RESEARCH ARTICLE

Late cardiac sodium current can be assessed using automated patch-clamp [v1; ref status: indexed, http://f1000r.es/4kj]

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Abstract

The cardiac late Na^+ current is generated by a small fraction of voltage-dependent Na^+ channels that undergo a conformational change to a burst-gating mode, with repeated openings and closures during the action potential (AP) plateau. Its magnitude can be augmented by inactivation-defective mutations, myocardial ischemia, or prolonged exposure to chemical compounds leading to drug-induced (di)-long QT syndrome, and results in an increased susceptibility to cardiac arrhythmias. Using CytoPatch™ 2 automated patch-clamp equipment, we performed whole-cell recordings in HEK293 cells stably expressing human Nav1.5, and measured the late Na^+ component as average current over the last 100 ms of 300 ms depolarizing pulses to -10 mV from a holding potential of -100 mV, with a repetition frequency of 0.33 Hz. Averaged values in different steady-state experimental conditions were further corrected by the subtraction of current average during the application of tetrodotoxin (TTX) 30 µM. We show that ranolazine at 10 and 30 µM in 3 min applications reduced the late Na^+ current to 75.0 ± 2.7% (mean ± SEM, $n = 17$) and 58.4 ± 3.5% ($n = 18$) of initial levels, respectively, while a 5 min application of veratridine 1 µM resulted in a reversible current increase to 269.1 ± 16.1% ($n = 28$) of initial values. Using fluctuation analysis, we observed that ranolazine 30 µM decreased mean open probability p from 0.6 to 0.38 without modifying the number of active channels n, while veratridine 1 µM increased n 2.5-fold without changing p. In human iPSC-derived cardiomyocytes, veratridine 1 µM reversibly increased APD90 2.12 ± 0.41-fold (mean ± SEM, $n = 6$). This effect is attributable to inactivation removal in Nav1.5 channels, since significant inhibitory effects on hERG current were detected at higher concentrations in hERG-expressing HEK293 cells, with a 28.9 ± 6.0% inhibition (mean ± SD, $n = 10$) with 50 µM veratridine.

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Introduction

The late cardiac Na$^+$ current can be recorded 10–100 milliseconds after membrane depolarization as a sustained inward current component (I_{sus}) in cardiomyocytes[1]. This late current represents a fraction of voltage-dependent Na$^+$ channels that fail to inactivate after the initial opening. Instead, these channels change to a conformation with frequent late re-openings in a so-called burst mode[2]. Among other mechanisms, calmodulin kinase II overexpression in chronic heart failure increases the rate of transition to the late bursting mode conformation[2]. The phenomenon is also observed in Na$_v$1.5 inactivation-deficient mutants, such as ΔKPQ[3] or 1795InsD[4,5], leading to a specific form of congenital long QT syndrome, LQT-3[6–8].

Given its role in cardiac arrhythmogenesis[9,10], pharmacological inhibition of the late Na$^+$ current component ($I_{Na\ late}$) is seen as an anti-arrhythmic strategy. Novel late sodium channel blockers, such as the partial fatty acid beta-oxidation inhibitor, ranolazine[11–15], are used for both myocardial ischemia and to alleviate neuropathic pain and show a preferential affinity for the burst mode conformation of Na$^+$ channels[16–21]. Other compounds, such as veratridine, a steroid-derived alkaloid extracted from rhizomes of *Veratrum album* or seeds of *Schoenocaulon officinale*[22,23], preferentially bind to activated Na$^+$ channels, impeding inactivation and leading to increased nerve excitability.

The objectives of this study were to (1) record the veratridine-dependent increase in the late Na$^+$ current using a HEK293 cell line stably transfected with human Na$_v$1.5[24], (2) measure the ranolazine-induced inhibition of basal and veratridine-activated $I_{Na\ late}$, and (3) asses the effects of veratridine at the same concentrations on action potentials (APs) in hiPSC-cardiomyocyte preparations externally paced in current-clamp mode. In parallel we also tested the inhibitory effects of veratridine on hERG1 current in stably transfected HEK293 cells. All these experiments were performed using CytoPatch™2 automated patch-clamp equipment.

Materials and methods

All experiments were performed using the CytoPatch™2, using standard dual-channel Cytocentrics chips with embedded quartz pipette tips 2 μM in diameter. For whole-cell late Na$^+$ current recordings, the voltage-clamp protocol consisted of repeated depolarizing pulses of -10 mV amplitude and 300 ms duration, from a holding potential of -100 mV, to allow a substantial removal from inactivation of Na$_v$1.5 channels. The peak and late Na$^+$ current were plotted and monitored over the entire duration of experiment, and extracted from recorded data for further analysis. Pharmacological compounds were applied in a predefined sequence using the dispensing needle of automated equipment. All experiments were performed at room temperature (21–22°C).

For whole-cell hERG current recordings, cells were held at -70 mV. After a brief 100-ms prepulse to -50 mV to determine the current leak, a 2-s depolarizing voltage step to +40 mV was followed by a 2-s step to -50 mV to elicit hERG tail currents every 10 s. Peak tail current amplitude was corrected by the leak current and the corrected peak tail current was averaged over the last three pulses of the control phase (I_{ctrl}) and the application phase (I_{cpd}), respectively.

From these averaged values the hERG tail current inhibition was calculated as follows:

$$\text{Inhibition} = 1 - (I_{cpd}/I_{ctrl})$$

iPSC-CM recordings were performed in current-clamp mode, using repeated sweeps consisting of 3 injected current pulses of 2000 pA amplitude, 0.5 ms duration, at 3-s intervals. During solution uptake the system was switched to voltage-clamp mode.

Cell cultures and preparations

HEK293 cells stably expressing either the human Na$_v$1.5 channel or the hERG K$^+$ channel were used as ready-to-use frozen Instant cells (product of Cytocentrics). Cells, kept in liquid nitrogen, were quickly thawed, centrifuged, resuspended in extracellular solution at a density of ~10^6 cells/ml, and used for experiments within 3 hours. For other experiments, Nav1.5-expressing cells were cultured in 25 cm^2 flasks in DMEM supplemented with 10% fetal bovine serum and 100 μg/ml zeozin, and kept at 37°C, 8% CO$_2$ in a humidified incubator. Adherent cell monolayers were detached with Versene (ethylenediaminetetraacetic acid – EDTA 0.02% in phosphate buffered saline - PBS), centrifuged for 2 min at 100 × g, and resuspended in hERG external solution.

hiPSC-derived iCell® Cardiomyocytes were kindly provided by Cellular Dynamics International (Madison, WI) as frozen samples, and cultured in monolayers in 12-well plates coated with 0.1% gelatin, for up to 41 days. The thawing/plating and maintenance media were provided by the cell supplier. For detachment, the monolayers were rinsed twice with calcium and magnesium-free PBS, then incubated for 2 min with trypsin 0.1% at 37°C. 1.5 ml of medium was added per well, the cells were suspended, centrifuged at 180 × g for 5 min, and resuspended in a 1:1 mixture of culture medium and hERG extracellular solution.

Solutions and chemicals

For recordings in Na$_v$1.5-transfected HEK293 cells the external solution had the following composition (in mM): NaCl 130, CsCl 5, CaCl$_2$ 2, MgCl$_2$ 1.2, HEPES 10, D-glucose 5, pH 7.4, osmolality 320 mOsm/kg. The internal solution contained (in mM): Cs-aspartate 70, CsCl 60, CaCl$_2$ 1, MgCl$_2$ 1, Na$_2$ATP 5, EGTA 11, HEPES 10, pH 7.2 with CsOH, osmolality 290 mOsm/kg. For recordings in iPSC-derived cardiomyocytes and hERG-transfected HEK293 cells the extracellular solution had the following composition (in mM): 140 NaCl, 2.5 KCl, 2 MgCl$_2$, 2 CaCl$_2$, 10 HEPES, 10 Glucose, 15 Sucrose. The pH was adjusted to 7.4 with NaOH 1M and the osmolality to 320 (± 5) mOsmol/kg with sucrose 1M, and the storage temperature was 4°C. The intracellular solution contained (in mM): 100 K Gluconate, 20 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 11 EGTA-KOH, 4 ATP-Mg^{2+}, 3 Phosphocreatine-Na$_2$-H$_2$O, 9 Sucrose. The pH was adjusted to 7.2 with KOH 1M, the osmolality to 295 (± 5) mOsmol/l, and then it was stored in 10-ml aliquots at -20°C, thawed and used within 4 hours. Veratridine (Sigma V5754) working solutions at 1, 5 and 50 μM were prepared from a 50 mM stock solution in ethanol. Ranolazine dihydrochloride (Sigma R6152) 10 and 30 μM was prepared from a 10 mM stock solution in DMSO. TTX (BN0518, Biotrend, Zurich, CH) was prepared from a 1 mM aqueous stock solution.

Data storage and analysis

The software files generated during the recordings were stored on computer hard disks. Patch clamp data were analyzed using the CytoPatch™ software and exported to Microsoft Excel or pClamp10 (Axon Instruments, part of Molecular Devices, Sunnyvale, CA) for further analysis, using a proprietary conversion tool. APD90 analysis for current-clamp recordings in iPSC-derived cardiomyocytes was performed with self-written software routines. APD90 was computed as the duration between the point of maximal AP upstroke speed during phase 0 to recovery of 90% of the difference between peak upstroke potential and resting potential. In the case of veratridine application, if recovery was incomplete during the 3-s interstimulus interval, APD90 was computed over several pacing cycles. For statistical analysis, one-way ANOVA for independent samples with Dunnett's post-hoc comparison, as well as Student's t tests where appropriate, were applied, at a level of significance of $p \leq 0.05$, using the GraphPad Prism software (La Jolla, CA).

Results

1. Late Nav1.5 sodium current component

Using automated voltage-clamp protocols applied to human Nav1.5-expressing HEK293 cells in the above mentioned conditions, we routinely recorded, in high-quality seal conditions (both seal and membrane resistance > 1 GΩ), and with stability for at least 20 min, whole-cell Na$^+$ currents. These currents were elicited by membrane depolarization to -10 mV from a holding potential of -100 mV, required for the proper removal from inactivation of cardiac Na$^+$ channels. The late Na$^+$ current component ($I_{\text{Na late}}$) was automatically computed and plotted as time average over the last 100 ms of the 300-ms depolarizing pulse of each sweep. Figure 1A shows the time course of 5 averaged experiments including repeated applications of veratridine 1 μM with different durations (2 min, 1 min, 5 min), as well as a 1-min application of TTX 30 μM, resulting in the rapid complete block of late Na$^+$ current. The average current level during TTX application in each experiment was subsequently subtracted from all other averaged steady-state levels to obtain unbiased estimations of $I_{\text{Na late}}$ in different experimental conditions. In general, reversibility was good and rapid upon TTX wash-out.

2. Late sodium current activation by veratridine and block by ranolazine in Nav1.5 cells

Under control conditions, a 5-min application of veratridine 1 μM induced an increase of the late Na$^+$ current by 269.1 ± 16.1% (mean ± SEM, $n = 28$) of initial values (Table 1 and Figure 2). Figure 1B shows representative current traces of selected sweeps, as well as
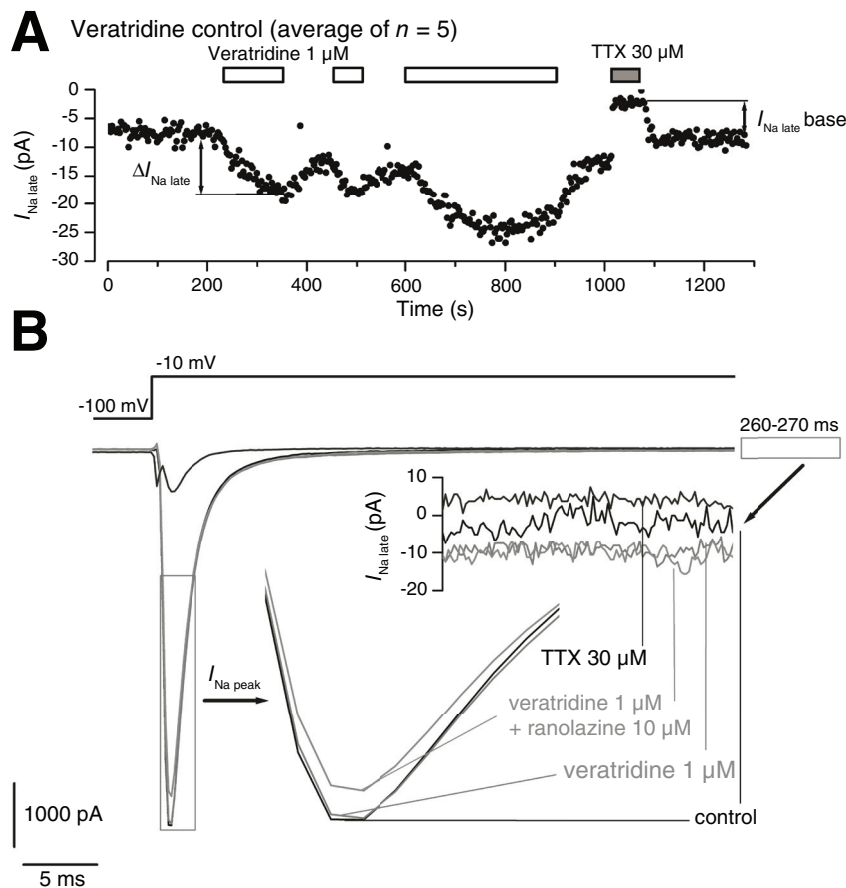

Figure 1. Activation by veratridine of the late Na$^+$ current in Nav1.5-expressing cells. A. Time course of $I_{\text{Na late}}$ (average transmembrane current during the last 100 ms of a 300-ms depolarizing pulse to -10 mV from a holding potential of -100 mV) during a typical experiment with repeated applications of veratridine 1 μM and a final application of TTX 30 μM. **B**. Overlap of individual sweeps showing the peak and late component of I_{Na} during the application of veratridine 1 μM, the co-application with ranolazine 10 μM, and with TTX 30 μM. The peak and late I_{Na} component are shown separately in inserts using magnified time and voltage scales, respectively.

Table 1. Effects of ranolazine 10 and 30 µM and veratridine 1 µM on TTX-sensitive* late Na+ current.

Experiment	Absolute values (mean ± SEM)			Relative values (mean ± SEM)				
Drug application (n = number of cells tested)	$I_{Na\ late}$ **initial** (pA)	$I_{Na\ late}$ **drug** (pA)	$I_{Na\ late}$ **final** (pA)	$I_{Na\ late}$ **initial** (%)	$I_{Na\ late}$ **drug** (%)	p value vs. initial§	$I_{Na\ late}$ **final** (%)	p value vs. initial§
Ranolazine 10 µM 3 min (n = 17)	-13.2 ± 2.6	-9.7 ± 1.8	-13.2 ± 2.3	100	75.0 ± 2.7	7.8E-8	105.6 ± 5.0	0.280
Ranolazine 30 µM 3 min (n = 18)	-10.6 ± 2.2	-6.1 ± 1.4	-11.9 ± 2.8	100	58.4 ± 3.5	1.1E-9	107.7 ± 7.7	0.330
Veratridine 1 µM 5 min (n = 28)	-12.4 ± 1.9	-36.3 ± 6.7	-15.8 ± 2.7	100	269.1 ± 16.1	4.7E-11	114.2 ± 8.2	0.095

* $I_{Na\ late}$ values were computed by subtraction of averaged amplitude in the presence of TTX 30 µM from averaged amplitudes in all other experimental conditions
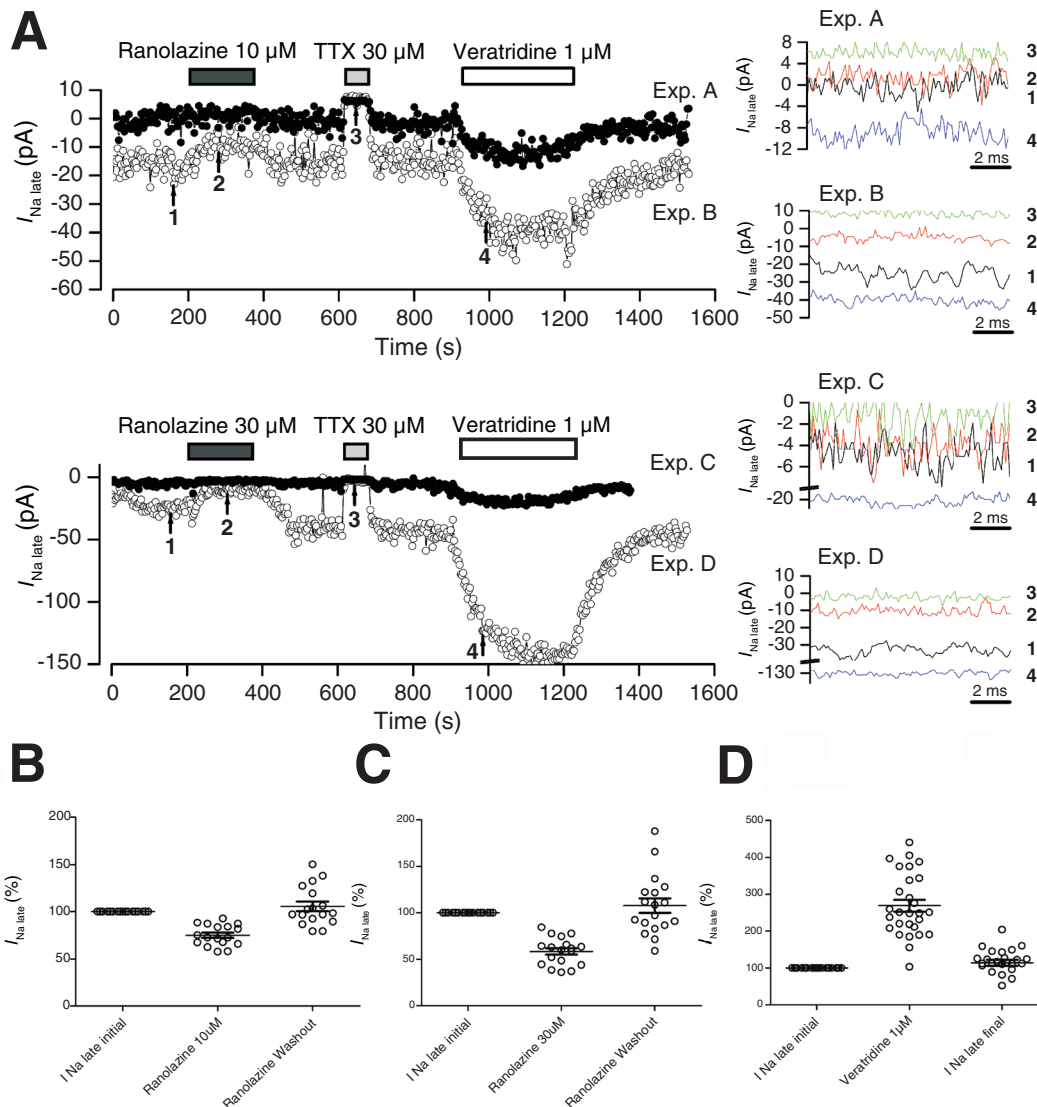
§ using two-tailed Student's t test for paired samples

Figure 2. Effects of ranolazine, TTX and veratridine on the late Na+ current in Nav1.5-expressing cells. A. Time plots of $I_{Na\ late}$ in four typical experiments. Intervals of application of ranolazine 10 or 30 µM, TTX 30 µM, and veratridine 1 µM are indicated. Right panels: $I_{Na\ late}$ traces from 260 to 270 ms at the time points marked with arrows and numbered in the left panels. **B** and **C**. Effects of ranolazine 10 and 30 µM, respectively, on relative levels of TTX-sensitive late Na+ current. **D**. Effect of veratridine 1 µM. Mean values are indicated for each condition, and error bars represent SEM.

magnified inserts of the peak and late phase, within an individual experiment. The application of veratridine 1 μM increased the late current amplitude by more than two-fold relative to the TTX 30 μM reference trace, without any noticeable effect on peak current, while the co-application of ranolazine 10 μM reduced the peak current with apparently no effect on the late component. Systematic experiments with ranolazine applied alone demonstrated $I_{Na\ late}$ reduction to 75.0 ± 2.7% (mean ± SEM, $n = 17$) of initial values at 10 μM, and 58.4 ± 3.5% (mean ± SEM, $n = 18$) at 30 μM (Table 1 and Figure 2).

Figure 3 shows the power density spectra of Fourier-transformed traces recorded under different conditions, as numbered in the upper time course of the experiments. Presumably, the opening and closure of Nav1.5 channels in the late gating mode conformation produces a Lorentzian component with corner frequency above 2 KHz. However, due to the reduced number of individual signal sources, the plateau spectral power density was small and difficult to distinguish. A better approach to fluctuation analysis is by the computation of single-channel parameters such as the mean open probability p and the average number of channels n, starting from the well-known estimations of current variance σ^2 and macroscopic current I[25]:

$$\sigma^2 = npi^2 - np^2i^2 = np(1-p)i^2 \text{ and } I = npi$$

We used a unitary Na+ current amplitude i at -10 mV of 1.43 pA, based on the unitary current amplitude of 2 pA at -40 mV observed in single-channel recordings of Nav1.5 late currents in HEK293 cells[26] and a reversal potential of +65 mV. In doing so, we could evaluate the mean open probability p and further the average number of Na+ channels n open in the late mode in selected traces, over the last 100 ms of activity during the 300-ms depolarizing pulses. It was observed that 30 μM ranolazine application resulted in a decrease of mean open probability from 0.6 to 0.38, without affecting the average number of channels contributing the late Na+ current ($n = 4$), in agreement with estimates obtained from averaged macroscopic currents. On the other hand, veratridine 1 μM increased the number of channels in late gating mode to $n = 10$, without influencing the mean open probability.

3. di-APD prolongation in iPSC-CM by application of veratridine

To get a better understanding of the role that the late Na+ current may play in arrhythmogenesis and in the complex mechanisms of di-LQT syndrome, we performed a series of automated patch-clamp
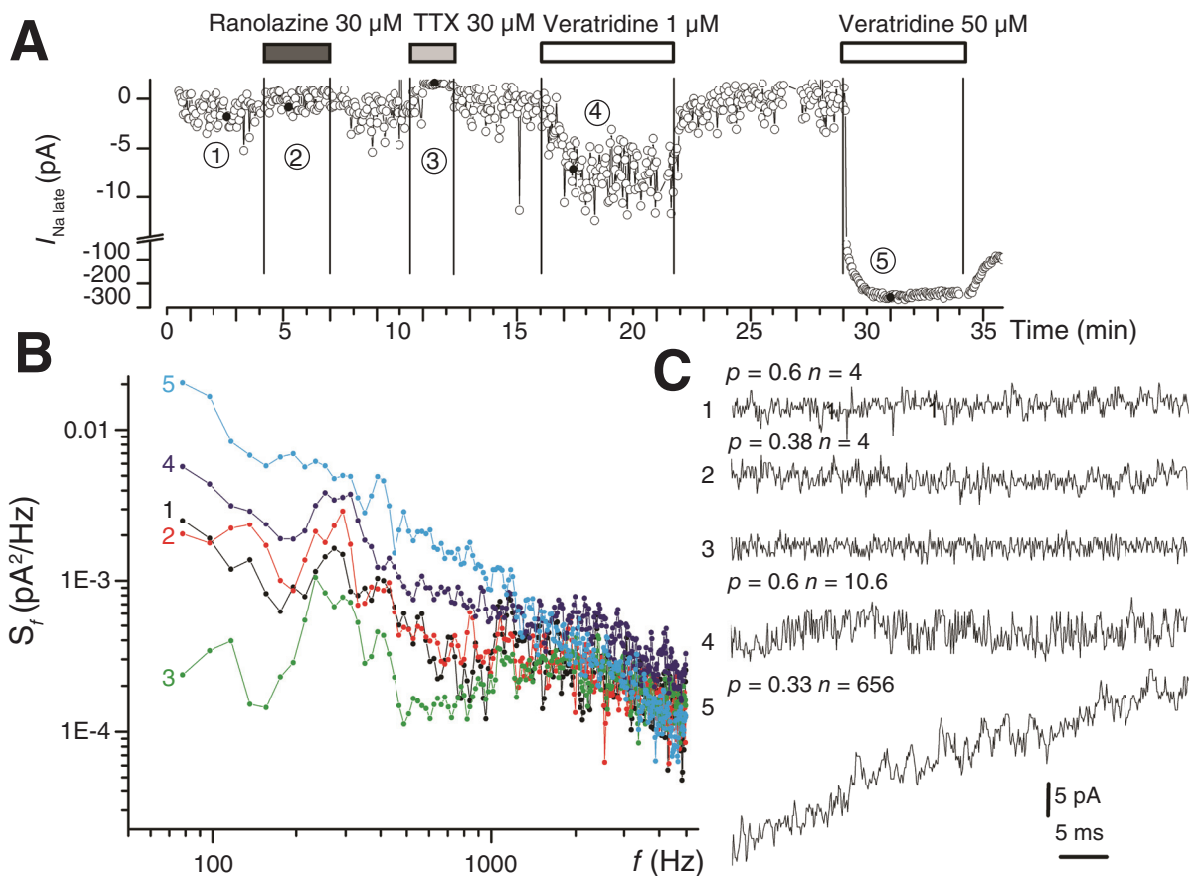

Figure 3. The late Na+ current presents fluctuations due to the opening and closure of individual channels. A. Time course of a typical experiment in Nav1.5-expressing cells showing the effects of ranolazine 30 μM, TTX 30 μM, and veratridine 1 μM on $I_{Na\ late}$. **B**. Averaged Fourier transforms of multiple traces recorded in different conditions in the experiment shown in **A**: 1. initial control; 2. ranolazine 30 μM; 3. TTX 30 μM; 4. veratridine 1 μM; 5. veratridine 50 μM. **C**. Fluctuation analysis of individual traces recorded during the five distinct periods shown in **A** allowed the distinction of individual channel gating events and the computation of the average open probability p and the average number of channels n for each condition.

experiments on hiPSC-derived cardiomyocytes with current-clamp recordings of stimulus-triggered APs. Each recorded sweep contained responses elicited by three injected current stimuli (0.5 ms duration, 2 nA amplitude). As shown in Figure 4, veratridine 1 μM induced a marked prolongation of AP duration, reaching saturation in less than 3 min. Further application of 5 μM veratridine resulted in further APD90 prolongation, exceeding the interstimulus interval. There was a slow trend to reversibility during the wash-out phase. Quantitative results for veratridine 1 μM application are summarized in Table 2. Thus, in $n = 6$ experiments at room temperature the relative APD90 prolongation induced by veratridine was 2.12 ± 0.41 fold (mean ± SEM), reversible to 1.21 ± 0.50 fold at wash-out (mean ± SEM, $n = 4$).

4. Weak hERG inhibition by veratridine

Last, we performed experiments using HEK293 cells stably expressing hERG1 to assess the effects of veratridine at different concentrations on this current component, in order to better understand the complex effects of this compound on the action potential of iPSC-derived cardiomyocytes. As shown in Figure 5, veratridine concentrations up to 5 μM did not produce levels of hERG inhibition significantly different than the diluting vehicle alone (ethanol 0.1%), while at 50 μM there was a $28.9 \pm 6.0\%$ (mean ± SD, $n = 10$) inhibition of peak hERG current, a statistically significant effect ($p < 0.0001$, one-way ANOVA for independent samples with Dunnett's multiple comparison test $vs.$ control).
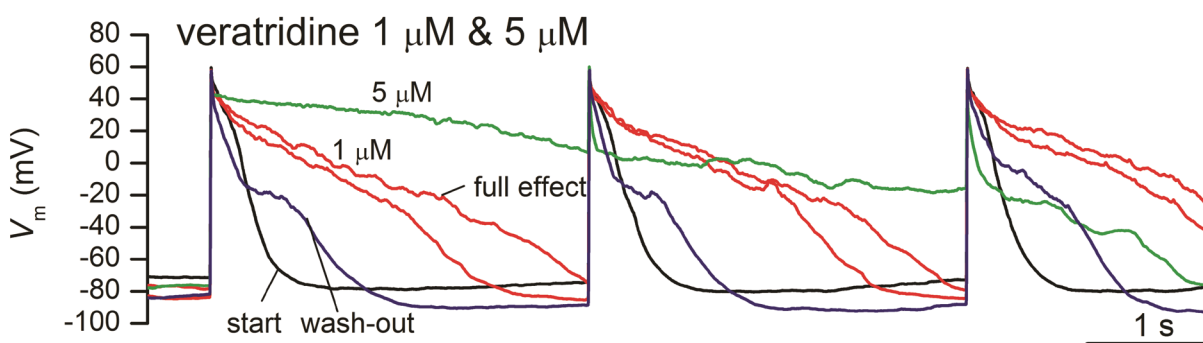
Figure 4. AP prolongation by veratridine in human iPSC-derived cardiomyocytes. Current-clamp AP recordings using a pacing protocol with injected current stimuli of 0.5-ms duration and 2 nA amplitude repeated at 3-s intervals. The overlap of traces in control conditions at the start of the experiment, with 1 μM veratridine (onset and full effect), with 5 μM veratridine, and during wash-out, shows partial recovery.

Table 2. Changes in APD90 duration (in ms, mean ± SEM) induced by veratridine in hiPSC-derived cardiomyocytes.

Experimental condition	APD90 initial	APD90 during appl.	APD90 wash-out	Relative APD90 during appl.	Relative APD90 wash-out
Veratridine 1 μM	777.5 ± 179.1	1429.3 ± 289.8	688.0 ± 86.9	2.12 ± 0.41	1.21 ± 0.50
p		0.02482	0.65037		
n	6	6	4	6	4
Ethanol 0.1% control	893.8 ± 343.8	1013.0 ± 408.4	917.3 ± 318.3	1.08 ± 0.07	1.17 ± 0.17
p		0.16664	0.75986		
n	4	4	4	4	4

Relative values are computed taking initial values as reference

p values for Student's t test for paired samples, two-tailed, for condition tested $vs.$ initial values
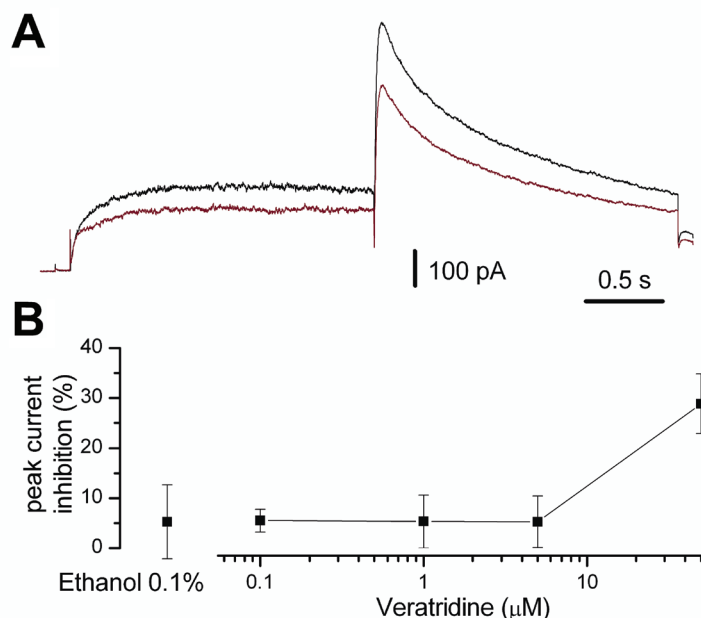
n = number of cells included in analysis

Figure 5. Effects of veratridine on hERG channels. A. Current traces in control conditions (upper) and during application of veratridine 50 μM (lower trace). **B**. Percentages of peak hERG current inhibition by different concentrations of veratridine and control ethanol 0.1% vehicle. Error bars represent SD. Number of experiments: control ($n = 6$), veratridine 0.1 μM ($n = 10$), 1 μM ($n = 5$), 5 μM ($n = 7$), 50 μM ($n = 10$).

Discussion

The main findings of the present study using the CytoPatch™2 instrument are the following: (1) inhibition of cardiac late Na$^+$ current to 75.0% and 58.4% of initial values by ranolazine 10 and 30 μM, respectively; (2) activation of the same current by veratridine 1 μM to 269.1% of initial values; and (3) prolongation of APD90 by veratridine 1 μM to 212% of initial values in human iPSC-derived cardiomyocytes. We have succeeded in recording and analysing the small (a few picoA) late Na$^+$ current component in a human cell line stably transfected with wild-type human SCN5A, using automated patch-clamp technology, with a voltage protocol different from that used in the first electrophysiology characterization of ΔKPQ LQT-3 mutant Na$^+$ channels[3]. We observed that instrumental noise does not significantly affect the average current value over the last 100 ms of a 300-ms depolarizing pulse from -100 to -10 mV, therefore this value can be computed and plotted in real-time, representing a sensitive indicator for evidencing pharmacological effects in automated patch-clamp assays. Another critical parameter is the frequency of repetition of depolarizing stimuli, because it may reveal use dependency for compounds exerting state-dependent binding effects[27]. Although in the present set of experiments we kept a fixed value of 0.33 Hz, inclusion of different pacing rates in future variants of the assay may offer supplementary information of pharmacological relevance. An important tool in the precise assessment of $I_{Na\ late}$ levels is the application of TTX 30 μM, which results in an almost complete late Na$^+$ current inhibition, and offers a current reference used for the correction by subtraction of all other values in different experimental conditions. An excellent seal stability and access resistance, such as those offered by the Cytocentrics microfluidic chips[28], is another prerequisite for successful late Na$^+$ pharmacology experiments.

Due to the limited amplitude of late Na$^+$ current under basal conditions, we increased it using low concentrations of veratridine, a steroid-derived natural alkaloid known to bind to an intramembrane site of Na$^+$ channel pore subunits and, in doing so, stabilize it in the open conformation[29,30], contributing to the $I_{Na\ late}$. Although veratridine at 1 μM resulted in a consistent 2.5 – 3-fold increase in late Na$^+$ current, compared to initial levels (Table 1 and Figure 1– Figure 3), we would not recommend this procedure for pharmacology assays because of possible interactions between veratridine and test compounds. Although we have not systematically explored this phenomenon, preliminary observations, as shown in Figure 1B, suggest a reduced effectiveness of ranolazine 10 μM when co-applied with veratridine, compared to application of ranolazine alone at the same concentration. The value of relative inhibition of the late Na$^+$ current by ranolazine 10 μM in the single compound application obtained in our study (75.0% of control levels) is larger than those obtained with the same concentration of ranolazine on late Na$^+$ current activated by veratridine 40 μM (89% and 81% for holding potentials of -110 mV and -90 mV, respectively)[31]. An elegant method that may offer pharmacologically relevant information concerning the site and mechanism of action of a certain compound is fluctuation analysis. As shown in Figure 3, when applied to selected traces, this method allows the computation of the mean open probability p and the average number of open channels n contributing to current variance. From this analysis, we hypothesize that ranolazine acts as an open pore blocker, reducing the mean open probability at 30 μM to 63% of initial values, from 0.6 to 0.38, without lowering the number of active channels, while veratridine 1 μM increases the number of active channels in late gating mode 2.5-fold, without influencing their mean open probability.

In agreement with the significant activatory effects of veratridine 1 μM on cardiac Nav1.5 channels in late gating mode in stably transfected cell lines, we found an important prolongation of action potential duration when the drug was applied to human iPSC-derived cardiomyocytes in current-clamp configuration, using CytoPatch™2 automated patch-clamp equipment. The APD90 was increased over twofold by veratridine 1 μM, with a good reversibility upon wash-out (Table 2), and an even higher prolongation with 5 μM (Figure 4), although in this case we were unable to accurately compute APD90 values for externally paced APs, due to their overlap with the next stimulus. This effect is consistent with the findings of previous studies, showing APD prolongation in bronchial smooth muscle cells upon veratridine application[32], and the enhancement of AP burst generation by veratridine in rat trigeminal sensory neurons[33]. Furthermore, we have demonstrated that hERG inhibition does not play any role in the observed APD prolongation by veratridine at 1 or 5 μM, since significant hERG inhibition occurred with higher veratridine concentrations (Figure 5). A recent combined *in vivo* and *in vitro* study proved that the augmentation of the myocardial late Na^+ current during ventricular remodeling induced by pulmonary arterial hypertension in rats was accompanied by significant APD90 increases, and a beneficial effect of repeated ranolazine administration via late Na^+ current block was demonstrated with a subsequent alleviation of induced intracellular Ca^{2+} overload[34].

In conclusion, the present study demonstrates that automated patch-clamp, as implemented by the CytoPatch™2 equipment using our proprietary CYTOCENTERING technology and quartz pipette tips embedded in silicon microfluidic chips[28,35], which allow high-quality stable seals, lead to reliable late Na^+ current pharmacology recordings, with results at least comparable to those obtained in manual patch-clamp experiments. In addition, automation may prove advantageous given the small amplitude of late Na^+ current, which therefore requires a large number of experiments for the accurate assessment of pharmacological effects.

Data availability

F1000Research: Dataset 1. Experimental data showing the effect of veratridine, ranolazine and TTX on late Na^+ currents in cultured cells, 10.5256/f1000research.5544.d36993[36]

Abbreviations

HEK: human embryo kidney
CHO: Chinese hamster ovary

hERG: human ether-á-go-go related gene
SEM: standard error of the means
SD: standard deviation
DMEM: Dulbecco's modified Eagle's medium
DMSO: dimethylsulfoxide
HEPES: N-2-hydroxyethylpiperazine-N´-2-ethansulfonic acid
EGTA: ethylene glycol-bis(ß-aminoethyl ether)-N,N,N,N´-tetraacetic acid
ATP: adenosine triphosphate
TTX: tetrodotoxin
IC: intracellular solution (pipette-filling solution)
EC: extracellular solution
PBS: phosphate-buffered saline
iPSC-CM: induced pluripotent stem cell-derived cardiomyocytes
APD: action potential duration

References

1. Clancy CE, Tateyama M, Liu H, *et al.*: **Non-equilibrium gating in cardiac Na+ channels: an original mechanism of arrhythmia.** *Circulation.* 2003; **107**(17): 2233–7.
   PubMed Abstract | **Publisher Full Text**

2. Grandi E, Puglisi JL, Wagner S, *et al.*: **Simulation of Ca-calmodulin-dependent protein kinase II on rabbit ventricular myocyte ion currents and action potentials.** *Biophys J.* 2007; **93**(11): 3835–47.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

3. Bennett PB, Yazawa K, Makita N, *et al.*: **Molecular mechanism for an inherited cardiac arrhythmia.** *Nature.* 1995; **376**(6542): 683–5.
   PubMed Abstract | **Publisher Full Text**

4. Bezzina C, Veldkamp MW, van Den Berg MP, *et al.*: **A single Na(+) channel mutation causing both long-QT and Brugada syndromes.** *Circ Res.* 1999; **85**(12): 1206–13.
   PubMed Abstract | **Publisher Full Text**

5. Veldkamp MW, Viswanathan PC, Bezzina C, *et al.*: **Two distinct congenital arrhythmias evoked by a multidysfunctional Na(+) channel.** *Circ Res.* 2000; **86**(9): E91–7.
   PubMed Abstract | **Publisher Full Text**

6. Abriel H: **Cardiac sodium channel Na(v)1.5 and interacting proteins: Physiology and pathophysiology.** *J Mol Cell Cardiol.* 2010; **48**(1): 2–11.
    PubMed Abstract | **Publisher Full Text**

7. Antzelevitch C, Nesterenko V, Shryock JC, *et al.*: **The role of late I Na in development of cardiac arrhythmias.** *Handb Exp Pharmacol.* 2014; **221**: 137–68.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

8. Grant AO: **Cardiac ion channels.** *Circ Arrhythm Electrophysiol.* 2009; **2**(2): 185–94.
    PubMed Abstract | **Publisher Full Text**

9. Clancy CE, Rudy Y: **Linking a genetic defect to its cellular phenotype in a cardiac arrhythmia.** *Nature.* 1999; **400**(6744): 566–9.
    PubMed Abstract | **Publisher Full Text**

10. Kleber AG, Rudy Y: **Basic mechanisms of cardiac impulse propagation and associated arrhythmias.** *Physiol Rev.* 2004; **84**(2): 431–88.
    PubMed Abstract | **Publisher Full Text**

11. Antzelevitch C, Belardinelli L, Zygmunt AC, *et al.*: **Electrophysiological effects of ranolazine, a novel antianginal agent with antiarrhythmic properties.** *Circulation.* 2004; **110**(8): 904–10.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

12. Belardinelli L, Shryock JC, Fraser H: **Inhibition of the late sodium current as a potential cardioprotective principle: effects of the late sodium current inhibitor ranolazine.** *Heart.* 2006; **92**(Suppl 4): iv6–iv14.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

13. Undrovinas AI, Belardinelli L, Undrovinas NA, *et al.*: **Ranolazine improves abnormal repolarization and contraction in left ventricular myocytes of dogs with heart failure by inhibiting late sodium current.** *J Cardiovasc Electrophysiol.* 2006; **17**(Suppl 1): S169–S77.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

14. Hale SL, Kloner RA: **Ranolazine, an inhibitor of the late sodium channel current, reduces postischemic myocardial dysfunction in the rabbit.** *J Cardiovasc Pharmacol Ther.* 2006; **11**(4): 249–55.
    PubMed Abstract | **Publisher Full Text**

15. Mahesh Kumar KN, Sandhiya S: **Ranolazine: A novel partial inhibitor of fatty acid oxidation for angina.** *Indian J Pharmacol.* 2006; **38**(4): 302–4.
    Publisher Full Text

16. Fredj S, Sampson KJ, Liu H, *et al.*: **Molecular basis of ranolazine block of LQT-3 mutant sodium channels: evidence for site of action.** *Br J Pharmacol.* 2006; **148**(1): 16–24.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

17. Luo A, Ma J, Song Y, *et al.*: **Larger late sodium current density as well as greater sensitivities to ATX II and ranolazine in rabbit left atrial than left ventricular myocytes.** *Am J Physiol Heart Circ Physiol.* 2014; **306**(3): H455–61.
    PubMed Abstract | **Publisher Full Text**

18. Maltsev VA, Undrovinas AI: **A multi-modal composition of the late Na$^+$ current in human ventricular cardiomyocytes.** *Cardiovasc Res.* 2006; **69**(1): 116–27.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

19. Moreno JD, Clancy CE: **Pathophysiology of the cardiac late Na current and its potential as a drug target.** *J Mol Cell Cardiol.* 2012; **52**(3): 608–19.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

20. Moreno JD, Yang PC, Bankston JR, *et al.*: **Ranolazine for congenital and acquired late I$_{Na}$-linked arrhythmias: *in silico* pharmacological screening.** *Circ Res.* 2014; **113**(7): e50–61.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

21. Zaza A, Belardinelli L, Shryock JC: **Pathophysiology and pharmacology of the cardiac "late sodium current".** *Pharmacol Ther.* 2008; **119**(3): 326–39.
    PubMed Abstract | **Publisher Full Text**

22. McKinney LC, Chakraverty S, De Weer P: **Purification, solubility, and pKa of veratridine.** *Anal Biochem.* 1986; **153**(1): 33–8.
    PubMed Abstract | **Publisher Full Text**

23. Kupchan SM, Lavie D, Deliwala CV, *et al.*: **Schoenocaulon alkaloids. I. Active principles of Schoenocaulon officinale. Cevacine and protocevine.** *J Am Chem Soc.* 1953; **75**(22): 5519–24.
    Publisher Full Text

24. Dice MS, Kearl T, Ruben PC: **Methods for studying voltage-gated sodium channels in heterologous expression systems.** *Methods Mol Med.* 2006; **129**: 163–85.
    PubMed Abstract | **Publisher Full Text**

25. Van Driessche W, Lindemann B: **Concentration dependence of currents through single sodium-selective pores in frog skin.** *Nature.* 1979; **282**(5738): 519–20.
    PubMed Abstract | **Publisher Full Text**

26. Lacerda AE, Kuryshev YA, Chen Y, *et al.*: **Alfuzosin delays cardiac repolarization by a novel mechanism.** *J Pharmacol Exp Ther.* 2008; **324**(2): 427–33.
    PubMed Abstract | **Publisher Full Text**

27. Rajamani S, El-Bizri N, Shryock JC, *et al.*: **Use-dependent block of cardiac late Na(+) current by ranolazine.** *Heart Rhythm.* 2009; **6**(11): 1625–31.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

28. Stett A, Burkhardt C, Weber U, *et al.*: **CYTOCENTERING: a novel technique enabling automated cell-by-cell-patch clamping with the CYTOPATCH chip.** *Recept Chann.* 2003; **9**(1): 59–66.
    PubMed Abstract

29. Catterall WA: **Cooperative activation of action potential Na$^+$ ionophore by neurotoxins.** *Proc Natl Acad Sci U S A.* 1975; **72**(5): 1782–6.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

30. Ulbricht W: **Effects of veratridine on sodium currents and fluxes.** *Rev Physiol Biochem Pharmacol.* 1998; **133**: 1–54.
    PubMed Abstract | **Publisher Full Text**

31. Le Grand B, Pignier C, Letienne R, *et al.*: **Sodium late current blockers in ischemia reperfusion: is the bullet magic?** *J Med Chem.* 2008; **51**(13): 3856–66.
    PubMed Abstract | **Publisher Full Text**

32. Bradley E, Webb TI, Hollywood MA, *et al.*: **The cardiac sodium current Na(v)1.5 is functionally expressed in rabbit bronchial smooth muscle cells.** *Am J Physiol Cell Physiol.* 2013; **305**(4): C427–35.
    PubMed Abstract | **Publisher Full Text**

33. Tsuruyama K, Hsiao CF, Chandler SH: **Participation of a persistent sodium current and calcium-activated nonspecific cationic current to burst generation in trigeminal principal sensory neurons.** *J Neurophysiol.* 2013; **110**(8): 1903–14.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

34. Rocchetti M, Sala L, Rizzetto R, *et al.*: **Ranolazine prevents I$_{Na}$L enhancement and blunts myocardial remodelling in a model of pulmonary hypertension.** *Cardiovasc Res.* 2014; **104**(1): 37–48.
    PubMed Abstract | **Publisher Full Text**

35. Scheel O, Himmel H, Rascher-Eggstein G, *et al.*: **Introduction of a modular automated voltage-clamp platform and its correlation with manual human ether-à-go-go related gene voltage-clamp data.** *Assay Drug Dev Technol.* 2011; **9**(6): 600–7.
    PubMed Abstract | **Publisher Full Text**

36. Chevalier M, Amuzescu B, Gawali V, *et al.*: **Experimental data showing the effect of veratridine, ranolazine and TTX on late Na$^+$ currents in cultured cells.** *F1000Research.* 2014.
    Data Source

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Current Referee Status: ☑ ☑

Version 1

Referee Report 29 October 2014

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In this study, Chevalier *et al.* had succeeded to record and analyse the late Na^+ current (I_{NaL}) in HEK293 cells stably transfected with wild-type human SCN5A, using automated patch-clamp technology. They were able to accurately assess the effects of pharmacological agents on I_{NaL}. They showed that the cardiac I_{NaL} can be inhibited by ranolazine and activated by veratridine. Using fluctuation analysis, they hypothesized that ranolazine would act as an open pore blocker, reducing the open probability without affecting the number of active channels, while veratridine would increase the number of active channels without influencing their open probability. In addition, they also provided additional functional data by performing a series of automated patch-clamp experiments showing that application of veratridine concentrations that increased I_{NaL} but had no effects on HERG current prolonged the action potential duration of human iPSC-derived cardiomyocytes.

This is an interesting and well-designed study that reports high quality data. Considering the role of late sodium current in the action potential duration, mainly under pathological conditions, and the difficulty to accurately record this small ionic current, the subject of this study is highly relevant. The demonstration of the suitability of the automated patch clamp technology to accurately measure the late sodium current and to assess the effects of pharmacological agents on this current is helpful. In addition, the use of the hiPSC–derived cardiomyoyctes with this approach is also interesting. I do not have any major issues to address. However, the paper could be improved by attention to the following points.

1. All experiments were performed at room temperature. Considering the limited amplitude of the late Na^+ current under basal conditions, it could have been useful to perform these experiments at a more physiological temperature to help increase the amplitude of the current.

2. In Figure 1B, it would have been interesting to also present the recordings with ranolaxine alone.

3. The *action potential* was elicited by the *injection* of 2 nA of *current*. This is a rather large current and is probably not just-suprathreshold depolarizing *current*. Do the hiPSC-derived cardiomyocytes usually require such a high current injection to elicit action potential?

4. For the HERG recordings, is there any specific reason why the external concentration of KCl was only 2.5 mM?

5. Data reported in Table 1 could have been normalized to the cell capacitance to report the current density instead of the current amplitude.

6. For some statistical analysis, an ANOVA would have been more appropriate that a Student's *t* tests as several groups were examined (eg., Tables 1 and 2).

7. There are few typographic errors in the Abstract: the units for the drugs ranolaxine, veratridine and TTX are all in millimolar concentrations but should be in the micromolar concentrations.

Overall, the study is interesting and describes a methodological approach that could help study the late sodium current. The experiments were well designed and presented and the subject of this manuscript is of importance to the field.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 28 October 2014

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The paper by Chevalier *et al*. analyzed whether late sodium current (I_{NaL}) can be assessed using an automated patch-clamp device. To this end, the I_{NaL} effects of ranolazine (a well known I_{NaL} inhibitor) and veratridine (an I_{NaL} activator) were described. The authors tested the CytoPatch automated patch-clamp equipment and performed whole-cell recordings in HEK293 cells stably transfected with human Nav1.5. Furthermore, they also tested the electrophysiological properties of human induced pluripotent stem cell-derived cardiomyocytes (hiPS) provided by Cellular Dynamics International. The title and abstract are appropriate for the content of the text. Furthermore, the article is well constructed, the experiments were well conducted, and analysis was well performed.

I_{NaL} is a small current component generated by a fraction of Nav1.5 channels that instead to entering in the inactivated state, rapidly reopened in a burst mode. I_{NaL} critically determines action potential duration (APD), in such a way that both acquired (myocardial ischemia and heart failure among others) or inherited (long QT type 3) diseases that augmented the I_{NaL} magnitude also increase the susceptibility to cardiac arrhythmias. Therefore, I_{NaL} has been recognized as an important target for the development of drugs with either antiischemic or antiarrhythmic effects. Unfortunately, accurate measurement of I_{NaL} is a time consuming and technical challenge because of its extra-small density. The automated patch clamp device tested by Chevalier *et al.* resolves this problem and allows fast and reliable I_{NaL} measurements.

The results here presented merit some comments and arise some unresolved questions. First, in some experiments (such is the case in experiments B and D in Figure 2) current recordings obtained before the ranolazine perfusion seem to be quite unstable. Indeed, the amplitude progressively increased to a maximum value that was considered as the control value (highlighted with arrows). Can this problem be overcome? Is this a consequence of a slow intracellular dialysis? Is it a consequence of a time-dependent shift of the voltage dependence of activation/inactivation? Second, as shown in Figure 2, intensity of drug

effects seems to be quite variable. In fact, experiments A, B, C, and D in Figure 2 and panel 2D, demonstrated that veratridine augmentation ranged from 0-400%. Even assuming the normal biological variability, we wonder as to whether this broad range of effect intensities can be justified by changes in the perfusion system. Has been the automated dispensing system tested? If not, we suggest testing the effects of several K^+ concentrations on inward rectifier currents generated by Kir2.1 channels ($I_{Kir2.1}$).

The authors demonstrated that the recording quality was so high that the automated device allows to the differentiation between noise and current, even when measuring currents of less than 5 pA of amplitude. In order to make more precise mechanistic assumptions, the authors performed an elegant estimation of current variance (σ^2) and macroscopic current (I) following the procedure described more than 30 years ago by Van Driessche and Lindemann [1]. By means of this method, Chevalier et al. concluded that ranolazine acts as an open pore blocker reducing the open channel probability, while veratridine increases the number of channels in the burst mode. We respectfully would like to stress that these considerations must be put in context from a pharmacological point of view. We do not doubt that ranolazine acts as an open channel blocker, what it seems clear however, is that its onset block kinetics has to be "ultra" slow, otherwise ranolazine would decrease peak I_{NaL} even at low frequencies of stimulation. This comment points towards the fact that for a precise mechanistic study of ionic current modifying drugs it is mandatory to analyze drug effects with much more complicated pulse protocols. Questions thus are: does this automated equipment allow to the analysis of the frequency-, time-, and voltage-dependent effects of drugs? Can versatile and complicated pulse protocols be applied? Does it allow to a good voltage control even when generated currents are big and fast? If this is not possible, and by means of its extraordinary discrimination between current and noise, this automated patch-clamp equipment will only be helpful for rapid I_{NaL}-modifying drug screening. Obviously it will also be perfect to test HERG blocking drug effects as demanded by the regulatory authorities.

Finally, as cardiac electrophysiologists, we would like to stress that it seems that our dream of testing drug effects on human ventricular myocytes seems to come true. Indeed, human atrial myocytes are technically, ethically and logistically difficult to get, but human ventricular are almost impossible to be obtained unless from the explanted hearts from patients at the end stage of cardiac diseases. Here the authors demonstrated that ventricular myocytes derived from hiPS generate beautiful action potentials that can be recorded with this automated equipment. The traces shown suggested that there was not alternation in the action potential duration. Is this a consistent finding? How long do last these stable recordings? The only comment is that resting membrane potential seems to be somewhat variable. Can this be resolved? Is it an unexpected veratridine effect? Standardization of maturation methods of ventricular myocytes derived from hiPS will be a big achievement for cardiac cellular electrophysiology which was obliged for years to the imprecise extrapolation of data obtained from a combination of several species none of which was representative of human electrophysiology. The big deal will be the maturation of human atrial myocytes derived from hiPS that fulfil the known characteristics of human atrial cells.

Minor points:
We suggest suppressing the initial sentence of section 3. We surmise that results obtained from the experiments described in this section cannot serve to understand the role of I_{NaL} in arrhythmogenesis.

References
1. Van Driessche W, Lindemann B: Concentration dependence of currents through single sodium-selective pores in frog skin. *Nature*. 1979; **282** (5738): 519-520 PubMed Abstract | Publisher Full Text

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.