Temperature dependent hERG channel pharmacology and kinetics on the IonFlux™ system

Introduction

The human ether-a-go-go-related gene (hERG) encodes a K^+ delayed rectifier channel that plays a critical role in the control of action potential repolarization in mammalian and human cardiac myocytes. Therapeutic compounds from a variety of chemical classes can potently block the hERG channel and cause the prolongation of the electrocardiographic QT interval, which may result in potentially fatal ventricular arrhythmia, torsade de pointes (TdP). A risk of TdP is one of the major causes of drug withdrawals from the market by regulatory agencies and pharmaceutical companies (Trudeau, M. C. 1995, Sanguinetti M. C. 1995).

The patch clamp technique is the gold standard in evaluating the inhibitory potential of a compound using *in vitro* hERG assays. Although technically more difficult, patch-clamp recording at the near physiological temperature is better suited for drug safety testing than room temperature because the temperature dependent process can contribute to the steady state inhibition of hERG channels. Reported data has showed that increased temperature accelerated the hERG activation, inactivation, recovery from inactivation, and deactivation kinetics, as well as steady-state level inhibition (Zhou, Z. 1998). Studies have also shown that some compounds were markedly temperature sensitive and showed significant potency shift at physiological temperature (Sale, H. 2008, Kirsch, G. E, 2004). Therefore, it is desirable to conduct functional hERG screening at physiological temperature. Automated patch clamp systems can achieve higher throughput than the conventional manual patch clamp and are less labor intensive, which allow greater capacity to conduct early stage functional hERG screening. However, the first generation of automated patch clamp systems to enter the market have been limited to room temperature screening only. To address this need, the IonFlux system integrates a temperature control module, enabling users to perform temperature dependent electrophysiology screening assays. In this report, we present pharmacology and kinetic data from the hERG channel, comparing room

Figure 1: The IonFlux system utilizes a "plate reader" format to simplify workflow and increase throughput. Systems are available with 16 and 64 amplifiers. Throughputs of 10,000 data points per day can be achieved. It integrates a temperature control module, which enables the user to perform temperature dependent electrophysiology assay screening.

temperature to physiological temperature recordings on the IonFlux platform.

Materials and Methods

Monitoring Temperature Equilibration

Temperature changes can be monitored by measuring changes in open hole resistance before the addition of cells. Buffer conductivity is temperature dependent and increases as the temperature is elevated. Figure 2 shows that the open hole resistance measurements can be used as a proxy for monitoring temperature equilibration. The IonFlux system allows temperature control from ambient to up to 40°C. A temperature change of 5°C is achieved within a minute, and the temperature remains stable once it reaches equilibrium.
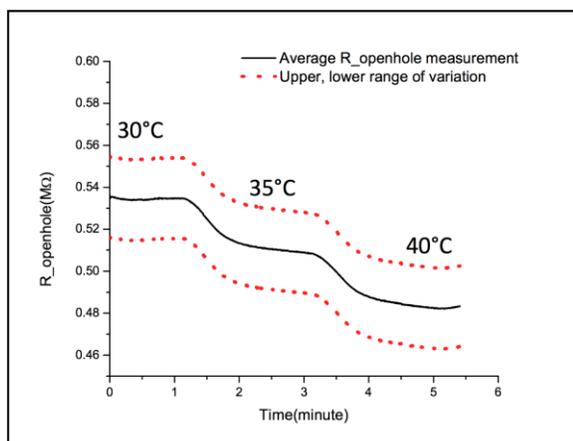
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Figure 2: The open hole resistance serves as a proxy for monitoring temperature equilibration. IonFlux system allows temperature control from ambient to up to 40°C. The temperature elevation of 5°C is achieved within a minute, and temperature is stable once it reaches equilibrium

Preparation of Cells

Chinese hamster ovary (CHO) cells expressing hERG1a channels were cultured in $175cm^2$ filter-top flasks containing DMEM+Glutamax-1 (Invitrogen 10565), 10% fatal bovine serum, and G-418 (500μg/ml) at 37°C and 5% CO2. The cells were kept below 90% confluency, and transferred to the 30°C incubator overnight before the experiment. For cell isolation, flasks were first washed with 2 ml of Ca- and Mg-free PBS, followed by 5ml of Detachin™ solution, after which cells were given two to five minutes of further Detachin™ treatment. After release, the cell suspension was spun for 90 seconds at 1000 rpm prior to being re-suspended in extracellular solution.

Solutions & Compounds

Extracellular solution (mM): 140 NaCl, 2 KCl, 3 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 9 glucose, pH 7.4 with NaOH. The intracellular solution for the whole cell voltage clamp contained (mM): 120 KCl, 5 $MgCl_2$, 10 $CaCl_2$, 5 EGTA, 4 Tris-ATP, 10 HEPES, pH 7.3 with KOH. Cell suspension in extracellular solution (5x106 cells/ml) was dispensed on an IonFlux plate. The hERG voltage pulse protocol used in this study was a conditioning pulse of +40mV for 1s, followed by the test pulse of -50mV for 1.5s repeated every 10s. The holding potential during the wait period was -80mV. The peak tail current was measured during the pulse step to -50mV.

hERG blocker compounds (fluoxetine, erythromycin and D.L-sotalol) were purchased from Sigma. They were dissolved in DMSO or deionized water to make stock solutions and diluted serially into extracellular buffer. The buffer with the highest concentration of DMSO(0.1%) was tested as negative control and showed no significant response. Data analysis and graphical presentation were performed using a combination of IonFlux software, Microsoft excel, and Origin Lab. All data are shown with arithmetic mean ± standard error of mean (SEM).

Results

Temperature dependent pharmacology

Erythromycin (ERM) has shown a marked increase in potency at physiological temperature. Time series plot for inhibition of hERG by ERM at room temperature versus physiological temperature is shown in Fig. 3A. Cells were trapped and sealed at room temperature (22°C), and the ERM block was recorded. No significant inhibition was observed till 300μM ERM was applied. The inhibition was reversible upon washing. Heater was turned on during the wash, and temperature was set at 35°C. Once the temperature and current were stable, the dose application of ERM was repeated. Current sweeps of the same cell ensemble (20 cells) at 22°C versus 35°C are shown in Fig. 3B using the IonFlux sweep overlay function. The peak amplitude of the current was almost double with a much faster decay phase at 35°C as compared with room temperature. 1000μM ERM inhibited about 50% hERG tail current at 22°C (Fig. 3C), whereas the same concentration of ERM blocked about 95% hERG current at 35°C (Fig. 3D). This result is in good agreement with literature values (Kirsch, G.E, 2004, Stanat, S, 2003)

Fluoxetine is a hERG inhibitor which does not show temperature dependent potency changes. In a different experiment, fluoxetine served as a control and tested within the same plate (IF-HT plate in 384 format) with two other compounds, erythromycin and D,L sotalol. Dose curves for fluoxetine at 25°C and 35°C are shown superimposed in Fig. 4A. The two dose response curves are largely overlapped, IC50s estimated at 3.8μM at 25°C and 5.1μM at 35°C. In contrast, the erythromycin dose response at 35°C was left shifted by over 3 fold from 831μM (at 25°C) to 251μM (at 35°C), as shown in Fig. 4B. D,L sotalol also showed a significant potency shift, from 681μM (25°C) to 215μM (35°C), as shown in Fig. 4C. The results indicate that while fluoxetine hERG inhibition is not temperature sensitive, erythromycin and sotalol are
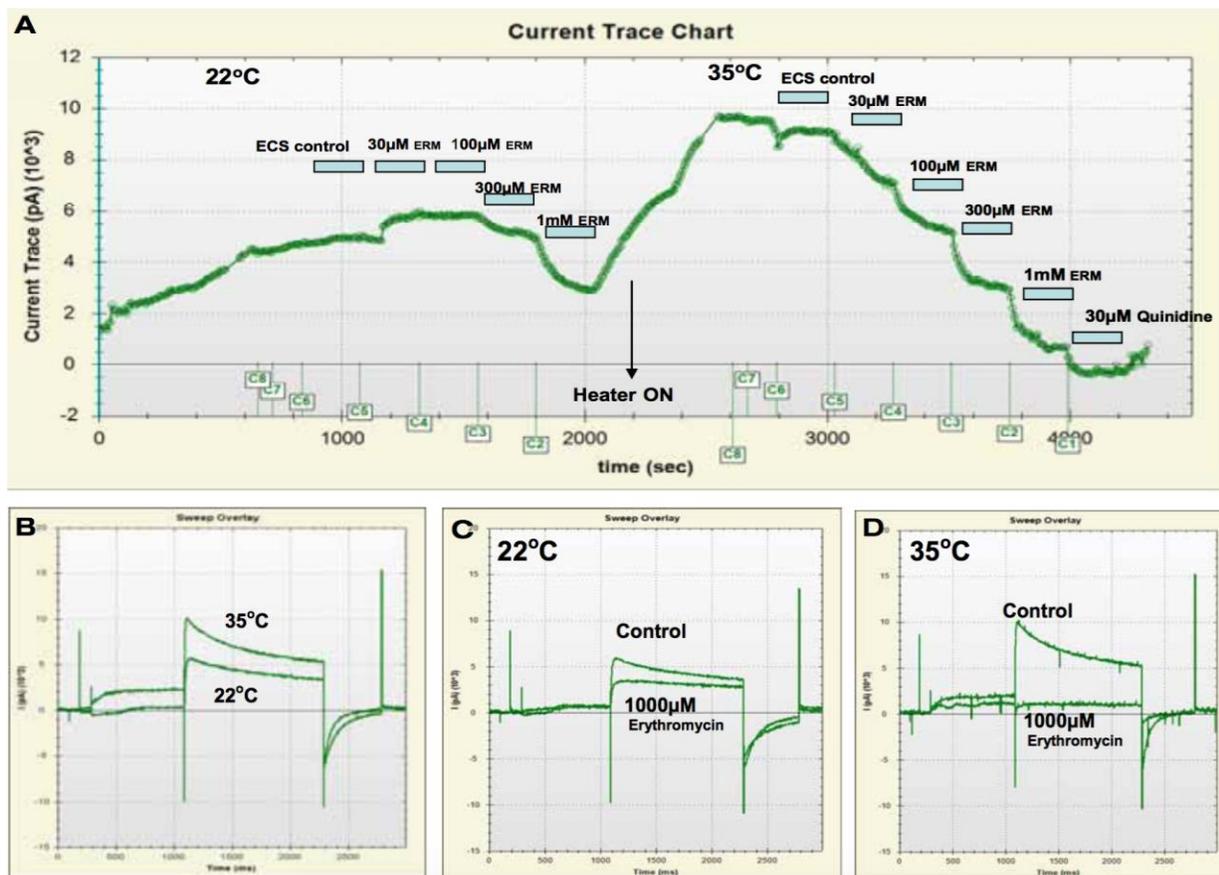
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Figure 3: A: Time series plot for inhibition of hERG by erythromycin at 22°C vs 35°C. The erythromycin showed markedly increased potency at physiological temperature. **B:** Current sweeps of the same cell ensemble (20 cells) at 22°C. vs 35°C. The peak amplitude of the current was al- most doubled with a much faster decay phase at 35°C as compared with 22°C. **C:** 1000µM ERM inhibited about 50% hERG current at 22°C. **D:** 1000µM ERM blocked about 95% hERG current at 35°C.
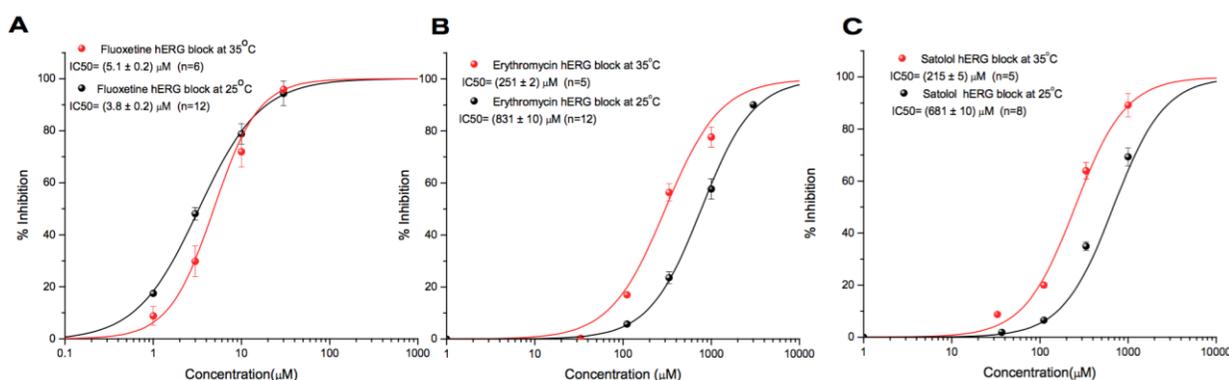

Figure 4: A: Dose curves for fluoxetine at 25°C and 35°C are shown superimposed. The two dose response curves are almost the same with IC50 estimated to be 3.8µM (at 25°C, n=12) and 5.1µM (at 35°C, n=6). **B:** Erythromycin dose response at 35°C was left shifted by more than 3 folds from 831µM (at 25°C, n=12) to 251µM (at 35°C, n=5). **C:** D,L sotalol showed temperature sensitive potency shift from 681µM (at 25°C, n=8) to 215µM (at 35°C, n= 5).

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temperature sensitive hERG blockers. These results are consistent with literature (Kirsch, G.E, 2004, Stanat, S, 2003, Rajamani, S, 2006, Thomas, D, 2002) in showing that significant temperature dependence is observed for some compounds.

Temperature Dependent Kinetics

Examination of kinetic transition provides more information regarding the influence from temperature on ion channel currents. We studied hERG current sweeps at room temperature as compared to physiological temperature (Fig 5). First, the current magnitude from the same ensemble of cells was almost doubled at 35°C as compared with 22°C. Second, the outward tail current from deactivation also decayed faster at 35°C. The time constant estimated from a single exponential equation decreased from 938±16ms at 22°C to 443±3ms at 35°C. Although the absolute value of the time constant depends on the activation voltage protocol, the fractional change (i.e. 2 ~3 times as compared to RT) agrees with the literature (Zhou, Z. 1998)

Discussion

Using the IonFlux temperature control module, the inhibitory effects of erythromycin and D,L sotalol were determined to be temperature sensitive. Measurements obtained at near physiological temperature yielded a much lower IC50 than room temperature, whereas the temperature variation did not

show a significant effect on fluoxetine, a negative control. The pharmacology shifts obtained using the IonFlux platform agree well with the manual patch clamp data (Table 1). A study of the kinetics showed that hERG tail current magnitude almost doubled at physiological temperature, with a 2~3 times faster decay of the outward tail current due to deactivation, which is also consistent with manual patch clamp data. Screening against hERG liability at physiological temperature yields data that is likely to provide a better correlation to cardiac risk in vivo. Therefore, experiments at temperature are a desirable drug safety testing modality. The IonFlux system is unique among automated patch clamp (APC) systems in providing a module for temperature control, a valuable feature for hERG safety screening and other temperature dependent ion channel targets.

Temperature	Compound	IonFlux IC50 (µM)	Literature IC50 (µM)
RT(22°C-25°C)	Fluoxetine	3.8	0.7-3
	Erythromycin	831	500-1410
	D,L-Sotalol	681	500-800
Physiological(35°C)	Fluoxetine	5.1	0.5-3
	Erythromycin	251	70-200
	D,L-Sotalol	215	269-280

Table 1: Effect of temperature on concentration-dependence of hERG blockade on IonFlux as compared with literatures.
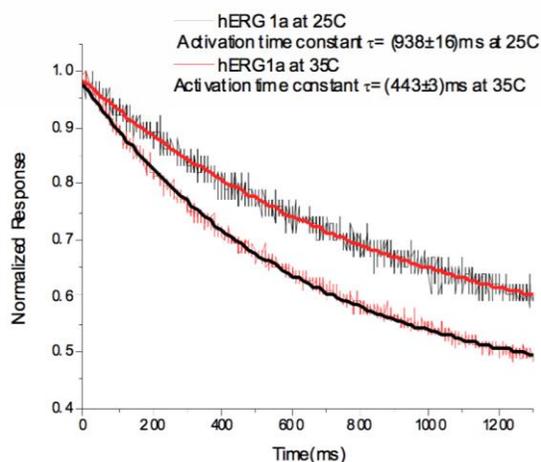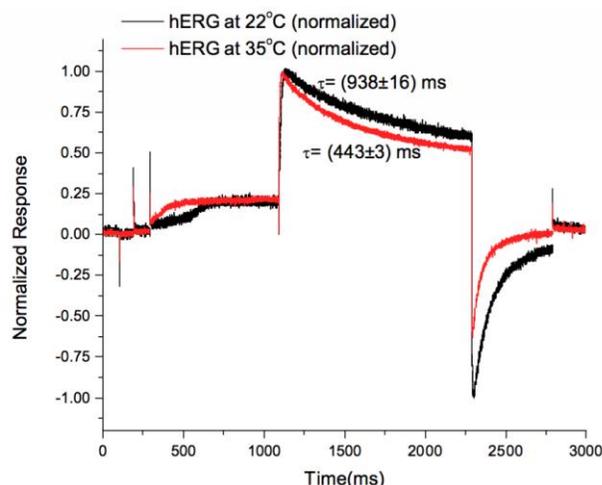

Figure 5: Normalized hERG current sweeps at room temperature vs. physiological temperature. The outward tail current from deactivation decayed much faster at 35°C. The time constant estimated from a single exponential fit decreased from 938 ± 16ms at 22°C to 443 ± 3ms. The fractional increase (i.e. 2 ~3 times as compared to RT) in kinetics agrees with the literature (Zhou, Z. 1998).

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Acknowledgement

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