Potency of Late-Nav1.5 Current Inhibition Depends on the Agonist Used to Augment It

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1 ABSTRACT

Late Nav1.5 current is an important component of the Comprehensive in vitro Proarrhythmia Assay (CiPA) in-silico model because block of Late Nav1.5 can offset hERG current inhibition thus preventing QTc prolongation and torsade de pointe. The magnitude of Late Nav1.5 current is small so different agonists have been used to increase the current in heterologous systems. We determined whether two late Nav1.5 agonists (veratridine (30 μM) vs. ATX-II (100 nM)) affected the blocking potencies of the pro- and non-arrhythmic compounds – amitriptyline (AMI), cisapride (CIS), domperidone (DOM), lidocaine (LID), nifedipine (NIF), ranolazine (RAN), risperidone (RIS), terfenadine (TER) and verapamil (VER). Experiments were performed using the IonWorks BarracudaTM automated patch-clamp (APC) system at ambient temperature. Currents were elicited using a step-ramp voltage protocol from a holding potential of -80 mV (step 1: 0 mV, 40 ms, step 2: 40 mV, 200 ms, ramp: 1.2 V/s , 100 ms). Late current was measured at the end of step 1 and during the ramp. Compound effects were dependent on the agonist used: 1) ATX II activated late Nav1.5 currents were 3 to 20 times more sensitive than veratridine activated currents. 2) The rank potency was also different: ATX II: AMI>(CIS, DOM)>(VER, TER)>(RAN,NIF)>LID>RIS, Veratridine: AMI>TER,>(CIS,NIF,VER,DOM)>RIS>RAN>LID. 3) ATX II activated peak currents were less sensitive than late ATX II activated currents by a factor of 1.2-14 whereas block of peak and veratridine modified currents was comparable. The notably different responses of both Late Nav1.5 activated currents are underscored by the 15 times different block potency measured for RAN (ATX II, 6 μM; veratridine, 91 μM). This difference was confirmed using the QPatch APC system (ATX II, 12 μM; veratridine, 57 μM). This work shows that there are significant differences between these two late Nav1.5 agonists. Based on the RAN results, ATX-II is the most appropriate agonist as it supports the clinical evidence that RAN has low pro-arrhythmic liability even though it shows comparable hERG block potency.

2 METHODS

Cell Lines: Cell lines stably expressing human Nav1.5 (Nav1.5-HEK293; SCN5A; Charles River Laboratories (Wilmington, MA); Catalog # CT6207) and Nav1.5 (Nav1.5-CHO; SCN5A; Charles River Laboratories (Wilmington, MA); Catalog #CT6007) ion channels were constructed[1]. The cells were maintained in 100-mm cell culture dishes in Ham's F-12/DMEM HEK293 media supplemented with 10% fetal bovine serum, 100 U/mL of penicillin G sodium, 100 mg/mL of streptomycin sulfate, and the appropriate selection antibiotics. Prior to experiments, the cells were passed in a medium free of selection antibiotics. Cells were harvested by washing twice with 15–20 mL of Hank's Balanced Salt Solution (HBSS) and treatment with Accutase solution. Detached cells were washed with HBSS and resuspended in HEPES-buffered physiological saline (HB-PS): 137 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH adjusted to 7.4 with NaOH, and osmolarity adjusted to 295 ± 5 mOsm. The final cells dilution was 1x10^6 cells per mL, approximately.

Solutions and Electrophysiological Procedures: Stock solutions of test articles were prepared in dimethyl sulfoxide (DMSO) and stored frozen. Each test article formulation was sonicated to facilitate dissolution. For experiments test article concentrations were prepared fresh daily by diluting stock solutions into extracellular solutions (HB-PS buffer) supplemented with 3 mM $CaCl_2$. The final solution composition for Nav1.5 channel was 137 mM NaCl, 4 mM KCl, 4.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10mM glucose, pH adjusted to 7.4 with NaOH. All test and control solutions contained 0.3% DMSO. The test article formulations were prepared in 384-well compound plates using the Cyclone automated liquid handling system. The internal HEPES-buffered solution consisted of 90 mM CsF, 50 mM CsCl, 5 mM MgCl2, 2.5 mM EGTA, and 10 mM HEPES, pH 7.2 adjusted with CsOH. A stock solution of Escin (perforating agent) was prepared in DMSO (14 mg/mL) and added to the internal solution at a final concentration of 14 μg/mL. Recordings were performed on IonWorks Barracuda™ system in Population Patch-Clamp™ (PPC) mode[2].

Test Article Administration and Test Procedures: Test article concentrations were applied to naïve cells (4 wells/concentration; one concentration per well). Each application consisted of addition of 20 μL of 2X concentrated test article solution to the total 40 μL of final volume of the extracellular well of the PPC planar electrode. Duration of exposure to each test article concentration was five (5) minutes. Onset and steady state block of Nav1.5 current was measured using a pulse pattern, repeated every 5 sec, consisting of depolarization to 0 mV amplitude for a 40 ms duration, followed by step to 40 mV for 200 ms and, finally, a 100 ms ramp (1.3 V/s) to a holding potential of -90 mV. The peak current was measured during the step to 0 mV. The late Nav1.5 current was measured using the same voltage protocol and all external solutions contained ATX-II or veratridine in order to activate the late current. The Nav1.5 late current was measured at 0 mV potential during the ramp. All data were uniformly corrected for run-down: %Block' = 100%- ((%Block - %PC)*(100% / (%VC - %PC)), where %VC and %PC are the mean values of the current inhibition with the vehicle and the positive control (Lidocaine, 3 mM), respectively. Individual well data were filtered according to electrical criteria and the experiments were accepted based on plate level acceptance criteria.

Well Acceptance Criteria: Seal Resistance (baseline): Rseal ≥ 500 MΩ; Leak current ≤ 25% peak current; Current amplitude (baseline): inward peak current ≥ 0.5 nA. Plate Acceptance Criteria: Z' factor (assay sensitivity) ≥ 0.5. Z' factor for each experiment was calculated as: Z' = 1 – ((3x SDVC + 3x SDPC)/ABS (MeanVC – MeanPC), Where MeanVC and SDVC were the Mean and Standard Deviation values for a vehicle control, MeanPC and SDPC were the Mean and Standard Deviation values for a positive control (3 mM Lidocaine).

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3 RESULTS

1) VERATRIDINE AND ATX-II MODIFIED CURRENTS SHOW DISTINCT KINETIC PROFILES

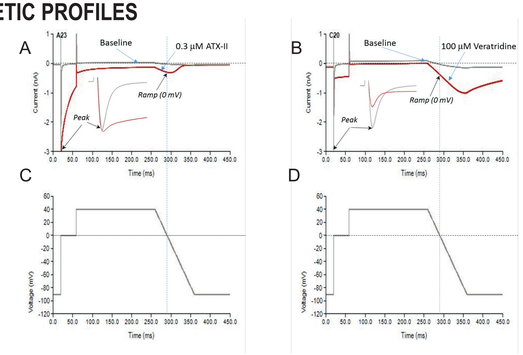


Figure 1. Nav1.5 current traces (6 traces before (baseline) and 6 min after stimulation) in the presence of either ATX-II (Panel A) or veratridine (Panel B). Current was elicited using the step-ramp voltage protocols repeated at a 0.1 Hz frequency (Panels C and D). 'Ramp' current was measured at 0 mV potential in order to minimize the contribution of leak.

2) ATX-II MODIFIED PEAK AND LATE CURRENTS SHOW STABLE TIME COURSES

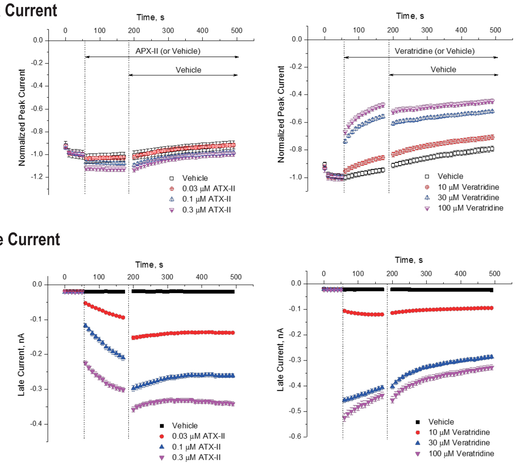
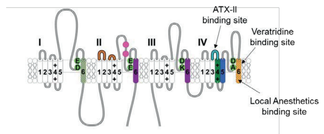


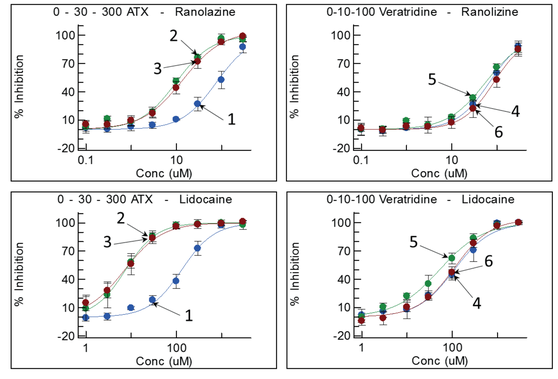
Figure 2. Time-course of Peak and Late Nav1.5 Current Activated by ATX-II and veratridine.
Nav1.5-HEK293; Protocol as in Figure 1, 0.1 Hz stimulation. Peak and Ramp (Currents were measured at 0 mV). Mean +- SEM, N=28-32

3) RANOLAZINE AND LIDOCAINE POTENCIES FOR ATX-II AND VERATRIDINE ACTIVATED LATE CURRENT BLOCK ARE INDEPENDENT OF THE AGONIST CONCENTRATION



Modified from Cestele S, Catterall WA. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. Biochimie 2000; 82:883 92

A



1 – 0 ATX-II, Peak
2 – 0.03 ATX-II, RAMP
3 – 0.3 ATX-II, RAMP

4 – 0 Veratridine, Peak
5 – 10 Veratridine, RAMP
6 – 100 Veratridine, RAMP

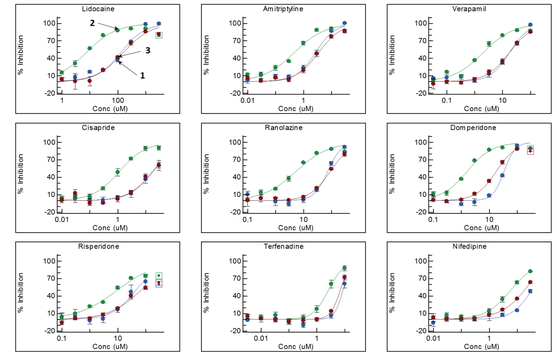
B

Compound	IC_{50} (μM)						
	Vehicle	0.03 μM ATX-II	0.1 μM ATX-II	0.3 μM ATX-II	10 μM Veratr.	30 μM Veratr.	100 μM Veratr.
	Peak	Ramp	Ramp	Ramp	Ramp	Ramp	Ramp
Ranolazine	77.07	9.85	10.50	12.10			
Lidocaine	130.51	6.94	7.79	7.13			
Ranolazine	68.12				56.23	76.24	85.70
Lidocaine	110.89				52.51	103.33	101.08

Figure 4. Veratridine and local anesthetic binding sites are in close proximity on S6 (Panel A). The Concentration-Response Relationship of Lidocaine and Ranolazine on Late Nav1.5 Current Elicited by Different Concentrations of ATX-II and veratridine is shown in Panel A and the IC_{50} values are listed in the table in Panel B.

4) LATE ATX-II ACTIVATED CURRENTS ARE MORE SENSITIVE TO CHANNEL BLOCK AND SHOW GATING DEPENDENCE

A



1 – Vehicle, Peak; 2 – 0.1 μM ATX-II, RAMP; 3 – 30 μM veratridine, RAMP

B

Compound	IC_{50} (μM)		
	Vehicle	0.1 μM ATX-II	30 μM Veratridine
	Peak	Ramp	Ramp
Lidocaine	128.22	7.42	148.00
Amitriptyline	2.71	0.47	3.48
Verapamil	17.58	2.03	17.67
Cisapride	18.63	1.14	17.41
Ranolazine	73.04	5.36	90.82
Domperidone	33.48	1.60	17.69
Risperidone	44.56	9.59	67.59
Terfenadine	8.58	2.60	6.88
Nifedipine	33.29	5.92	17.55

C

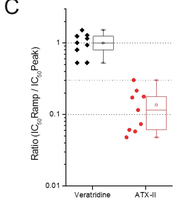


Figure 5. A) Concentration-response relationship of compounds on ATX-II and veratridine activated peak and late Nav1.5 currents. B) Table showing IC_{50} values obtained in ATX-II and veratridine. Note that the block of veratridine activated peak and late currents is comparable C) Plots the ratio of the IC_{50} measured during the ramp divided by the IC_{50} measured at the peak. The boxes indicated the 25, 50 and 75 percentile; whiskers indicate the 10 and 90 percentiles, and filled diamond mean values.

4 CONCLUSIONS

- Effects on Late Nav1.5 current can be successfully measured in the IonWorks Barracuda™ high throughput patch system.
- Veratridine and ATX-II do not compete for the lidocaine and ranolazine binding sites.
- ATX II activated late Nav1.5 currents were 3 to 20 times more sensitive than veratridine activated currents.
- The ranked potencies for ATX-II activated late current block are AMI>(CIS, DOM)>(VER, TER)>(RAN,NIF)>LID>RIS.
- The ranked potencies for veratridine activated late current block are AMI>TER, > (CIS,NIF,VER,DOM) > RIS>RAN>LID.
- Peak currents were less sensitive than ATX II activated late currents by a factor of 3.2-14 whereas block of peak and veratridine modified currents was comparable.