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Ethnic-Related Sodium Voltage-Gated Channel α Subunit 5 Polymorphisms Shape the In Vitro Pharmacological Action of Amiodarone upon $Na_v1.5$

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ABSTRACT

$Na_v1.5$-derived Na^+ current (I_{Na}) exerts a pivotal role in the depolarization phase of cardiomyocytes' action potential, and, therefore, changes in I_{Na} can contribute to fatal arrhythmias. $Na_v1.5$ displays naturally occurring ethnicity-related polymorphisms, which might alter the functioning and pharmacology of the channel. Some studies have shown how single-nucleotide polymorphism can change the response to antiarrhythmic drugs. Investigations on the role of $Na_v1.5$ in arrhythmogenesis associated with its functional polymorphisms are currently growing as well as the possible variability in the antiarrhythmic pharmacotherapy among ethnic groups. The influence of the ethnicity-related polymorphisms (S524Y, S1103Y, R1193Q, V1951L) on the responsiveness, selectivity, and pharmacological efficacy of the clinically used antiarrhythmic amiodarone (AMIO) is not completely known. Our objectives were to analyze biophysical and pharmacological aspects of four ethnicity-related polymorphisms before and after exposure to AMIO. Polymorphisms caused reduced AMIO potency compared with wild type (WT), which can vary by up to 4× between them. AMIO shifted the voltage dependency for current inactivation without significant effect in voltage-dependent activation to a similar extent in WT and polymorphisms. The recovery from inactivation was altered between the polymorphisms when compared with WT. Finally, the use dependency of AMIO differed between studied groups, especially at a more depolarized cell membrane. Thus, our work may guide future studies focusing on the efficiency of AMIO in treating different arrhythmias and establish more individualized guidelines for its use depending on the $Na_v1.5$ polymorphism after validating our findings using in vivo studies.

SIGNIFICANCE STATEMENT

Sodium voltage-gated channel α subunit 5 (*SCN5A*) gene encodes the α subunit of $Na_v1.5$, the main cardiac voltage-gated Na^+ channel. Interestingly, ethnicity-related polymorphisms are found in *SCN5A*. Amiodarone is used in clinical practice, and some of its effects are attributed to interaction with $Na_v1.5$. Important, amiodarone efficacy is variable among patients. Here we show that ethnicity-related *SCN5A* polymorphisms lead to altered $Na_v1.5$-amiodarone interaction, which may be the cause for the variable efficacy observed in clinical usage of amiodarone.

Introduction

Sodium voltage-gated channel α subunit 5 (*SCN5A*) is the gene responsible for encoding the pore-forming α subunit of the human cardiac voltage-gated Na^+ channel, $Na_v1.5$ (Zimmer et al., 2014). Changes in $Na_v1.5$ sequence can lead to modification of its biophysical properties (Tan et al., 2005; Hu et al., 2015). $Na_v1.5$-derived Na^+ current (I_{Na}) exerts a pivotal role in the depolarization phase of cardiomyocytes' action potential (AP), and, therefore, modifications in the I_{Na} can cause electrical disturbances of myocytes and contribute to the occurrence of cardiac arrhythmias (Veerman et al., 2015, Veltmann et al., 2016)). Indeed, cardiac arrhythmias, such as long QT syndrome type 3, atrial fibrillation, and Brugada syndrome, have been associated with abnormal biophysical properties of $Na_v1.5$ (Veerman et al., 2015, Veltmann et al., 2016; Kruger and Isom, 2016).

An interesting aspect of $Na_v1.5$ is the existence of naturally occurring polymorphisms that might alter the functioning and pharmacology of the channel (Makielski et al., 2003; Tan et al., 2005). Single-nucleotide polymorphism is the most frequent genetic alteration, and mutation of a single nucleotide may or may not trigger the formation of a new product with loss or gain of $Na_v1.5$ function. Intriguing, some of these polymorphisms can be ethnicity-related, such as S524Y, S1103Y, R1193Q, and V1951L (Ackerman et al., 2004; Tan et al., 2005; Tester and Ackerman, 2009).

The ethnicity-related polymorphisms S524Y and S1103Y have a prevalence of 6% and 13%, respectively, in the Afro-descendent population (Splawski et al., 2002; Tan et al.,

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ABBREVIATIONS: AMIO, amiodarone; AP, action potential; G, conductance; G_{MAX}, maximum conductance; HEK293, Human Embryonic Kidney 293; I, current; I_{MAX}, maximum current; I_{Na}, $Na_v1.5$-derived Na^+ current; IV, current-voltage relationship; SCN5A, sodium voltage-gated channel α subunit 5; V_a, voltage at which half of the maximum conductance is achieved; V_h, potential corresponding to half the maximum inactivation; WT, wild type.

2005). Considering the V1951L and R1193Q polymorphisms, they are prevalent (7% and 0.3%, respectively) in Whites and Hispanic people. Moreover, the V1951L polymorphism has a prevalence of 16% in the Asian population (Ackerman et al., 2004). Each one of these polymorphisms might lead to specific phenotypes with a singular consequence for heart function and therapy. For example, some studies have shown how a single-nucleotide polymorphism can change the response to antiarrhythmic drugs commonly used in clinical practice (Shinlapawittayatorn et al., 2011; Sardar et al., 2014, 2016).

The interest in biomedical investigations on the role of $Na_v1.5$ in arrhythmogenesis associated with its functional polymorphisms is currently growing as well as the possible variability in the antiarrhythmic pharmacotherapy among ethnic groups (Sardar et al., 2014, 2016). To date, the influence of the ethnicity-related polymorphisms on the responsiveness, selectivity, and pharmacological efficacy of antiarrhythmic drugs targeting $Na_v1.5$ is not completely known. The antiarrhythmic drug amiodarone (AMIO), for example, is a classic antiarrhythmic agent belonging to class III and has diverse pharmacological action mechanisms because it also inhibits peak $Na_v1.5$ current (class I activity), I_k (class III activity), I_{Ca} (class IV activity), and α-β adrenoceptors (class II activity) being used in patients with ventricular tachyarrhythmias, heart failure, and recurrent fibrillation (Sardar et al., 2014, 2016; Lafuente-Lafuente et al., 2015). Clinically, AMIO represents about 45% of all prescriptions in the United States (Zimetbaum, 2012). Prospective clinical trials demonstrated that among all antiarrhythmic drugs currently used in the management of fibrillation, AMIO has the greatest (65%) potential for maintaining sinus rhythm compared with other antiarrhythmics, such as sotalol and propafenone (Vamos and Hohnloser, 2016).

Considering AMIO's clinically intense use and the ethnicity-related polymorphisms, our objectives in this study were to analyze biophysical and pharmacological aspects of S524Y, S1103Y, R1193Q, and V1951L before and after exposure to AMIO. We understand that our data can contribute to advances in the knowledge about how ethnicity-related polymorphisms affect the interaction of AMIO with $Na_v1.5$, which can provide evidence to design a more accurate and individualized pharmacological strategy to treat arrhythmic syndromes.

Materials and Methods

Ethics. All experimental protocols were approved by our institutional committee that regulates the use of laboratory procedures (Research Ethics Committee number:1869170619).

Cell Culture, Polymorphisms, and Transfection. For cellular procedures, Human Embryonic Kidney 293 (HEK293) cells were used that lack functional macroscopic I_{Na}. Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Burlington, ON) with 4.5 g/l of D-(+) glucose and 10% FBS and 1% penicillin/streptomycin supplementation. The cells were maintained at 37°C and 5% CO_2 atmosphere in an incubator. Human wild-type (WT) $Na_v1.5$ and all polymorphisms studied (S524Y, S1103Y, R1193Q, and V1951L) were cloned into the BamH1 and HindIII restriction sites of the pcDNA 3.1 vector (Invitrogen) purchased from GenScript Limited (Hong Kong). In this study, all $Na_v1.5$ variants were evaluated using the backbone of *SNC5A* isoform National Center for Biotechnology Information reference number XM_006713282.2 (containing 2016 residues, including Q1077).

For transfection procedure, HEK293 cells were kept in 60 mm culture at 60%–80% confluence and maintained for 5 hours in 800 μl Opti-MEM (Thermo Fisher Scientific) supplemented with 4 μl LipofectAMINE 2000, 1 μl PLUS Reagent (Invitrogen), and 1 μg of each plasmid cDNA. After 5-hour period, the Opti-MEM was replaced by Dulbecco's modified Eagle's medium culture medium. Patch-clamp recording was performed after 48 hours.

Whole-Cell Patch-Clamp Recording. All procedures were performed at room temperature (22–25°C). The EPC-10.2 patch-clamp amplifier (HEKA Elektronik, Germany) and Axopatch 200B amplifier (Molecular Devices) in the voltage-clamp mode were used to record the macroscopic I_{Na} via the PACTHMASTER acquisition software (HEKA Elektronik, Germany) or Clampex 10.7 (Molecular Devices). During experiments, transfected cells were replated onto glass coverslips and mounted in a recording chamber filled with extracellular solution containing (in mM) 130 $NaCl_2$, 1.8 $CaCl_2$, 5 CsCl, 1.2 $MgCl_2$, 10 HEPES, and 5 glucose, pH = 7.4. Patch pipette tip resistance was set to 2.5–3.0 MΩ, and series resistance was typically 3–5 MΩ. Cells with series resistance >7 MΩ were not considered to prevent voltage-clamp errors. Electronic compensation of series resistance (60%–70%) was applied given the fast kinetics and the steep voltage dependence of I_{Na}. In addition, patch pipettes were filled with an intracellular solution containing (in mM) 50 aspartic acid, 60 CsCl, 5 Na_2ATP, 11 EGTA, 10 HEPES, 4.27 $CaCl_2$, and 1 $MgCl_2$, pH = 7.2. After the whole-cell configuration stabilization, cells were kept for 2 minutes at rest to allow proper intracellular solution dialysis. Records were low-pass-filtered (cutoff frequency 2 kHz) and digitalized at 10–20 kHz. HEK293 cells typically fall under 10–30 pF cell capacitance range.

The pharmacological potency of AMIO was evaluated for WT and $Na_v1.5$ ethnic polymorphisms S524Y, S1103Y, R1193Q, and V1951L. The following crescent concentrations of the drug were applied (in μM): 1, 10, 30, 100, and 1000. A square pulse to −20 mV from a holding potential of −120 mV and 1 Hz stimulation frequency was used to access the tonic effect of AMIO, which is commonly achieved after 2 minutes stimulation. Peak inward current level prior to AMIO challenge was considered 100%, which was our control for the dose-response curve analysis. Percentage of drug blockage was determined as the fraction of reduced peak inward current after steady-state level after AMIO exposition was achieved and normalized by control peak inward current. After normalization, the equation y = A1 + (A2 − A1)/(1 + 10^((LOGx0 − x)*p)) was fitted for WT and each sodium channel polymorphism curve in which A1 (bottom asymptote) and A2 (top asymptote) were fixed at 0 and 1, respectively. Data were analyzed using OriginPro 2018. Voltage dependency for current activation was accessed by 25-millisecond square pulses ranging from −80 to +37 mV, with 3 mV increment and at 1 Hz frequency and from a holding potential of −120 mV. Boltzmann equation $I = (E_m - E_{rev})\left((G_{max})/\left[1 + e^{\left(\frac{V_{50} - E_m}{k}\right)}\right]\right)$ was fitted to the current-voltage relationship (IV) in which E_{rev} is the predicted reversal potential of the current, V_{50} is the voltage at which half of the current is activated, G_{MAX} is the estimated maximum conductance, and k is the slope of the curve. Conductance at each tested potential (G) was normalized by (G_{max}). G/G_{max} plots were then fitted using a Boltzmann equation: $G/G_{max} = 1/\left[1 + e^{\left(\frac{E_m - V_a}{k_a}\right)}\right]$. E_m is the membrane potential, V_a is the voltage at which half of the maximum conductance is achieved, and k_a represents the slope of the curve. Data were analyzed using OriginPro 2018.

Time to peak of I_{Na} was computed as the time of pulse beginning until the maximum inward current was achieved in each tested potential. Time to I_{Na} decay was calculated as the time to peak I_{Na} to decay 50% of its value for each tested membrane potential.

Voltage-dependent steady-state inactivation was studied by a 50-millisecond duration and −20 mV amplitude test pulse immediately after a long prepulse (2000 milliseconds) ranging from −120 mV to 20 mV, with 5 mV increment at each step with 1-second interval

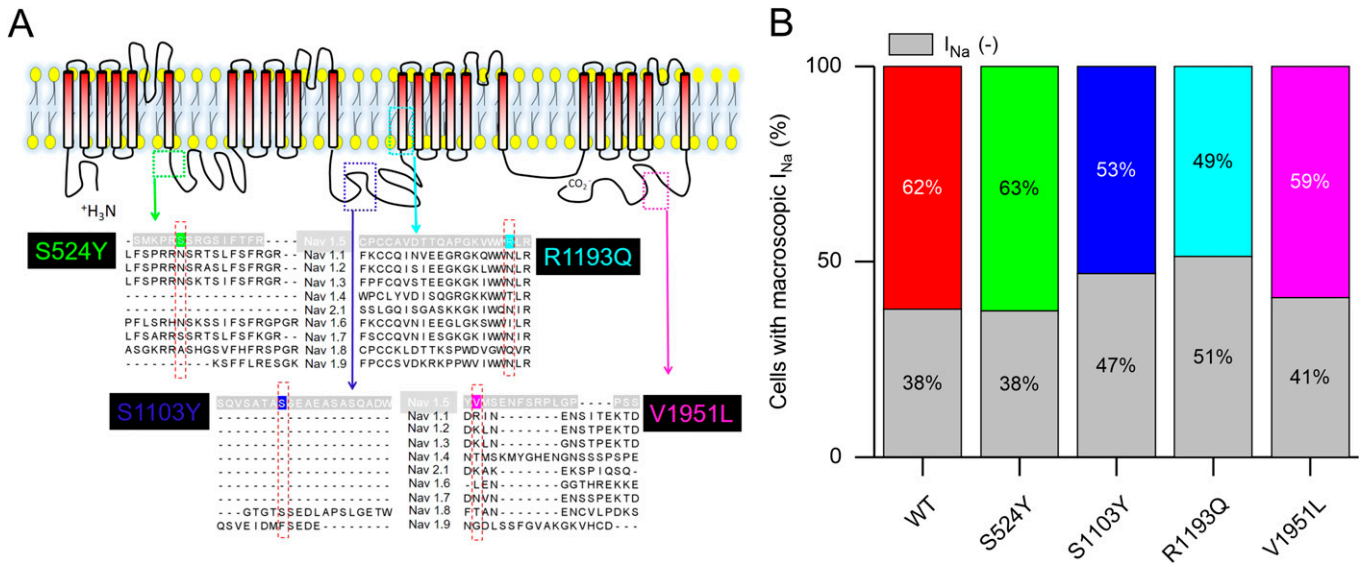

Fig. 1. Ethnicity-related $Na_v1.5$ polymorphisms. (A) Schematic representation of the ethnicity-related single nucleotide mutations distribution in the $Na_v1.5$ channel. Sequences are compared between $Na_v1.5$ channel isoforms (B) Percentage of cells showing macroscopic voltage-dependent currents. All $Na_v1.5$ polymorphisms are expressed in the Q1077 splice variant. N = (WT: 58, S524Y: 32, S1103Y: 32, R1193Q: 37, V1951L: 44), $^*P < 0.05$. Chi-Square Test.

between sweeps to achieve a stationary voltage-dependent inactivation. Peak currents obtained from the −20 mV test pulse after each prepulse were normalized by maximum current (I/I_{max}) and fitted using a Boltzmann equation $\frac{I}{I_{max}} = 1/\left[1 + e^{\left(\frac{E_m - V_h}{k_h}\right)}\right]$. In this equation, E_m represents the membrane voltage applied during the conditioning pulses. V_h is the potential in which half of the maximum inactivation is achieved and k_h represents the slope factor of the curve.Data were analyzed using OriginPro 2018.

Window current was calculated from the area under the intersection of fitted voltage-dependent activation, and steady-state

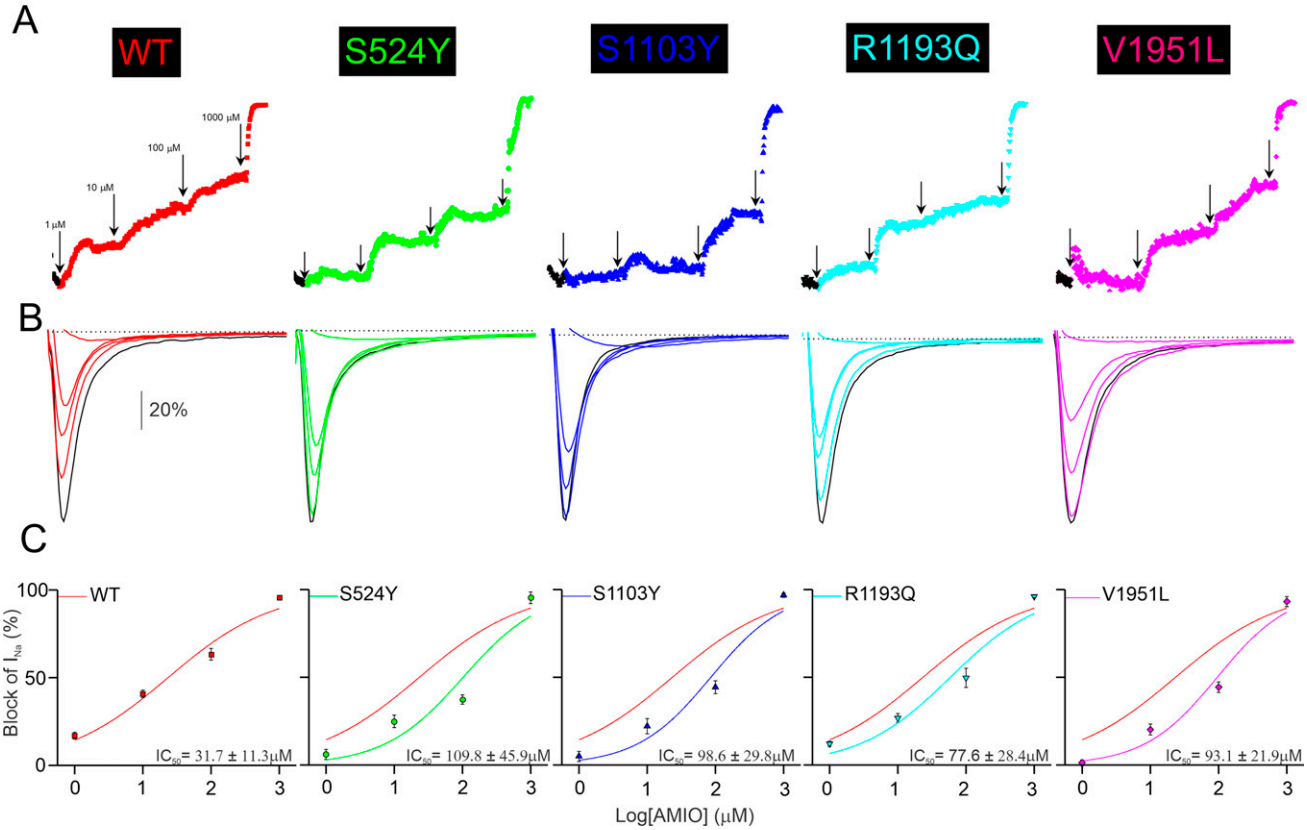

Fig. 2. Dose-response blockage of I_{Na} by amiodarone. (A) Time course and (B) representative traces of amiodarone blockage of I_{Na} on the ethnicity-related $Na_v1.5$ polymorphisms. (C) Summarized dose-response of amiodarone and estimated concentration to reach 50% of the current block (IC_{50}). N = 5–11/points. * $P < 0.05$. Two-way ANOVA; comparing WT and polymorphism.

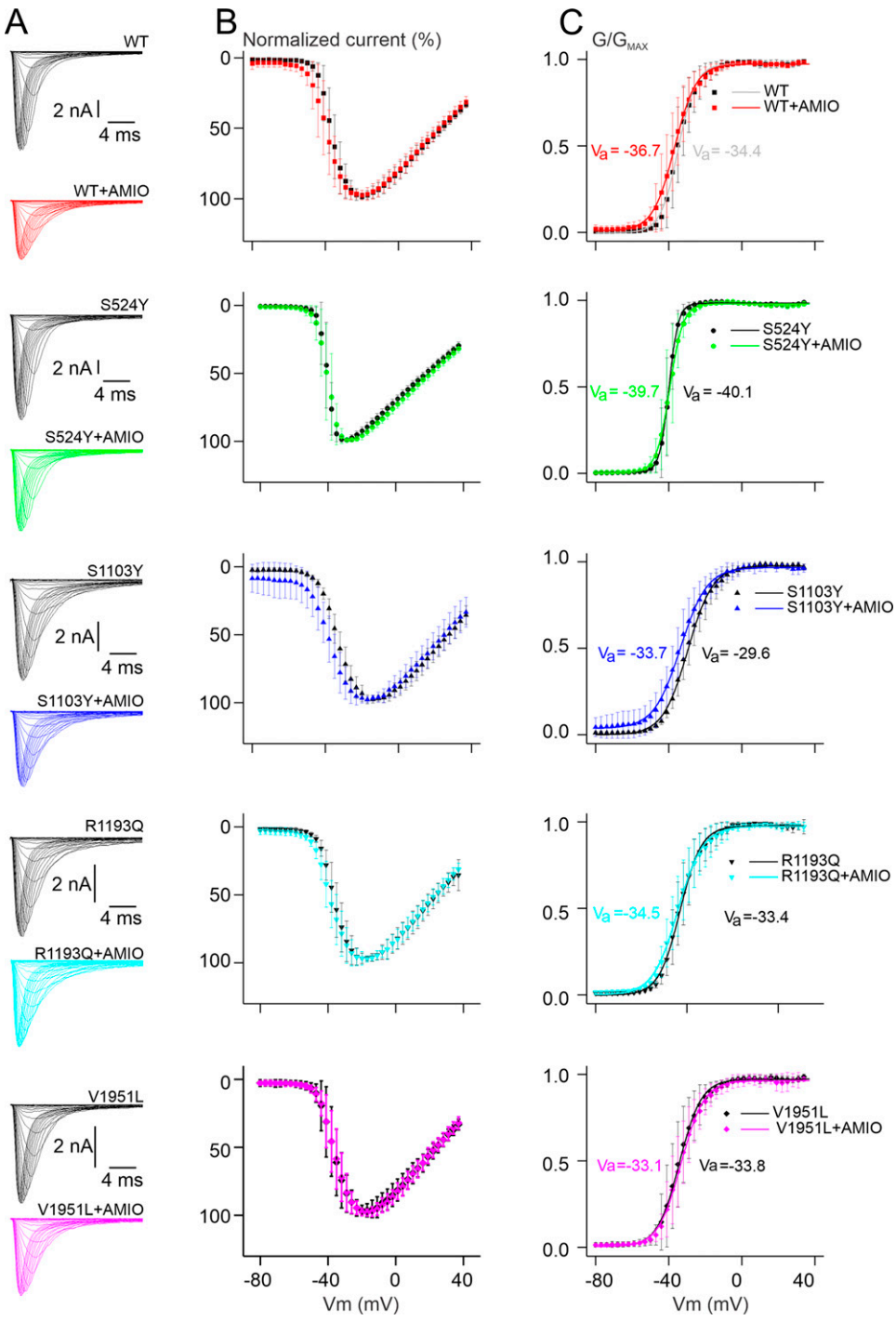

Fig. 3. Comparison of the voltage-dependent activation of I_{Na} among $Na_v1.5$ ethnicity-related polymorphisms. (A) Representative traces of the voltage-dependent activation of the current before (upper panel, black traces) and after exposure to amiodarone (lower panel, colored traces). Only first 25 traces are shown. Scale is the same within the same group (-) amiodarone and (+) amiodarone. Current and time scale values are the same for all representative traces. (B) Normalized IV plots by the peak current are shown for each tested potential before (black traces) and after exposure to amiodarone (colored traces). (C) Voltage-dependent activation curves as a function of the tested membrane potential. V_a: voltage in which half of the maximum conductance was achieved. N = (WT: 10, S524Y: 6, S1103Y: 7, R1193Q: 6, V1951L: 7). Two-way ANOVA comparing WT and polymorphism, seen in the Table 1 for statistics.

inactivation curves were normalized by peak I_{Na}. Boltzmann equation was fit to the curves.

Recovery from inactivation was recorded as double-squared pulses (50 milliseconds and 100 milliseconds) to −20 mV from a holding potential of −120 mV with a variable interinterval pulse: 1–10 milliseconds with 1-millisecond increment, 12–98 milliseconds with 2-millisecond increment, and 800–1000 milliseconds with 100-millisecond increment. A two-exponential equation was fitted to the recovery-from-inactivation curves as the following: $Y = A_1*(1 - \exp(X/\tau_1)) + A_2*(1 - \exp(X/\tau_2))$, from which fast and slow time constants' (τ_1 and τ_2) current recovery was obtained. A_1 and A_2 represent the initial quantities, the maximum fractional current recovery for the fast and slow components, respectively. Data were analyzed using OriginPro 2018.

The estimated time constants were compared between groups before and after exposure to AMIO. In addition, the normalized fractional contribution of each component (A_1 and A_2) obtained from the exponential equations was compared between the experimental groups. Data were analyzed using OriginPro 2018.

The use-dependent block of I_{Na} was evaluated by varying the stimulation frequency (1 Hz, 2 Hz, and 5 Hz) of square pulses (−20 mV, 100 milliseconds). This protocol was run from a holding potential of −120 mV and −80 mV. In all protocols described above, $Na_v1.5$-WT and polymorphisms were recorded before and after reaching a stable

tonic effect of AMIO (typically after 2–3 minutes) that was accompanied with square pulses to −20 mV from a holding potential of −120 mV. In all experiments, AMIO was solubilized from a stock of 20 mM solution in DMSO.

Statistical Analysis. OriginPro 2018 was used for all statistical analyses. Normally distributed data (using the Kolmogorov-Smirnov test) were analyzed using one-way, two-way ANOVA followed by Tukey's post hoc test or Student's *t* test, as indicated in the figure legend or table. Proportion was evaluated using chi-square test. A significance level of 5% was set in all analyses ($P < 0.05$). Data are expressed as mean ± S.D.

Results

Comparing the Pharmacological Potency of AMIO in $Na_v1.5$ Ethnicity-Related Polymorphisms. As seen in Fig. 1A, the sequence of $Na_v1.5$ ethnicity-related polymorphisms was aligned with other isoforms of Na_v in the region of interest in which each polymorphism occurs. The schematic representation of $Na_v1.5$ channels is marked with dashed squares that highlight the region from which each variant is located and was constructed according to Pan et al. (2018). The S524 is located in the DI-DII linker. The S1103 residue is positioned in the DII-DIII linker. The R1193 residue is located in the S1 transmembrane segment of the DIII domain, and finally, the V1951 residue is placed in the C-terminal domain of the $Na_v1.5$ channel. Each one of the $Na_v1.5$ polymorphisms was individually transfected to HEK293 cells, and the whole-cell patch-clamp technique was performed to record I_{Na} in the presence or absence of AMIO. Figure 1B displays the percentage of transfected cells in which macroscopic I_{Na} was observed, and no difference between all studied groups was observed, as expected since these $Na_v1.5$ variants per se are not pathologic ($P > 0.05$).

Then the pharmacological potency of AMIO was evaluated for WT and $Na_v1.5$ ethnicity-related polymorphisms. Figure 2A shows the time course of AMIO effects on peak I_{Na} elicited from a square pulse to −20 mV from a holding potential of −120 mV. Transfected HEK293 cells were exposed to AMIO, and the representative traces of the time-course effect of AMIO on I_{Na} are presented in Fig. 2B. The summarized effects of AMIO percentage block of I_{Na} are plotted in Fig. 2C and fitted with a symmetrical sigmoidal curve. The pharmacological potency of AMIO was estimated as the concentration required to reach 50% of the current block (IC_{50}). We can observe that the highest pharmacological potency was detected in the control group (WT-I_{Na}; IC_{50} = 31.7 ± 11.3 μM, 95% confidence interval, 9.5 to 53.9 uM). On the other hand, the potency of AMIO was lower in all polymorphisms compared with WT, the lowest being found in S524Y (IC_{50} = 109.8 ± 45.9 μM, 95% confidence interval, 19.8 to 199.7 uM, $P < 0.05$ compared with control using two-way ANOVA), which displayed almost 4× less sensitivity to AMIO. R1193Q showed the highest potency among the studied polymorphisms (IC_{50} = 77.6 ± 28.4 μM, 95% of confidence interval, 21.9 to 133.3 μM, $P < 0.05$ compared with control using two-way ANOVA), whereas S1103Y and V1951L had intermediate values (IC_{50} = 98.6 ± 29.8 μM, 95% confidence interval, 40.2 to 157.1 μM, $P < 0.05$ compared with control using two-way ANOVA, and IC_{50} = 93.1 ± 21.9 μM, 95% of confidence interval, 50.1 to 136.1 μM, $P < 0.05$ compared with control using two-way ANOVA, respectively). Hence, all ethnicity-related polymorphisms showed a significant reduction in AMIO sensitivity compared with WT ($P < 0.05$). With these results, we conclude that AMIO displays different pharmacological sensitivity among the ethnicity-related polymorphisms studied.

Effects of AMIO in the Voltage Dependency for Current Activation of $Na_v1.5$ Ethnicity-Related Polymorphisms. A series of square pulses ranging from −80 to +37 mV (3 mV increments on each step at 1 Hz frequency) from −120 mV holding potential was applied to generate an IV. Representative current traces of I_{Na} activation followed by depolarizing steps are displayed in Fig. 3A. The normalized IV plots can be seen in Fig. 3B, the middle column. From the IV plots, the G_{MAX} of each cell was estimated using a Boltzmann equation fitted to the data (see *Materials and Methods*). The calculated conductance at each voltage step (G) was normalized by the maximum conductance, and a Boltzmann equation was fitted to the G/G_{MAX} curves (Fig. 3C). G/G_{MAX} is the distribution of the fractional conductance among the tested voltages, which represents the voltage dependency for channel activation. We observed that the voltage at which half of G_{MAX} is achieved (V_a) was similar in WT and all polymorphisms, although the slope factor was smaller in S524Y and larger in S1103Y when compared with WT (Table 1). However, challenging groups with 10 μM of AMIO altered neither the slope factor nor V_a. Using the same patch-clamp recordings from Fig. 3, we evaluated the impact of AMIO on the time to peak current (Supplemental Fig. 1A) and on the time to 50% of current inactivation (Supplemental Fig. 1B). AMIO was not able to change either time to current peak or time to 50% of current inactivation in WT and in the studied polymorphisms. However, it is interesting to note that prior to AMIO challenge we found a slower time for I_{Na} peak and decay in S1103Y and R1193Q when compared with WT (Supplemental Fig. 2, A and B) and faster I_{Na} decay for

TABLE 1
Voltage-dependent activation parameters of I_{Na} among $Na_v1.5$ ethnicity-related polymorphisms obtained using Boltzmann equation fitted to the data. Kolmogorov-Smirnov test, two-way ANOVA followed by Tukey's post-test.

	AMIO (−)	AMIO (−)	AMIO (+)	AMIO (+)
	Slope	V_a (mV)	Slope	V_a (mV)
WT (n = 10)	4.1 ± 0.4	−34.4 ± 1.3	4.9 ± 0.3	−36.7 ± 1.6
S524Y (n = 6)	1.9 ± 0.1*	−40.1 ± 1.1	2.7 ± 0.2[&]	−39.7 ± 1.5
S1103Y (n = 7)	6.3 ± 0.4*	−29.6 ± 1.3	6.7 ± 0.6	−33.7 ± 1.6
R1193Q (n = 6)	5.3 ± 0.4	−33.4 ± 1.7	6.2 ± 0.4	−34.5 ± 2.1
V1951L (n = 7)	4.9 ± 0.5	−33.9 ± 2.1	5.5 ± 0.3	−33.1 ± 1.7

V_a, voltage in which half of the maximum conductance was achieved.
*$P < 0.05$, comparing polymorphism (−) AMIO to WT (−) AMIO.
[&]$P < 0.05$, comparing polymorphism (+) AMIO to WT (+) AMIO.

S524Y when compared with WT (Supplemental Fig. 2, A and B). Thus, these data suggest that AMIO has no significant impact on the transition from the closed to open state of the cardiac sodium channel in WT and in the studied polymorphisms.

Effects of AMIO in the Stationary Voltage-Dependent Inactivation of I_{Na} of $Na_v1.5$ Ethnicity-Related Polymorphisms. A series of preconditioning pulses ranging from −120 to 0 mV (6 mV increment on each step, at 1 Hz) from a −120 mV holding potential and with 2000 milliseconds was applied to establish a stationary voltage-dependent inactivation of I_{Na} (the remaining noninactivated current was measured from a second test pulse, −20 mV). Figure 4 displays the representative traces from the test pulse, before (Fig. 4A, left column) and after (Fig. 4B, middle column) exposure to 10 μM AMIO. The orange, thick trace is the remaining current at −80 mV, highlighting the effects of AMIO in the stationary voltage-dependent inactivation of I_{Na} in WT and tested polymorphisms. The I_{Na} obtained after each preconditioning voltage (I) was normalized by the maximum current when the conditioning pulse was at −120 mV (I_{MAX}) and plotted as a function of the tested conditioning voltages

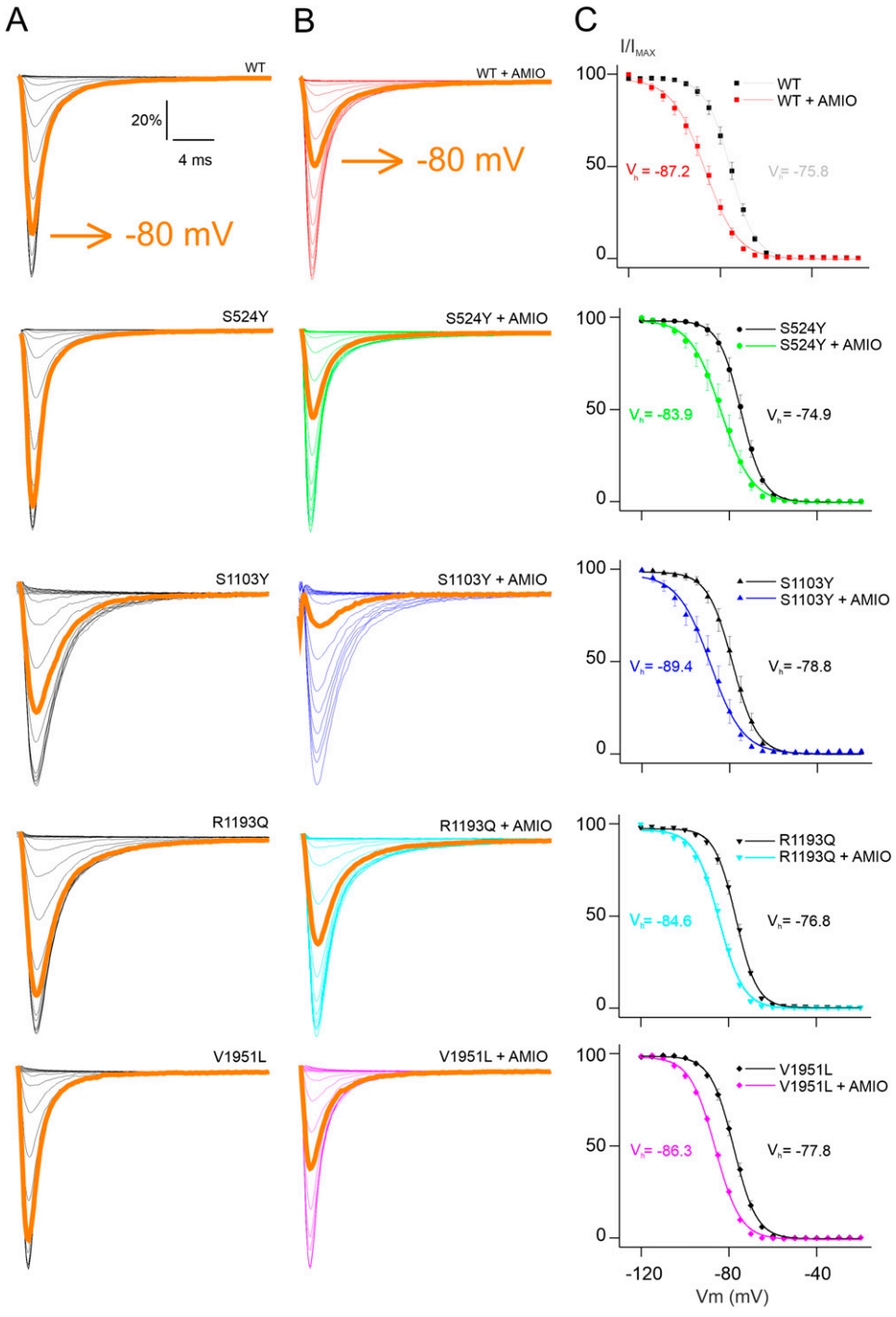

Fig. 4. Comparison of the stationary voltage-dependent inactivation of I_{Na} among $Na_v1.5$ ethnicity-related polymorphisms. (A) Representative traces of the voltage-dependent activation of the current before (black traces) and (B) after exposure to amiodarone (colored traces). The orange thick traces highlight the current recorded at −80 mV conditioning pulse (C) Voltage-dependent channel availability curves as a function of the tested membrane potential. V_h: voltage in which half of the maximum channel availability is achieved N = (WT: 11, S524Y: 7, S1103Y: 5, R1193Q: 7, V1951L: 7). Two-way ANOVA comparing WT and polymorphism, seen in the specific Table.

(I/I_{MAX}) in Fig. 4C, right column. I/I_{MAX} is the distribution of the fractional current among the tested voltages, which represents the voltage dependency for channel inactivation. This stationary voltage-dependent inactivation can be compared with the voltage at which half of I_{MAX} is achieved (V_h). A Boltzmann-like equation was fitted to the data (see *Materials and Methods*).

No difference was observed in V_h from the $Na_v1.5$ polymorphisms compared with WT before exposure to AMIO ($P > 0.05$). Moreover, in all variants, after AMIO exposure there was a negative shift in the V_h of I_{Na} similar to that recorded from the WT and polymorphisms. Interestingly, slope factor is higher in WT (from 5.1 ± 0.7 to 6.4 ± 0.5, $P < 0.05$) and S1103Y (from 5.2 ± 0.6 to 7.1 ± 1.3, $P < 0.05$) after challenging with AMIO. Additional fitting parameters from all groups are listed in Table 2. From these results, we conclude that AMIO induced a negative shift on the voltage-dependent inactivation of WT isoform of $Na_v1.5$ and among the $Na_v1.5$ polymorphisms .

Since AMIO caused a negative shift on the inactivation curve of I_{Na}, we decided to calculate the window I_{Na} before and after AMIO exposure. Window current is considered as the area under the curve of intersection between voltage-dependent activation and steady-state inactivation curves. Boltzmann equation was fitted to both curves. Prior to fitting, data were normalized by maximum I_{Na} using experimental data from Figs. 3 and 4. As shown in Supplemental Fig. 3, WT and polymorphisms had a small but detectable window I_{Na}. AMIO did not change the magnitude of the window I_{Na} in any of the tested groups.

Effects of AMIO in the Recovery from Inactivation of I_{Na} of $Na_v1.5$ Ethnicity-Related Polymorphisms. The fast and slow recovery of I_{Na} from inactivation was accessed through a series of twin pulses (to −20 mV, from a holding potential of −120 mV) varying the interval between the test and the conditioning pulse (1–1000 milliseconds). Since the conditioning pulse brings the majority of sodium channels to the inactivation state after being activated, we evaluated the fractional current that is recovered as a function of the time interval between the twin pulses. Figure 5, A and B display the representative traces of I_{Na} recovery from inactivation before (5A, left column) and after (5B, middle column) exposure to 10 μM AMIO. Only the first 10 sweeps, in which interval time was between 1 and 10 milliseconds, were displayed to highlight the effects of AMIO in the fast component (τ_1) of inactivation recovery. The summarized data regarding the fast and slow recovery from inactivation are presented in Fig. 5C, right column. A two-component exponential equation was used to estimate the fast (τ_1) (Table 3) and slow (τ_2) (Table 4) time constants of the current recovery. In addition, the relative contribution (A_1 and A_2, see Table 5 and Table 6) of each recovery component to the peak current was studied.

S1103Y and R1193Q variants displayed slower τ_1 compared with WT ($P < 0.05$, Table 3), whereas comparable τ_2 was found in all groups prior to AMIO challenge. Moreover, AMIO slowed both the τ_1 and τ_2 in WT and all sodium channel variants, and S1103Y and R1193Q displayed slower kinetics of τ_1 compared with WT. Also, AMIO exerted a significant effect on the relative contribution of τ_1 and τ_2 to the total current recovery, especially for S524Y, S1103Y, and R1193Q, in which the relative contribution of τ_1 was larger when compared with WT. From these data, we conclude that AMIO strongly slows the recovery from inactivation kinetics of all variants. In addition, AMIO changes the contribution of τ_1 and τ_2 to inactivation in an ethnicity-related fashion.

Use-Dependent Block of I_{Na} by AMIO among the $Na_v1.5$ Ethnicity-Related Polymorphisms. The use-dependent block of I_{Na} after exposure to 10 μM of AMIO was evaluated by square pulses (−20 mV) varying the stimulation frequency from 1 Hz, 2 Hz, and 5 Hz from a holding potential of −120 mV (Fig. 6). Figure 6A shows the representative traces of I_{Na} recorded after exposure to AMIO. Current traces were normalized by the maximum current (at 1 Hz) for each group. Normalized currents were plotted as a function of the log of stimulation frequency (Fig. 6B), and a linear regression was fitted to the data, from which the slope of the curve was used to compare the use-dependent block of AMIO among them. As shown in Fig. 6B and Table 7, WT and all polymorphisms were well fitted after linear regression, with strong correlation with R2 typically >0.85 as well as with Pearson's correlation (typically below −0.9), indicating that there is a strong inverse correlation between increased frequency and the current block and that they are comparable between all groups.

Finally, the use-dependent block of I_{Na} by AMIO was further evaluated using a more depolarized holding potential of −80 mV (which can mimic the membrane potential of atrial cardiomyocytes). Normalized current traces at the recorded frequencies are shown in Fig. 7A for WT and $Na_v1.5$ polymorphisms. Figure 7B displays the summarized plots of the normalized currents recorded at −80 mV holding potential as a function of the stimulation frequency. As can be seen in Table 8, R1193Q showed no significant use dependency when membrane holding potential was −80 mV, whereas the other sodium channel variants showed a similar profile to WT.

TABLE 2
Voltage-dependent inactivation parameters of I_{Na} among $Na_v1.5$ ethnicity-related polymorphisms obtained using Boltzmann equation fitted to the data. Kolmogorov-Smirnov test, two-way ANOVA followed by Tukey's post-test.

	AMIO (−)	AMIO (−)	AMIO (+)	AMIO (+)
	Slope	V_h (mV)	Slope	V_h (mV)
WT (n = 11)	5.1 ± 0.7	−75.9 ± 3.7	6.4 ± 0.5**	−87.3 ± 6.3**
S524Y (n = 7)	4.6 ± 0.6	−74.9 ± 4.1	5.7 ± 0.6	−83.9 ± 4.1**
S1103Y (n = 5)	5.2 ± 0.6	−78.8 ± 3.8	7.1 ± 1.3**	−89.4 ± 5.9**
R1193Q (n = 7)	4.7 ± 0.6	−76.8 ± 1.9	5.5 ± 0.8	−84.6 ± 2.3**
V1951L (n = 7)	5.1 ± 0.5	−77.9 ± 2.2	5.7 ± 0.3	−86.3 ± 1.2**

V_h, voltage in which half of the maximum channel availability is achieved.
**$P < 0.05$, comparing (−) AMIO vs. (+) AMIO.

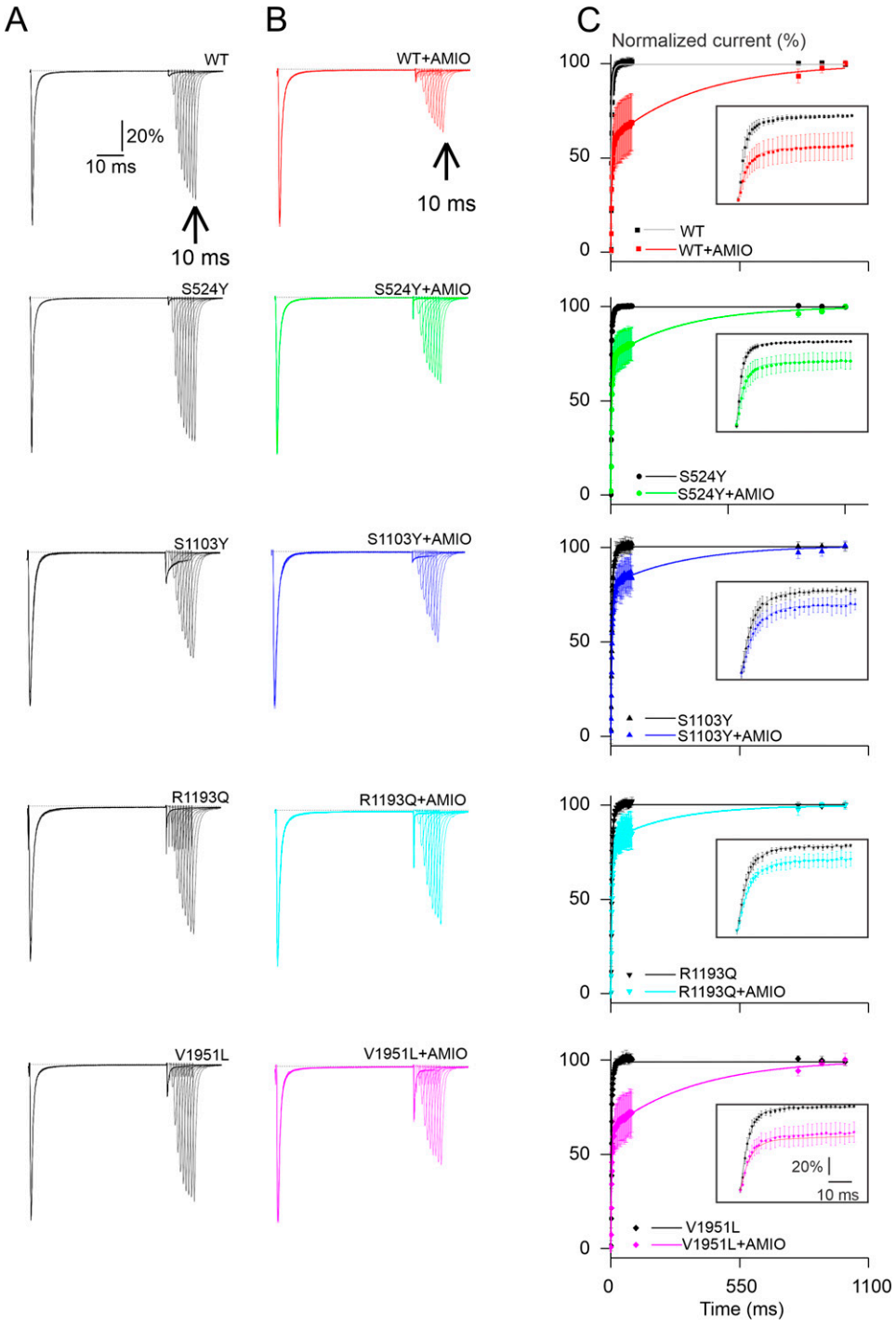

Fig. 5. Comparison of the recovery from inactivation of I_{Na} among $Na_v1.5$ ethnicity-related polymorphisms. (A) Representative traces of I_{Na} the recovery from inactivation before (black traces) and (B) after exposure to amiodarone (colored traces). Only the initial, fast component of the recovery is shown for better visualization. (C) Fractional recovery of I_{Na} from inactivation is plotted as a function of the interval between the conditioning and test pulses. The recovery is better fitted with a two-exponential function that implicates a fast and slow component of the current recovery. N = (WT: 11, S524Y: 7, S1103Y: 7, R1193Q: 7, V1951L: 7). Two-way ANOVA comparing WT and polymorphism, seen in the specific Table.

Combined, these data indicate I_{Na} blockage by AMIO is use-dependent, and overall studied polymorphisms displayed a similar behavior as observed for WT except for R1193Q variant.

Discussion

It is known that AMIO is a classic type III antiarrhythmic drug (Vaughan Williams, 1984) that was initially characterized as a blocker of rectifying K^+ currents, therefore prolonging the AP duration (Vaughan Williams, 1975) and the QT interval (Torres et al., 1986). The blockage of Na^+ channels can also decrease the probability of ectopic activity through the reduction of cardiomyocytes' excitability (Heger et al., 1981; Hamer et al., 1983; Heijman et al., 2017). Then the class I action of AMIO can also substantially contribute to its antiarrhythmic efficacy. Today, it is known that AMIO has a broader action on ion channels found in myocytes displaying antiarrhythmic properties of classes I, II, and IV (Heijman et al., 2017).

Here, we accessed the pharmacological potency of AMIO in blocking I_{Na} among naturally occurring $Na_v1.5$ ethnicity-

TABLE 3
Time constant (t_1) of I_{Na} recovery among $Na_v1.5$ ethnicity-related polymorphisms obtained using a two-exponential decay equation. Kolmogorov-Smirnov test, two-way ANOVA followed by Tukey's post-test.

	Time Constant	AMIO (−)	AMIO (+)
		Millisecond(s)	*Millisecond(s)*
WT (n = 11)	τ_1	1.91 ± 0.61	2.62 ± 0.74
S524Y (n = 7)	τ_1	1.53 ± 0.31	2.64 ± 1.46
S1103Y (n = 7)	τ_1	3.04 ± 0.86*	3.66 ± 1.27**
R1193Q (n = 7)	τ_1	3.12 ± 0.36*	3.49 ± 0.75**
V1951L (n = 7)	τ_1	2.21 ± 0.41	2.91 ± 1.41

*$P < 0.05$, comparing polymorphism AMIO (−) to WT AMIO (−).
**$P < 0.05$, comparing polymorphism AMIO (+) to WT AMIO (+).

TABLE 5
Initial quantities (A_1) relative to the fast component of I_{Na} recovery among $Na_v1.5$ ethnicity-related polymorphisms obtained using a two-exponential decay equation. Kolmogorov-Smirnov test, two-way ANOVA followed by Tukey's post-test.

		AMIO (−)	AMIO (+)
		%	%
WT (n = 11)	A1	0.78 ± 0.15	0.64 ± 0.16
S524Y (n = 7)	A1	0.71 ± 0.18	0.81 ± 0.08**
S1103Y (n = 7)	A1	0.68 ± 0.32	0.80 ± 0.08**
R1193Q (n = 7)	A1	0.67 ± 0.32	0.82 ± 0.05**
V1951L (n = 7)	A1	0.74 ± 0.17	0.71 ± 0.11

**$P < 0.05$, comparing polymorphism (+) to WT AMIO (+)

related polymorphisms. AMIO potency can be reduced up to 4× in variants when compared with WT. Taking as an example the African-selective polymorphisms S1103Y and S524Y, both displayed 3–4× less sensitivity to AMIO when compared with WT. This feature can become clinically relevant if such difference in the pharmacological potency prevents or at least significantly attenuates the AMIO class I activity, which may lower the efficacy of AMIO in treating ventricular or supraventricular arrhythmias in individuals harboring these polymorphisms. This also can be particularly important when considering the lower bioavailability and dosing of long-term oral treatments with AMIO (Hamilton et al., 2020). Following this rationale, some long-term AMIO antiarrhythmic effects are partially associated with the prevention of triggered mechanisms (Singh et al., 1984), and the blockage of I_{Na} is known to create stabilization of the membrane potential, as driven by the reduction of overall cell excitability (Shaw and Rudy, 1997a,b; Kahlig et al., 2014).

Next, we compared the electrophysiological phenotype of four common ethnicity-related $Na_v1.5$ polymorphisms and the effects of AMIO. Some biophysical aspects of the $Na_v1.5$ polymorphisms analyzed here have been investigated in other studies. Using Q1077 splice variants, previous studies reported no change in the V_a of V1951L (Tan et al., 2005), which is consistent with our data. The same work reported no change in the V_a of S524Y, a positive shift of S1103Y, and a negative shift of R1193Q. However, in our study, V_a from all variants was comparable to WT. Our data are consistent with other works both for the African-related S1103Y (Plant et al., 2006) and for the R1193Q (Abe et al., 2018). However, despite similar V_a of S1103Y and S524Y compared with WT, the slope factor was larger for the former and smaller for the latter when compared with WT. The slope of the Boltzmann curve represents the voltage sensitivity that can be converted to the gating charge (Hodgkin and Huxley, 1952; Spray et al., 1981), indicating that the voltage sensitivity can be modulated. The exposure to 10 μM AMIO did not change the voltage-dependent activation of WT and all studied polymorphisms. Interestingly, S1103Y has relatively high frequency in the African-descendent population, and it was linked to a proarrhythmic phenotype in the presence of antiarrhythmic drugs that lead to QT prolongation, like AMIO (Splawski et al., 2002). Although it is not clear which splice variant was used in the referred study, this phenomenon can be associated with a stronger voltage dependency for channel activation, which is in accordance with the increased voltage sensitivity observed in our study that is further accentuated by exposure to AMIO.

Moreover, the voltage dependency for stationary inactivation was not different among all variants compared with WT, according to previous findings (Plant et al., 2006; Shinlapawittayatorn et al., 2011; Abe et al., 2018), whereas it is not clear from those reports which splice variant was used. Tan et al. (2005), however, observed no differences in V_h of all polymorphisms described in Q1077 background, whereas S1103Y displayed a positive shift and R1193Q displayed a negative shift of V_h (using Q1077 background). It is important to recognize that biophysical properties of I_{Na} can sometimes vary depending on several experimental factors, including temperature, solutions, presence of β subunit, and pH (Plant et al., 2006; Abe et al., 2018). Nevertheless, in our experimental conditions, which were standardized for all variants, no significant differences were observed among the V_h of $Na_v1.5$ polymorphisms. Besides, exposure to AMIO displayed a significant negative shift in V_h of all variants compared with its respective value before exposure to the drug, although there was a comparable magnitude between all of them.

Block of I_{Na} by AMIO has long been recognized to be dependent upon binding primarily to the closed and inactivated

TABLE 4
Time constant (t_2) of I_{Na} recovery among $Na_v1.5$ ethnicity-related polymorphisms obtained using a two-exponential decay equation. Kolmogorov-Smirnov test, two-way ANOVA followed by Tukey's post-test.

	Time Constant	AMIO (−)	AMIO (+)
		Millisecond(s)	*Millisecond(s)*
WT (n = 11)	τ_2	9.57 ± 10.17	199.28 ± 59.07
S524Y (n = 7)	τ_2	6.85 ± 5.79	290.89 ± 86.68**
S1103Y (n = 7)	τ_2	19.16 ± 22.97	229.08 ± 66.23
R1193Q (n = 7)	τ_2	16.81 ± 22.06	156.98 ± 109.33
V1951L (n = 7)	τ_2	7.21 ± 7.29	270.61 ± 124.86

**$P < 0.05$, comparing polymorphism (+) AMIO to WT AMIO (+)

TABLE 6
Initial quantities (A_2) relative to the slow component of I_{Na} recovery among $Na_v1.5$ ethnicity-related polymorphisms obtained using a two-exponential decay equation. Kolmogorov-Smirnov test, two-way ANOVA followed by Tukey's post-test.

		AMIO (−)	AMIO (+)
		%	%
WT (n = 11)	A2	0.21 ± 0.15	0.35 ± 0.16
S524Y (n = 7)	A2	0.28 ± 0.18	0.18 ± 0.08**
S1103Y (n = 7)	A2	0.31 ± 0.32	0.19 ± 0.08**
R1193Q (n = 7)	A2	0.32 ± 0.32	0.17 ± 0.05**
V1951L (n = 7)	A2	0.25 ± 0.17	0.28 ± 0.11

**$P < 0.05$, comparing polymorphism (+) AMIO to WT AMIO (+).

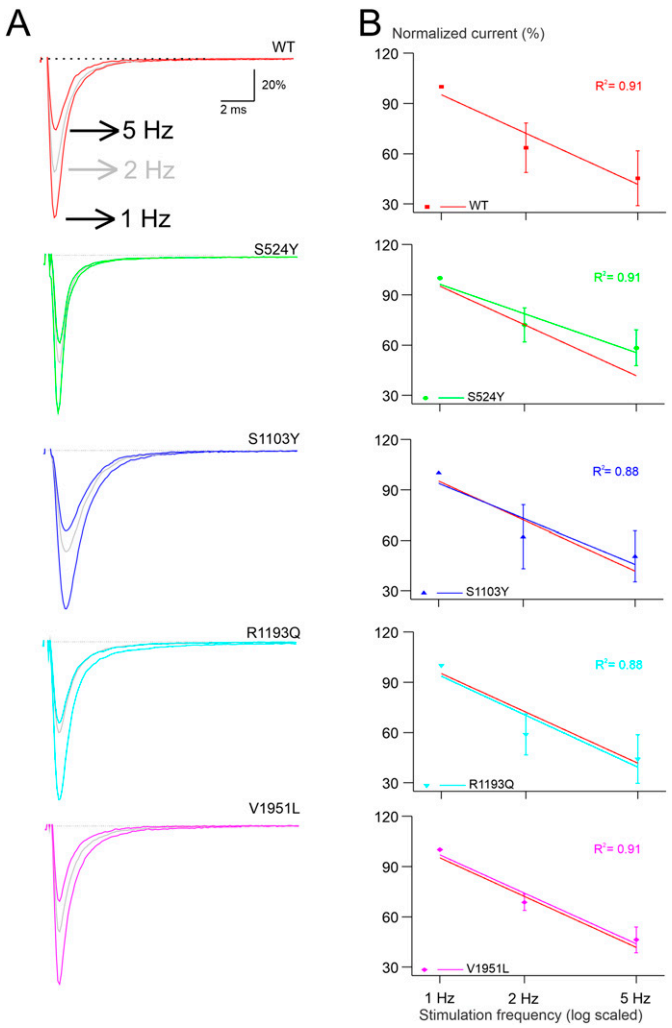

Fig. 6. Use-dependent block of I_{Na} by amiodarone in membrane holding potential at −120 mV. (A) Representative traces of I_{Na} recorded at 1, 2, and 5 Hz and normalized by 1 Hz. Holding potential was set to −120 mV (B) summarized data of the use-dependent block. Data were linearly regressed and the slope was used to compare the use-dependent effects of amiodarone between variants. N = (WT: 6, S524Y: 7, S1103Y: 8, R1193Q: 8, V1951L: 8). Two-way ANOVA comparing WT and polymorphism, seen in the specific Table.

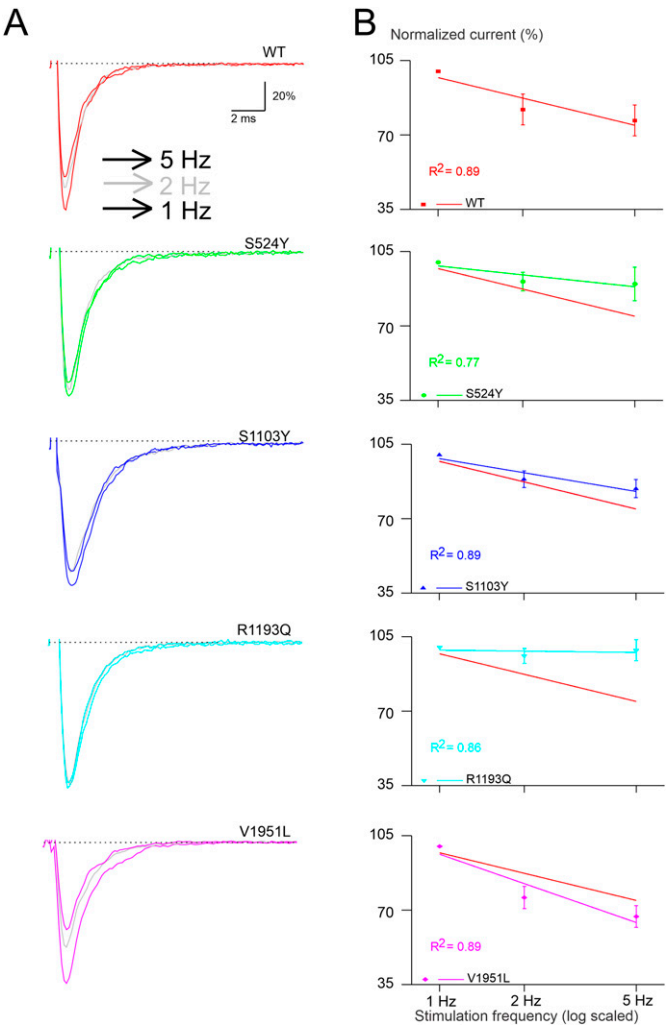

Fig. 7. Use-dependent block of I_{Na} by amiodarone in membrane holding potential at −80 mV. (A) Representative traces of I_{Na} recorded at 1, 2, and 5 Hz and normalized by 1 Hz. Holding potential was set to −80 mV (B) summarized data of the use-dependent block. Data were linearly regressed and the slope was used to compare use-dependent effects of amiodarone between variants. N = (WT: 6, S524Y: 7, S1103Y: 6, R1193Q: 7, V1951L: 6). Two-way ANOVA comparing WT and polymorphism, seen in the specific Table.

states of the channel (Follmer et al., 1981). This is consistent with an increased time to recovery from inactivation, as reported here. Among polymorphisms, the African-related S1103Y and the Asian-related R1193Q displayed slower kinetics for the fast component of the recovery from inactivation compared with WT. This observation is discrepant from previous reports in which no difference was observed for both variants (Tan et al., 2005). Nevertheless, the S1103Y and R1193Q polymorphisms are located in the DII-DIII linker, and this region of $Na_v1.5$ has been implicated in the channel inactivation (Camacho et al., 2006). Also, slower kinetics of the fast component for recovery from inactivation were

TABLE 7

Linear regression of frequency-dependent block of I_{Na} among $Na_v1.5$ ethnicity-related polymorphisms, at −120 mV holding potential. Kolmogorov-Smirnov test, Student's *t* test.

	Pearson	R2	slope
WT (n = 6)	−0.95 ± 0.04	0.91 ± 0.09	−0.76 ± 0.23
S524Y (n = 7)	−0.95 ± 0.03	0.91 ± 0.05	−0.58 ± 0.15
S1103Y (n = 8)	−0.93 ± 0.06	0.88 ± 0.10	−0.68 ± 0.21
R1193Q (n = 8)	−0.93 ± 0.03	0.88 ± 0.06	−0.77 ± 0.21
V1951L (n = 8)	−0.97 ± 0.16	0.91 ± 0.06	−0.75 ± 0.11

$P > 0.05$ comparing to WT.

TABLE 8

Linear regression of frequency-dependent block of I_{Na} among $Na_v1.5$ ethnicity-related polymorphisms, at −80 mV holding potential. Kolmogorov-Smirnov test, Student's *t* test.

	Pearson	R2	Slope
WT (n = 6)	−0.91 ± 0.08	0.84 ± 0.15	−0.32 ± 0.24
S524Y (n = 7)	−0.30 ± 0.82	0.67 ± 0.32	−0.14 ± 0.30
S1103Y (n = 6)	−0.79 ± 0.17	0.65 ± 0.27	−0.22 ± 0.14
R1193Q (n = 7)	0.22 ± 0.91*	0.76 ± 0.27	−0.015 ± 0.18*
V1951L (n = 8)	−0.91 ± 0.11	0.84 ± 0.19	−0.45 ± 0.17

*$P < 0.05$, comparing to WT.

maintained after exposure to AMIO. On the other hand, AMIO leads to a substantial increase in the time constant for the slow component of the inactivation recovery in all variants, which predictably contributes to the marked use dependency that is typical for this drug. Besides, the magnitude of increasing in the slow time constant is comparable between WT and all variants except for S524Y, which displayed a significant increase compared with the other variants exposed to AMIO.

AMIO also interfered with the fraction of the current (and probably the channel) that undergoes a fast and slow recovery from inactivation. S524Y, S1103Y, and R1193Q showed a higher fraction of the current that recovers by fast kinetics. This effect could contribute to the less use-dependent block observed for R1193Q, particularly at more depolarized holding potential. Besides, it can strongly contribute to the reduced pharmacological potency of AMIO that is observed when compared with WT. Interestingly, in a previous study using Nav1.4 it was shown that DIV-S6 has a pivotal role in the ability of lidocaine to modulate slow recovery from sodium channel inactivation (Gawali et al., 2015).

AMIO has a marked use dependency that happens with a combination of the stimulation frequency and the duration of the depolarizing pulses (Follmer et al., 1987; Kodama et al., 1997). The use dependence of class I antiarrhythmic drug is a classic phenomenon providing important implications to the treatment of arrhythmic diseases. Although the use dependency of all variants was comparable to WT at more negative potentials, one variant was different when membrane holding potential was set at −80 mV. R1193Q, an Asian-related polymorphism, displayed almost no frequency dependency. Differences in membrane holding (or diastolic) potential are found across the human myocardium, as expressed, for example, by the more depolarized environment of the atria compared with ventricles (Li et al., 2002). Such contrast could lead to a heterogeneous profile of AMIO action. Importantly, it could interfere with AMIO effects on treating supraventricular arrhythmias, especially reentrant tachyarrhythmia or electrical disturbances associated with atrial ectopic focus, including atrial flutter and fibrillation. As discussed above, ectopic activity is more dependent on membrane excitability and, therefore, on the I_{Na} (Heger et al., 1981; Hamer et al., 1983; Heijman et al., 2017). The clinical relevance of more accurate knowledge and prescription of AMIO to treat supraventricular arrhythmias becomes more evident with the understanding that AMIO is already used to treat atrial fibrillation and other supraventricular arrhythmias (Hamilton et al., 2020). Besides, other studies report a relatively higher efficiency of AMIO to treat atrial fibrillation when compared with other antiarrhythmic drugs (Vitolo et al., 1981; Kochiadakis et al., 1998), whereas the efficiency of AMIO to restore an acute onset of atrial fibrillation can vary greatly (35%–65%) (Chevalier et al., 2003; Hamilton et al., 2020). However, additional factors probably contribute to the atrial AMIO selectivity. For example, the slower repolarization phase of atrial action potential leads to failure of the AP to restore maximum resting potential at rapid pacing rates. Also, the slower repolarization further contributes to significantly shortening of the diastolic interval in atria but not ventricles, thus reducing the rate of dissociation of sodium blockers from the channel.

Taken together, our data indicate that AMIO has reduced pharmacological potency among the ethnicity-related $Na_v1.5$ polymorphisms when compared with WT, which is consistent with specific modulation of the variant's biophysical properties. In addition, R1193Q displayed a reduction in the use dependency induced by AMIO at relative depolarization of cell membrane, comparing cells at −120 mV and −80 mV. We understand that our work may guide future studies focusing on identifying the efficiency of AMIO in treating different types of arrhythmias as well as establishing more accurate and specific guidelines for its use depending on the $Na_v1.5$ polymorphisms. However, proof-of-concept studies using in vivo experiments are still necessary.

Authorship Contributions

Participated in research design: Joviano-Santos, Santos-Miranda, Roman-Campos.

Conducted experiments: Joviano-Santos, Santos-Miranda, Sarmento.

Contributed new reagents or analytic tools: Roman-Campos.

Performed data analysis: Joviano-Santos, Santos-Miranda, Sarmento, Roman-Campos.

Wrote or contributed to the writing of the manuscript: Joviano-Santos, Santos-Miranda, Sarmento, Roman-Campos.

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Manuscript title: Ethnic-related SCN5A polymorphisms shape the *in vitro* pharmacological action of amiodarone upon Nav1.5

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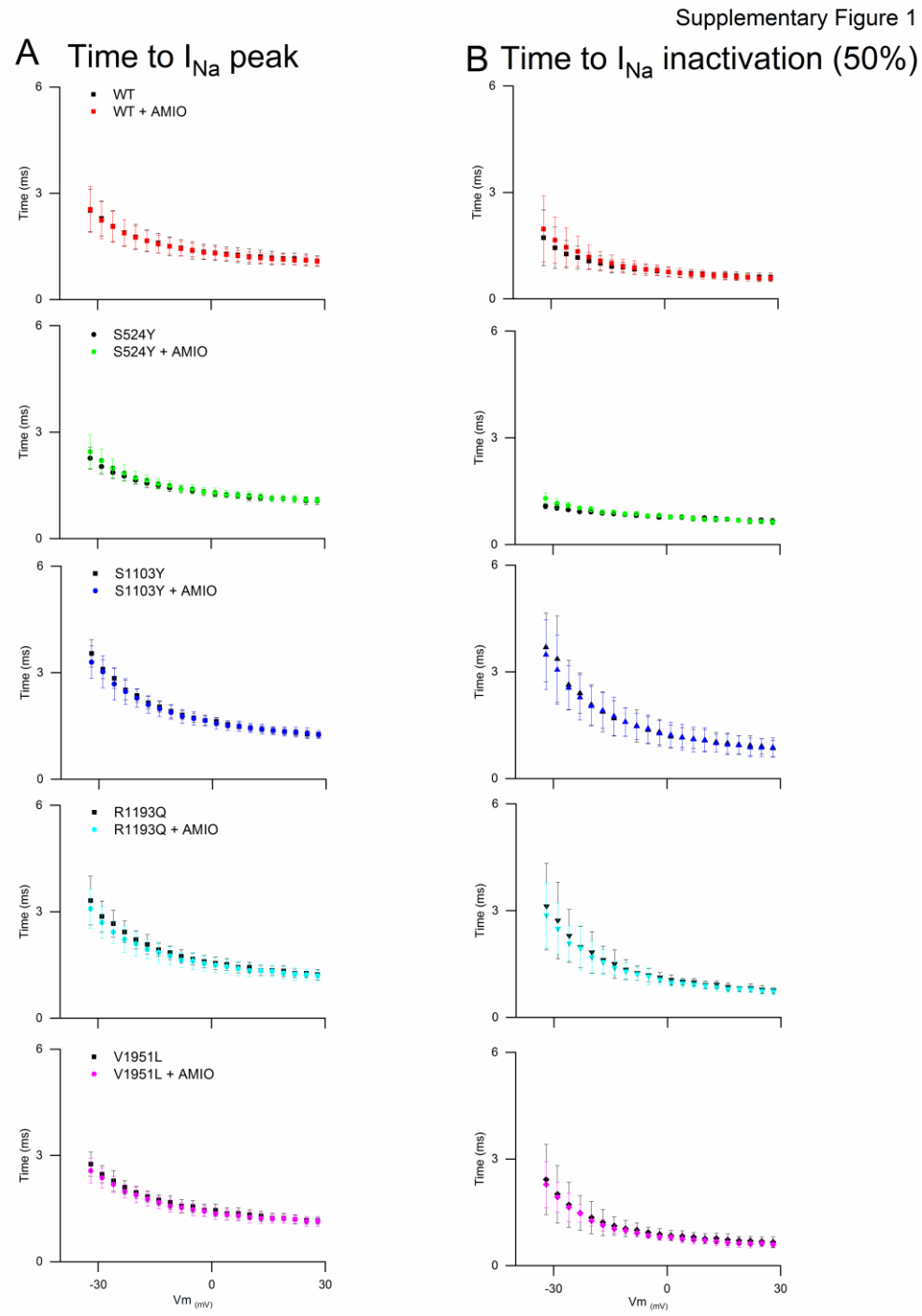
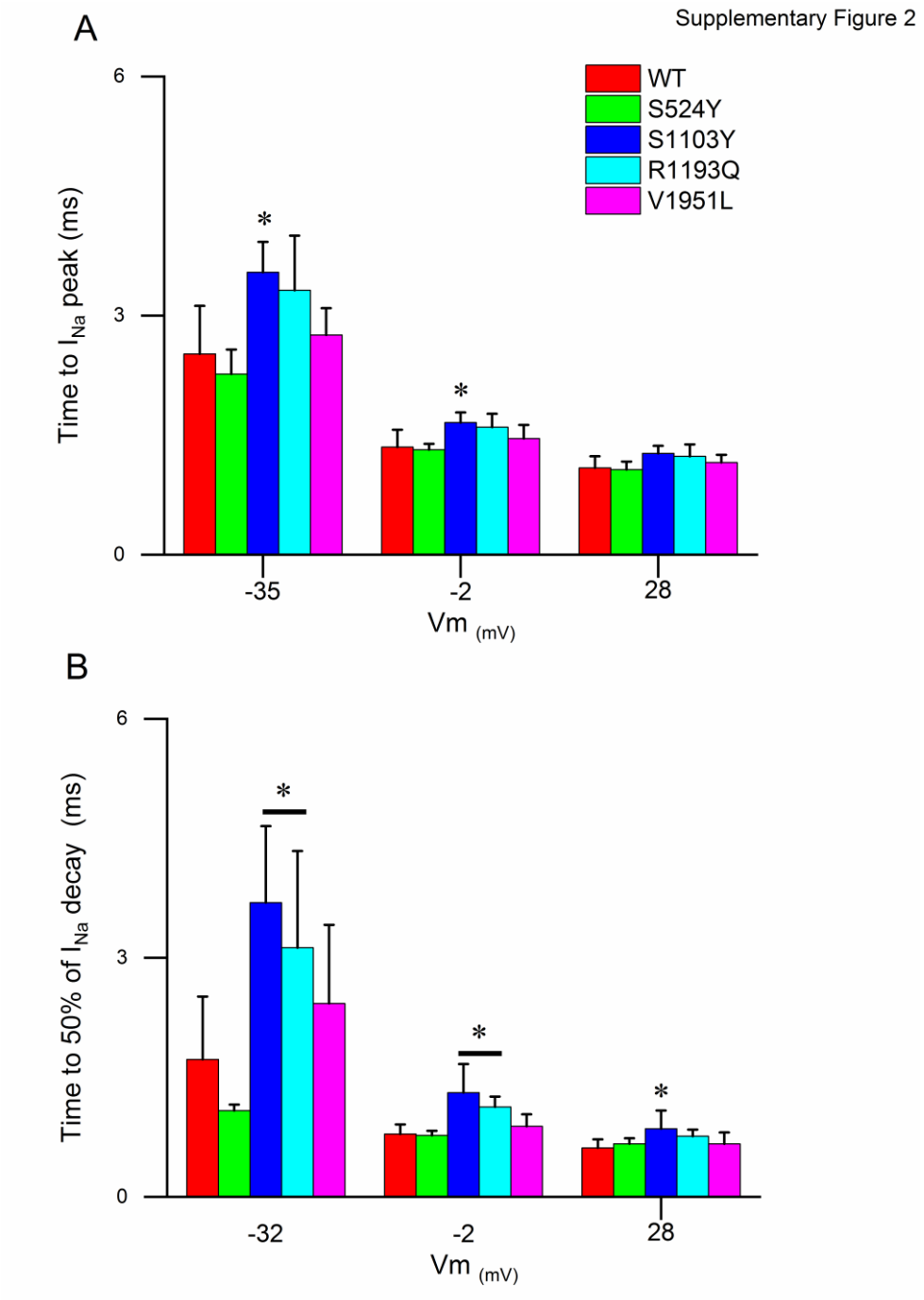
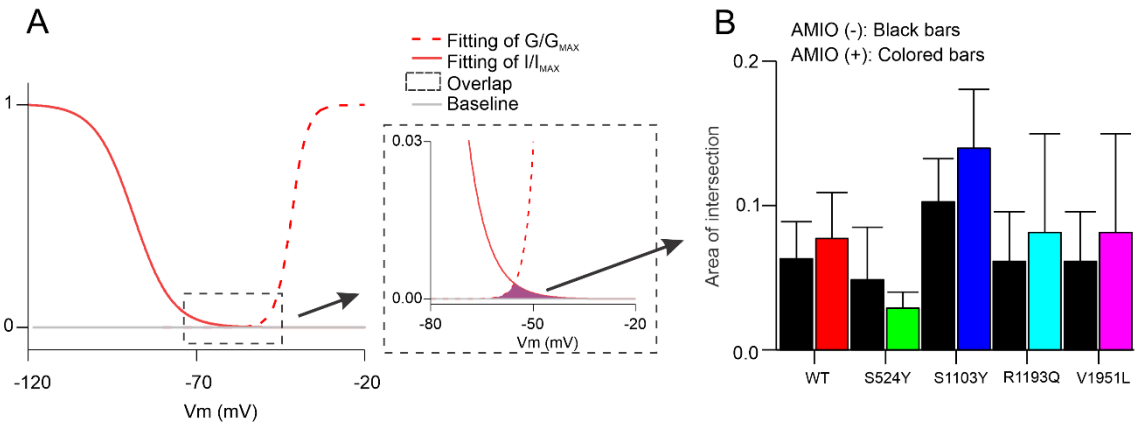


Supplementary Figure 1: Kinetics of I_{Na} and impact of AMIO. (A) Time to Na^+ current peak in tested membrane potentials. (B) Time to 50% of peak Na^+ current decay in tested membrane potentials. Parameters were evaluated in WT and studied polymorphisms. N = (WT - 10, S524Y - 6, S1103Y - 7, R1193Q - 6, V1951L - 7). Two-way ANOVA comparing WT and polymorphism.



Supplementary Figure 2: Kinetics of I_{Na} in WT and polymorphisms. (A) Time to Na^+ current peak in tested membrane potentials. (B) Time to 50% of peak Na^+ current decay in tested membrane potentials. N = (WT - 10, S524Y - 6, S1103Y - 7, R1193Q - 6, V1951L - 7). One-Way ANOVA comparing WT and polymorphism for each membrane potential. *<0.05.

Supplementary Figure 3



Supplementary Figure 3: Window I_{Na}. (A) Superimposed fit of steady-state inactivation (I/I_{MAX}) and activation (G/G_{MAX}) curves for WT I_{Na}. The overlap between curve fittings is zoomed as indicated by the dashed box. Filled area in violet is calculated window I_{Na}, measured as the area of intersection between I/I_{MAX} and G/G_{MAX} fittings. (B) Mean window I_{Na} area for studied groups. N = (WT - 10, S524Y - 6, S1103Y - 5, R1193Q - 6, V1951L – 7). Two-way ANOVA followed by Tukey's post-test comparing WT to each polymorphism, (-) Amiodarone and (+) Amiodarone.