 0.11 mV ($n = 33$). The mutants, F1760K and N406K, of $hH1_\alpha$ had no effect ($P > 0.05$, vs the wildtype) on the steady-state inactivation of I_{Na} with the $V_{1/2}$ and k values of −95.9 ± 0.19 mV and 7.8 ± 0.17 mV, respectively, for F1760K ($n = 25$) and of −94.0 ± 0.27 mV and 7.4 ± 0.24 mV, respectively, for N406K ($n = 18$). In contrast, the point mutation Y1767K of $hH1_\alpha$ significantly shifted the $V_{1/2}$ of inactivation to hyperpolarizing direction with a value of −100 ± 0.17 mV ($n = 26$, $P < 0.05$, vs the wildtype) and with a k value of 8.0 ± 0.15 mV (Fig. 2D). These results show that the mutants, F1760K and N406K, of $hH1_\alpha$ do not significantly alter the steady-state inactivation of $I_{Na,\alpha}$, but a point mutation (Y1767K) does.

Alteration of Voltage-Dependent Inhibits the Mutant Y1767K

Depolarization of the cell membrane causes an inactivation of voltage-activated Na^+ channels. To assess whether single point mutations of $hH1_\alpha$ Na^+ channels affected the characteristic of voltage-dependent inactivation, an experimental protocol was applied to patched HEK293t cells. The voltage protocol consisted of a 10-s conditioning pulse ranging from −180 mV to −50 mV with 10-mV increments followed by a 100 ms (Fig. 3A) or 5 ms (Fig. 3C) interval at the holding potential of −150 mV and a subsequent test pulse to 30 mV. Figure 3 shows that with the 100 ms recovery interval, I_{Na} of the mutant Y1767K, was significantly reduced when voltages of conditioning pulses were depolarized above −120 mV, whereas I_{Na} of the wildtype, F1760K, and N406K of $hH1_\alpha$ was not affected by the voltages of conditioning pulses (Fig. 3B). With the 5 ms recovery interval, the reduction of I_{Na} of the mutant Y1767K was even more profound when the voltages of conditioning pulses were depolarized above −120 mV (Fig. 3D). With the 5 ms recovery interval, the normalized amplitude of I_{Na} for the wildtype, F1760K, and N406K also showed a 10–15% reduction

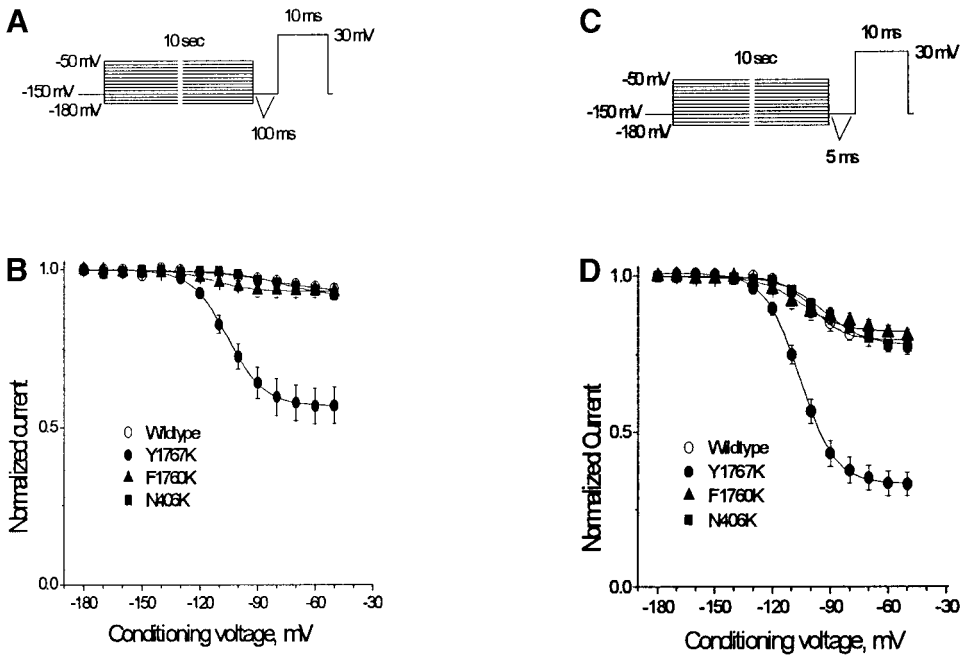

FIG. 3. Voltage-dependent block of I_{Na} in HEK293t cells expressing the wildtype or three mutants of hH1$_\alpha$ Na^+ channels. (A) The experimental protocol is shown. Currents were elicited by 10-ms test pulses to 30 mV following a 10-s conditioning pulse varying from −180 to −50 mV with 10-mV increments. 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. (B) Whole-cell currents were normalized by I_{Na} recorded with the conditioning voltage of −180 mV for the wildtype (○, n = 11), Y1767K (●, n = 10), F1760K (▲, n = 7), and N406K (■, n = 19). (C) The voltage protocol is similar to that in A with a recovery interval of 5 ms. (D) Normalized currents were shown for the wildtype (○, n = 21), Y1767K (●, n = 15), F1760K (▲, n = 15), and N406K (■, n = 12). The data were fit with a Boltzmann equation.

after the conditioning pulses depolarized above −100 mV. These results indicate that the single point mutation Y1767K in the D4-S6 region of hH1$_\alpha$ enhances the characteristics of voltage-dependent inactivation of hH1$_\alpha$ Na^+ channels

Development of Inactivation and Recovery from the Inactivated State of the Wildtype and the Three Mutants

Voltage-gated Na^+ channels can directly enter into an inactivated state from the resting state. This process occurs without opening of the channel and is referred as resting inactivation (11–15). To assess the effects of single point mutations on the development of resting inactivation of hH1$_\alpha$ Na^+ channels, a conditioning pulse to −70 mV with variable durations was followed by a 10-ms test pulse to 30 mV (Fig. 4A). We selected −70 mV as the conditioning voltage to reduce the chance of channel activation. Fig. 4B shows that the amplitudes of $I_{Na\alpha}$ dramatically decreased as the duration (Δt) of conditioning pulses was prolonged, indicating that an increasing proportion of channels entered the inactivated state. The decay time constant of inactivation development was 45.4 ± 4.4 ms (open circle, n = 8) for the wildtype, 52.5 ± 4.8 ms for Y1767K (solid circle, n = 9), 30.4 ± 2.6 ms for F1760K (solid triangle, n = 7, $P < 0.05$, vs wildtype), and 22.1 ± 3.6 ms for N406K (solid square, n = 8, $P < 0.01$, vs wildtype), respectively (Fig. 4B). Our results indicate that a single point mutation at the 1767 site (Y1767K) of hH1$_\alpha$ does not significantly alter the development of inactivation, but the mutants, F1760K and N406K, do.

To determine whether the mutants of hH1$_\alpha$ affected recovery from inactivation, a double-pulse protocol was used to test recovery from resting inactivation at −70 mV. A 10-s depolarizing conditioning pulse to −70 mV was followed by a variable recovery interval at −150 mV and then a subsequent test pulse to 30 mV (Fig. 5A). The 10-s conditional pulse to −70 mV was to ensure all Na^+ channels entered the inactivated state without opening channels. The time course of recovery from inactivation of both $I_{Na,\alpha}$ was fit by a logistical equation (see the Methods). The time for 50% recovery from inactivation was 3.4 ± 0.45 ms for the wild type (n = 8), 8.3 ± 1.80 ms for Y1767K (n = 11, $P < 0.05$, vs the wildtype), 1.0 ± 0.04 ms for F1760K (n = 7, $P < 0.01$, vs the wildtype), 3.0 ± 0.46 ms for N406K (n = 7, $P > 0.05$, vs the wildtype), respectively (Fig 5B). These results show that the mutant Y1767K of hH1$_\alpha$ delayed the recovery from inactivation and, that in contrast, the mutant F1760K accelerated the

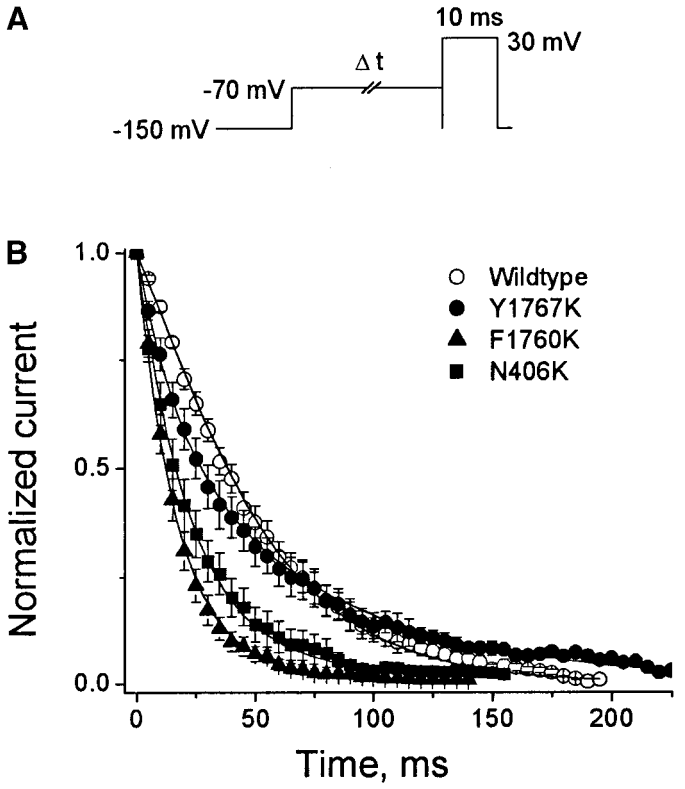

FIG. 4. Effects of point mutations on development of resting inactivation of $hH1_\alpha$ Na^+ channels. (A) The pulse protocol was composed of a depolarizing pulse from a holding potential of −150 to −70 mV with various duration followed by a 10-ms test pulse to 30 mV. The pulse rate was 0.1 Hz. (B) Prolonging the duration of prepulse reduced the amplitude of normalized currents for the wildtype (○, $n = 8$), Y1767K (●, $n = 9$), F1760K (▲, $n = 7$), and N406K (■, $n = 8$) of $hH1_\alpha$ Na^+ channels. Data were fit with a single exponential function.

process of recovery from inactivation. This is consistent with a recently reported finding with the isoform Y1586K of a rat skeletal muscle sodium channel (μ1) (16).

Effects of Coexpression of the β_1-Subunit on the Kinetics of Na^+ Currents

Our previous data showed that coexpression of the β_1-subunit with $hH1_\alpha$ significantly modified the kinetics of the Na^+ current (2). To examine the effects of the β_1-subunit on the kinetics of Na^+ currents, we coexpressed the β_1-subunits with $hH1_\alpha$ or with its N406K mutant in HEK293t cells. The reason for using N406K in this experiment is that we have found that the single point mutation N406K of $hH1_\alpha$ significantly reduced the inhibitory effect of the polyunsaturated fatty acids on Na^+ currents (Xiao *et al.*, unpublished data). In HEK293t cells cotransfected with β_1-subunits, the densities of I_{Na} were significantly increased, 33% for the wildtype plus the β_1-subunit ($n = 7$) and 68% for N406K plus the β_1-subunit ($n = 19$), respectively (Fig. 6).

We also compared the effects of coexpression of the β_1-subunit with the wildtype or N406K mutant on the fast steady state inactivation. Currents were evoked by a two-pulse protocol. The average $V_{1/2}$ of the fast steady-state inactivation curve of I_{Na} for the wildtype was -95 ± 0.4 mV with a k value of 8.5 ± 0.2 mV ($n = 33$). The wildtype plus β1-subunit was -76 ± 1.5 mV with a k value of 5.8 ± 0.3 mV ($n = 9$, $P<0.01$, a shift of $V_{1/2}$ by 19 ± 0.7 mV. On the other hand, the average $V_{1/2}$ of the fast steady-state inactivation curve of I_{Na} for the mutant N406K was -94 ± 1.0 mV with a k value of 6.5 ± 0.5 mV (n = 18). Coexpression of N406 and the β_1-subunit shifted the $V_{1/2}$ of the I_{Na} to -82 ± 1.4 mV with a k value of 6.9 ± 0.7 mV ($n = 11$, $P < 0.01$), a shift of 12 ± 0.8 mV. These results suggest that functional association of the β_1-subunit with $hH1_\alpha$ or its mutant N406K causes a significant shift of the fast steady-state inactivation to the depolarizing direction and the shift for the wildtype of $hH1_\alpha$ is even greater.

To test whether coexpression of the β_1-subunit with $hH1_\alpha$ or with its mutant N406K affected voltage-dependent inactivation, we depolarized patched HEK 293t cells to various voltages. With a 100 ms recovery interval, the inactivation process of I_{Na} for N406K and

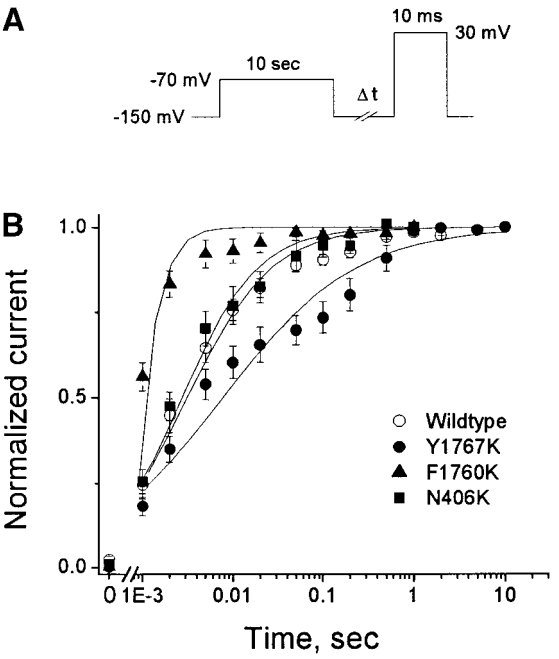

FIG. 5. Effects of point mutations on recovery from inactivation. (A) The pulse protocol was composed of a 10-s depolarizing pulse from −150 mV to −70 mV followed by a hyperpolarizing pulse to −150 mV with progressively longer durations and then a 10-ms test pulse to 30 mV. The membrane holding potential was −150 mV and the rate of pulses was 0.1 Hz. (B) The time course of recovery of peak $I_{Na,\alpha}$ from inactivation is shown for different isoforms of $hH1_\alpha$ Na^+ channels. Data were fit with a logistical function.

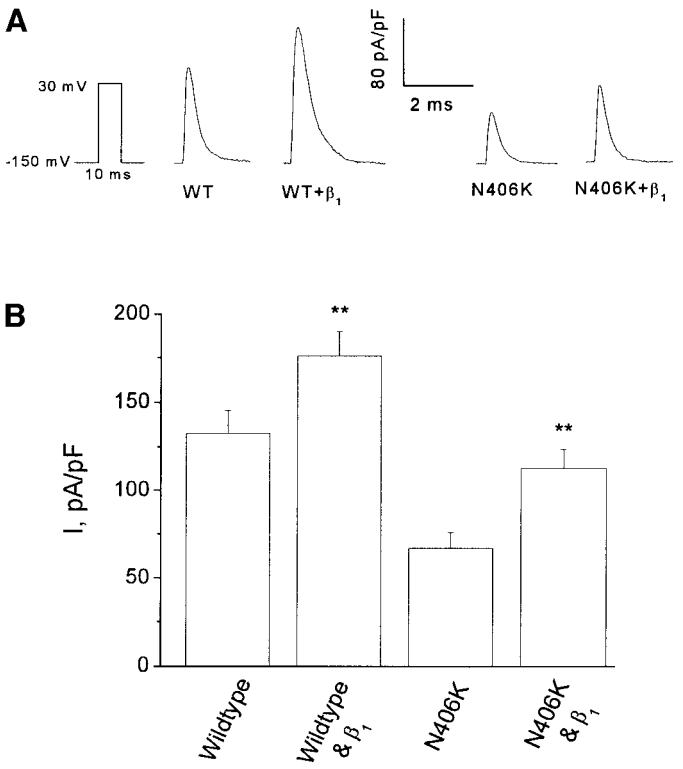

FIG. 6. Current densities of voltage-activated human cardiac Na^+ channels coexpressing just α- or both α- and β_1-subunits. (A) Current traces were evoked by depolarizing test pulses from a holding potential of −150 to 30 mV (see the protocol of inset). (B) Peak current densities evoked by test pulses were averaged for the wildtype 132 ± 14 pA/pF (n = 69), wildtype + β_1-subunit 176 ± 14 pA/pF (n = 7), N406K 73 ± 9 pA/pF (n = 32, and N406K + β_1 113 ± 11 pA/pF (n = 19). It is evident that coexpression with the β1-subunit increases the current density of N406K by the same amount as for the wildtype, 44 and 40 pA/pF, respectively.

for N406K plus the β_1-subunit was similar to that of the wildtype. In contrast, coexpression of the β_1-subunit and $hH1_\alpha$ enhanced the process of inactivation when the conditioning voltage was more positive than −90 mV. The portion of inactivated channels for $hH1_\alpha$ plus β_1 was significantly different than that for $hH1_\alpha$ alone when the conditioning voltages were at −60 and −50 mV. These results indicate that coexpression of $hH1_\alpha$ and the β_1-subunit accelerates the slow voltage-dependent inactivation, but not for the mutant N406K.

DISCUSSION

Dietary long chain polyunsaturated fatty acids, especially from fish oils, prevent ischemia-induced fatal cardiac arrhythmias in animals and probably in humans (reviewed in 1). This protective action has been shown to result from inhibition of ion currents for Na^+, $Ca^{2+}$and K^+, of which the Na^+ and Ca^{2+} currents now seem most important (1). The present study seeks to extend our understanding of the protective action by seeking the primary site of interaction of the antiarrhythmic PUFAs with the cardiac myocytes, perhaps a possible binding or interactive site for the PUFAs on the affected ion channel protein. We chose the α-subunit of the human cardiac Na^+ channel, $hH1_\alpha$, which is the large component of the channel protein through which Na^+ ions enter the cardiac cell to initiate an action potential. Three mutated single amino acid substitutions were made in the α-subunit at D4-S6 of F1760, Y1767 and at D1-S6 of N406 and transiently expressed in HEK293t cells. At each of these sites a lysine (K) was substituted for the phenylalanine (F), tyrosine (Y) and asparagine (N), respectively, transiently expressed in HEK293t cells. Our choice of these mutants was based on noted similarities between the actions of local anesthetics and those of the anti arrhythmic fatty acids we have been studying. These similarities include: (a) LAs have a long clinical usage as antiarrhythmic agents and n-3 PUFAs are also cardiac antiarrhythmic agents (1); (b) both inhibit the cardiac voltage-dependent fast Na^+ current (1, 17); (c) both cause this inhibition by shifting the steady state inactivation of the Na^+ channel to more negative, hyperpolarized potentials (12, 18, 19); (d) both prolong the inactivated state of the Na^+ channel (2, 20); (e) both displace bound batrachotoxin from its binding site in the activated, open state of the Na^+ channel by noncompetitive inhibition (5, 21, 22); (f) both reversibly reduce the spontaneous beating rate of cultured neonatal rat cardiomyocytes (1-Fig. 2).

With so many similar functional effects of local anesthetics and anti-arrhythmic fatty acids, it seemed possible that they might both be sharing similar mechanisms of action. Since the site of binding or interaction of local anesthetics has been localized to be near the middle of D4-S6 of the α-subunit of the voltage-dependent Na^+ channel of rat brain RBIIA isoforms (23, 24), we have studied the homologous residues hH1-F1760 and hH1-Y1767 of human heart Na^+ channels involved in LA binding (8). Point mutations at these sites have inhibited the action of the local anesthetics on the Na^+ channel (23, 24). We have studied point mutations at these sites to learn if the same point amino acid mutations will likewise inhibit the action of the PUFAs on that channel. If a point amino acid mutation in an ion channel protein can significantly inhibit the expected effect of an agent on the conduction through that ion channel, it is interpreted as suggesting that the amino acid which has been replaced constitutes a site for the binding of that agent to the channel protein. That explains the choice of F1760 and Y1767 of D4-S6.

The reason for also testing N406 is that D1-S6 has been shown to contain the putative binding site for batrachotoxin (21) an alkaloid neurotoxin, which

causes the Na^+ channel to be persistently in the open state. It has also been shown that batrachotoxin can be displaced from its binding site by local anesthetics (22, 25, 26). Wang and Wang (26) reported 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 include the residues of μ1-I433, N434, and L437 of the rat skeletal muscle Na^+ channel α-subunit. In this study we have used the N406 residue in the human cardiac Na^+ channel α-subunit, which is homologous to the N434 residue in the rat μ1 skeletal muscle Na^+ channel α-subunit.

With the α-subunit in its folded channel configuration, however, these D1-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 local anesthetics on conductance of Na^+ channels, also block the binding of BTX to the Na^+ channel (27, 28). Likewise, mutations in D1-S6, which block the binding of BTX to the Na^+ channel, also block the inhibiting action of local anesthetics on the Na^+ channel (29).

Prior to testing the action of the anti-arrhythmic fatty acids on the mutated human Na^+ channel α-subunit, however, it was important to learn how much the mutations themselves were affecting the conductance of the Na^+ channels. This is what is documented and reported in this communication.

ACKNOWLEDGMENTS

We thank Dr. R. G. Kallen for the $hH1_\alpha$ clone, Drs. L. L. Isom and W. A. Catterall for the rat brain β_1 subunit clone, and Dr. S. C. Cannon for the CD8 clone and the HEK293t cell line. We are very grateful to JingBo Cai for her excellent job of cell culture and channel expression. This study was supported in part by research grants 9930254N (Y.F.X.) from the American Heart Association and DK38165 and HL62284 (A.L.), DA11762 (J.P.M.), and GM35401 (G.K.W.) from the National Health Institute of the U.S. Public Health Service.

REFERENCES

1. Leaf, A., Kang, J. K., Xiao, Y-F., Billman, G. E., and Voskuyl, R. A. (1999) The antiarrhythmic and anticonvusant effects of dietary n-3 fatty acids. *J. Membr. Biol.* **172,** 1–11.
2. Xiao, Y-F, Wright, S. N., Wang, G. K., Morgan, J. P., and Leaf, A. (2000) Coexpression with the β_1-subunit modifies the kinetics and fatty-acid block of $hH1_\alpha$ Na^+ channels. *Am. J. Physiol. Heart Circ. Physiol.* **729,** H35–H46.
3. Xiao, Y-F., Gomez, A. M., Morgan, J. P., Lederer, W. J., and Leaf, A. (1997) Suppression of voltage-gated L-type Ca^{2+} currents by polyunsaturated fatty acids in adult and neonatal rat cardiac myocytes. *Proc. Natl. Acad. Sci. USA* **94,** 4182–4187.
4. Pound, E., Kang, J. X., and Leaf, A. (2001) Partition of antiarrhythmic polyunsaturated fatty acids into red blood cell ghosts. *J. Lipid Res.*, in press.
5. Kang, J. X., and Leaf, A. (1996) Evidence that free polyunsaturated fatty acids modify Na^+ channels by directly binding to the channel protein. *Proc. Natl. Acad. Sci. USA* **93,** 1542–1546.
6. Hallaq, H., Smith, T. W., and Leaf, A. (1992) Modulation of dihydopyridine-sensitive calcium channels by fish oil fatty acids. *Proc. Natl. Acad. Sci. USA* **89,** 1760–1764.
7. Gellens, M. E., George, A. L., Chen, L., Chahine, M., Horn, R., Barchi, R. L., and Kallen, G. (1992) Primary structure and functional expression of the human cardiac tetrodotoxin-insensitive voltage-dependent sodium channel. *Proc. Natl. Acad. Sci. USA* **89,** 554–558.
8. Wang, S. Y., and G. K. Wang. (1997) A mutation in segment I-S6 alters slow inactivation of sodium channel. *Biophys. J.* 72, 1633–1640.
9. Wright, S. N., Wang, S-Y., Xiao, Y-F., and Wang, G. K. (1999) State-dependent cocaine block of sodium channel isoforms, chimeras, and channels coexpressed with the β1 subunit. *Biophys. J.* **76,** 233–245.
10. Konnerth, A., Lux, H. D., and Morad, M. (1987) Proton-induced transformation of calcium channels in chick dorsal root ganglion cells. *J. Physiol. (London)* **386,** 603–633.
11. Horn, R., Patlak, J., and Stevens, C. F. (1981) Sodium channels need not open before they inactivate. *Nature* **291,** 426–427.
12. Berman, M. F., Camardo, J. S., Robinson, R. B., and Siegelbaum, S. A. (1989) Single sodium channels from canine ventricular myocytes: Voltage dependence and relative rates of activation and inactivation. *J. Physiol. (London)* **415,** 503–531.
13. Scanley, B. E., Hanck, D. A., Chay, T., and Fozzard, H. A. (1990) Kinetic analysis of single sodium channels from canine cardiac Purkinje cells. *J. Gen. Physiol.* **95,** 411–437.
14. Lawrence, J. H., Yue, D. T.,. Rose, W. C., and Marban, E. (1991) Sodium channel inactivation from resting states in guinea-pig ventricular myocytes. *J. Physiol. (London)* **443,** 629–650.
15. Goldman, L. (1995) Sodium channel inactivation from closed states: Evidence for an intrinsic voltage dependency. *Biophys. J.* **69,** 2369–2377.
16. O'Reilly, J, P. Wang, S-Y., and Wang, G. K. (2000) A point mutation in domain 4-segment 6 of the skeletal muscle sodium channel produces an atypical inactivation state. *Biophys. J.* **78,** 773–784.
17. Hille, B. (1992) Ionic Channels of Excitable Membranes, 2nd ed., pp. 403–414. Sinauer Associates Inc., Sundeland, MA.
18. Xiao, Y-F., Kang, J. X., Morgan, J. P., and Leaf, A. (1995) Blocking effects of polyunsaturated fatty acids on Na^+ channels of neonatal rat ventricular myocytes. *Proc. Natl. Acad. Sci. USA* **92,** 11000–11004.
19. Hondeghem, L. M., and Katzung, B. J. (1977) Time- and voltage-dependent interactions of antiarrhythmic drugs with cardiac sodium channels. *Biochim. Biophys. Acta* **472,** 373–398.
20. Hille, B. (1977) Local anesthetics: Hydrophilic and hydrophobic pathways for the drug-receptor action. *J. Gen. Physiol.* **69,** 497–515.
21. Trainer, V., Brown, G. B., and Catterall, W. A. (1996) Site of covalent labeling of photoreactive batrachotoxin derivative near transmembrane segment I S6 of the sodium α-subunit. *J. Biochem,* **271,** 11261–11267.17.
22. Postma, S. W., and Catterall, W. A. (1984) Inhibition of binding of [^{3}H]batrachotoxinin A 20-α-benzoate to sodium channels by local anesthetics. *Mol. Pharmacol.* **25,** 219–227.

23. Ragsdale, D. S., McPhee, J. C., Scheur, T., and Catterall, W. A. (1994) Molecular determinants of state-dependent block of Na^+ channels by local anesthetics. *Science* **265,** 1724–1728.
24. Wright, S. N., Wang, S-Y., and Wang, G. K. (1998) Lysine point mutations in Na^+ channel D4-S6 reduce inactivated channel block by local anesthetics. *Mol. Pharmacol.* **54,** 733–739.
25. Sheldon, R. S., Cannon, N. J., and Duff, H. J. (1987) A receptor for type I antiarrythmic drugs associated with rat cardiac sodium channels. *Circ. Res.* **61,** 492–497.
26. Wang, S-Y., and Wang, G. K. (1998) Point mutations in I-S6 render voltage-gated Na^+ channels resistant to batrachotoxin. *Proc. Natl. Acad. Sci USA* **95,** 2653–2658.20.
27. Linford, N. J., Cantrell, A. R., Qu, Y., Scheur, T., and Catterall, W. A. (1998) Interaction of batrachotoxin with the local anesthetic receptor site in transmembrane segment IVS6 of the voltage-gated sodium channel. *Proc. Natl. Acad. Sci. USA* 95,13947–13952.
28. Wang, G. K., and Wang, S-Y. (1999) Batrachotoxin-resistant Na^+ channels derived from point mutations in transmembrane segment D4-S6. *Biophys. J.* **76,** 3141–3149.
29. Nau, C., Wang, S-Y., Strichartz, R., and Wang, G. K., (1999): Point mutations at N434 in D1-S6 of μ1 Na^+ channels modulate binding affinity and stereoselectivity of local anesthetic enantiomers. *Mol. Pharmacol.* **56,** 404–413.