Action Potential Clamp and Pharmacology of the Variant 1 Short QT Syndrome T618I hERG K⁺ Channel

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Abstract

Background: The familial Short QT Syndrome (SQTS) is associated with an increased risk of cardiac arrhythmia and sudden death. Gain-of-function mutations in the hERG K⁺ channel protein have been linked to variant 1 of the SQTS. A hERG channel pore (T618I) mutation has recently been identified in families with heritable SQTS. This study aimed to determine effects of the T618I-hERG mutation on (i) hERG current (I_{hERG}) elicited by ventricular action potentials; (ii) the sensitivity of I_{hERG} to inhibition by four clinically used antiarrhythmic drugs.

Methods: Electrophysiological recordings of I_{hERG} were made at 37°C from HEK 293 cells expressing wild-type (WT) or T618I hERG. Whole-cell patch clamp recording was performed using both conventional voltage clamp and ventricular action potential (AP) clamp methods.

Results: Under conventional voltage-clamp, WT I_{hERG} peaked at 0±10 mV, whilst for T618I I_{hERG} maximal current was rightward shifted to ~ +40 mV. Voltage-dependent activation and inactivation of T618I I_{hERG} were positively shifted (respectively by +15 and ~+25 mV) compared to WT I_{hERG}. The hERG ‘window’ was increased for T618I compared to WT hERG. Under ventricular AP clamp, maximal repolarising WT I_{hERG} occurred at ~ -30 mV, whilst for T618I hERG peak I_{hERG} occurred earlier during AP repolarisation, at ~+5 mV. Under conventional voltage clamp, half-maximal inhibitory concentrations (IC₅₀) for inhibition of I_{hERG} tails by quinidine, disopyramide, D-sotalol and flecainide for T618I hERG ranged between 1.4 and 3.2 fold that for WT hERG. Under action potential voltage clamp, T618I IC₅₀s ranged from 1.2 to 2.0 fold the corresponding IC₅₀ values for WT hERG.

Conclusions: The T618I mutation produces a more modest effect on repolarising I_{hERG} than reported previously for the N588K-hERG variant 1 SQTS mutation. All drugs studied here appear substantially to retain their ability to inhibit I_{hERG} in the setting of the SQTS-linked T618I mutation.

Introduction

The rapid delayed rectifier K⁺ channel current (I_K) is an important determinant of ventricular AP repolarisation and, consequently, of the duration of the QT interval on the electrocardiogram [1,2]. Channels mediating I_K are formed by proteins encoded by hERG (human Ether-à-go-go Related Gene; alternative nomenclature KCNH2 [3,4]). Native I_K and hERG channels exhibit sensitivity to pharmacological blockade by diverse drugs, including both Class Ia and Class III antiarrhythmic agents; excessive pharmacological inhibition of I_K/hERG leads to acquired long QT syndrome (LQTS [5-8]). Loss-of-function KCNH2 mutations are responsible for the LQT2 form of heritable long QT syndrome [9,10], whilst gain-of-function mutations are responsible for the SQTS1 form of heritable Short QT syndrome (SQTS [11,12]).

The KCNH2 mutations first identified in SQTS patients led to a common asparagine to lysine (N→K) substitution within the external S5-Pore linker region of the hERG channel protein [13,14]. hERG current (I_{hERG}) carried by N588K-hERG mutant channels failed to rectify normally, due to a substantial (+60 to +90 mV) rightward shift in voltage-dependent inactivation [13,15,16]. The use in vitro of the action potential (AP) voltage clamp technique showed that the impaired inactivation of N588K hERG channels altered significantly the profile of I_{hERG} during the plateau and repolarisation phases of ventricular APs, leading to increased I_{hERG} occurring much earlier during the ventricular AP waveform [13,15,16]. Additionally, SQTS1 patients with the N588K mutation were found to be refractory to treatment with Class III antiarrhythmic drugs (sotalol, ibutilide), but did respond to the Class Ia agents quinidine and disopyramide [13,17–19]. This differential influence of the N588K mutation on clinical effectiveness of Class Ia and III drugs correlates with changes in I_{hERG} blocking potency seen in vitro [13,18,20] and is explicable on the basis of the comparatively greater dependence of Class III than Class Ia drugs on I_{hERG} inactivation in order to bind to the...
channel [21]. A second gain-of-function hERG mutation, identified in the S5 domain of zebrafish EρG (zERG; L499P; hERG homologue L532P) in regga mutant zebrafish with accelerated cardiac repolarisation [22], has been found to produce marked kinetic alterations including to voltage and time-dependent inactivation [22,23]. The L532P hERG homologue also exhibits altered sensitivity to Class III drug block [25].

Recently, a novel SQT1 mutation has been identified in a Chinese family with a history of nocturnal sudden death [24]. Four of eleven family members evaluated exhibited shortened rate-corrected QT intervals (with a mean QTc, interval of 316 ms) [24]. Genotyping of the proband identified a base transition (C1853T) that led to a threonine to isoleucine substitution at position 618 (located in the hERG channel pore helix) of hERG; this was absent in 200 ethnically matched controls [24]. Pharmacological experiments with single high concentrations of quinidine or sotalol (producing 70% or greater inhibition of wild-type (WT) IhERG) were suggestive of retained IhERG kinetics, including a ~450 mV shift in voltage dependent inactivation [24]. Conformational changes were seen with the T618I hERG mutant, possibly due to the effect of the T618I mutation on the profile of hERG during dynamic physiological waveforms (ventricular APs) has not yet been reported. The present study was conducted to address both of these issues, through experiments on recombinant WT and T618I channel IhERG conducted at human physiological temperature.

Materials and Methods

Wild-type and T618I hERG

Human Embryonic Kidney (HEK-293) cells stably expressing WT and T618I were grown as described previously [23,26]. Cells were transfected with either the full-length of the hERG insert to ensure that only the correct hERG construct was expressed (Eurofins MWG Operon). Maintenance of Cells and Cell Transfection

Experiments employed HEK-293 cells stably or transiently expressing WT or T618I hERG constructs. Cells were passaged and maintained as described previously [23,26]. For transient transfection experiments, 24 hours after plating cells out they were transfection experiments, 24 hours after plating cells out they were transiently transfected with 0.3 μg of T618I hERG construct using Lipofectamine™ LTX (Invitrogen) according to the manufacturer's instructions. Expression plasmid encoding CD8 was also transiently transfected with 0.3 μg of CD8 (Invitrogen). Successfully transfected cells were identified using Dynagene™ mutagenesis. The following forward primer sequence was used: 5’CGG CGC TCT ACT TCA TCT TCA GCA GCC TCACG’ DNA was sequenced for the full length of the hERG insert to ensure that only the correct mutation had been made (Eurofins MWG Operon).

Electrophysiology

Once in the recording chamber, cells were superfused at 37°C with an external solution containing (in mM): 140 NaCl, 4 KCl, 2.5 CaCl2, 1 MgCl2, 10 Glucose and 5 HEPES (titrated to pH 7.45 with NaOH). Patch-pipettes (Corning 7052 glass, AM Systems) were pulled and heat-polished (Narishige MF83) to 2.5–4 MF2, pipette dialysate contained (in mM): 130 KCl, 1 MgCl2, 5 EGTA, 5 MgATP, 10 HEPES (titrated to pH 7.2 using KOH) [26,27]. hERG current (IhERG) recordings were made using an Axopatch 200, 200A or 200B amplifier (Axon Instruments, now Molecular Devices) and a CV201, CV202A or CV203BU headstage. Between 70–80% of pipette series resistance was compensated. Voltage-clamp commands were generated and data recorded using ‘WinWCP’ (John Dempster, Strathclyde University) or pClamp 9.0 and 10.0 (Molecular Devices). The ventricular action potential (AP) command used for AP clamp experiments was identical to that used in other recent studies from our laboratory [23,27].

Drugs

Disopyramide-phosphate powder (Sigma-Aldrich) was dissolved in Milli-Q water to produce an initial stock solution of 400 mM which was diluted further to produce stock solutions ranging down to 1 mM. Quinidine gluconate salt (Sigma-Aldrich) was dissolved in MilliQ water to produce an initial stock solution of 100 mM, which was diluted further to produce stock solutions ranging down to 30 μM. Flecainide acetate salt (Sigma-Aldrich) was dissolved in MilliQ water to produce an initial stock solution of 10 mM, which was diluted further to produce stock solutions ranging down to 1 mM. D-sotalol (Sequoia) was dissolved in DMSO to produce an initial stock solution of 100 mM, with further dilution of stocks to solutions ranging down to 10 μM. Disopyramide and quinidine containing stock solutions were diluted at least 1:1000-fold with Tyrode’s solution to achieve the final concentrations stated in the Results text. For D-sotalol, dilutions of 1:1000 fold were achievable for all final concentrations except 300 μM, for which a dilution of only 5:1000 fold was possible. During recordings all external solutions were applied using a home-built, warmed and rapid solution exchange device [28].

Data Analysis

Concentration-response data were fitted by a standard Hill equation in order to obtain half-maximal inhibitory concentration (IC50) and Hill-coefficient (nH) values (±95% confidence intervals (C.I.)). Mean data are otherwise presented as mean ± SEM. The voltage dependence of IhERG activation was determined by fitting the values of IhERG tail currents (normalised to peak IhERG tail value and plotted against voltage) with a Boltzmann equation of the form:

\[ I = I_{Max} / (1 + \exp((V_{0.5} - V)/k))] \]

where \( I \) is the IhERG tail amplitude following test potential \( V \), \( I_{Max} \) is the maximal IhERG tail observed during the protocol, \( V_{0.5} \) is the potential at which IhERG was half-maximally activated, and \( k \) is the slope factor for the relationship.

The voltage dependence of IhERG inactivation (assessed by studying availability) was determined by fitting normalised peak IhERG currents elicited by the third step of a three-step protocol (Figure 3A) by the equation:

\[ I/I_{Max} = 1 - (1 + \exp((V_{0.5} - V)/k))] \]

where \( I \) is transient current elicited by the third step of the protocol, following a brief (2 ms) conditioning step \( V_{0.5} \) to relieve inactivation induced by the first step; \( I_{Max} \) is the maximal transient current observed during the protocol and \( V_{0.5} \) and \( k \) denote, respectively, half-maximal inactivation voltage and slope factor for the fit to the plotted relation.
Statistical analysis (SigmaPlot 12) utilised, as appropriate, an unpaired t-test, a Welch’s t-test not assuming equal variances, or a two way repeated measures ANOVA test. P values of less than 0.05 were taken as statistically significant.

Results

Effects of the T618I hERG Mutation on the Voltage-dependence of I_{hERG} and its Activation

Figures 1Aii and Aiii show representative current traces for WT and T618I I_{hERG} elicited by the voltage protocol shown in the lower panels (Figures 1Bii and Bi). WT I_{hERG} increased progressively with the magnitude of the applied voltage command up to ~0/+10 mV, positive to which the current during the applied command declined in amplitude. Prominent I_{hERG} tails were observed on repolarisation to ~40 mV after each voltage command, with tail current amplitude exceeding that of the preceding current during the depolarising step, particularly for positive command voltages. These features are typical of WT I_{hERG} [3,4,25,29]. The traces shown in Figure 1Aii indicate that at potentials negative to 0 mV, T618I I_{hERG} resembled WT I_{hERG}. However, at test potentials between ~0 and +40 mV (over which WT I_{hERG} elicited by depolarising commands became reduced in amplitude), T618I I_{hERG} continued to increase in magnitude. T618I I_{hERG} began to decline in amplitude at test potentials positive to +40 mV. Notably, with positive test commands, T618I I_{hERG} did not exhibit tail currents (I_tails) that exceeded pulse current in amplitude ([24]). Figure 1C shows mean end-pulse current voltage (I-V) relations for WT and T618I I_{hERG}, demonstrating maximal current for WT I_{hERG} at ~+10 mV and an area of negative slope in the I-V relation at more positive potentials. For T618I I_{hERG}, rectification of the I-V relation was positively voltage-shifted, with the area of negative slope in the I-V relation at more positive potentials. For T618I I_{hERG}, rectification of the I-V relation was positively voltage-shifted, with the area of negative slope in the I-V relation at more positive potentials. For T618I I_{hERG} this was ~8.0±3.4 mV (n = 7; p<0.01 versus WT). The corresponding k values were 7.8±1.5 and 8.5±0.9 mV respectively (p>0.05).

Effects of the T618I Mutation on I_{hERG} Activation and Deactivation Time-course

In order to investigate effects of the T618I mutation on the time-course of I_{hERG} activation, we used an “envelope of tails protocol” in which I_tails were measured at ~40 mV following activating commands of different durations from ~80 to 0 mV (see inset to Fig. 2A). I_tails elicited by commands of different duration were normalised to the maximum current during the protocol and plotted as a function of command pulse duration, as shown in Figure 2A ([23,30]). Mono-exponential fits to the data yielded a t_{activation} of 104.1±8.3 ms for WT I_{hERG} and of 112.0±13.0 ms for T618I I_{hERG} (n = 5 and 6 cells respectively; p>0.5), indicating that activation time-course was similar for WT and T618I I_{hERG} during this protocol. In order to compare I_{hERG} deactivation time-course between WT and T618I hERG, I_tails elicited at ~40 mV following voltage commands to +20 mV were fitted with a standard bi-exponential function. Figures 2Bi and Bii show respectively the mean fast and slow time-constants of deactivation (\tau_f ‘fast tau’ and \tau_s ‘slow tau’, respectively) for WT and T618I I_{hERG}. Both fast and slow phases of deactivation were faster for T618I than WT I_{hERG} (evidenced by smaller tau values plotted in Figure 2B; p<0.05 and p<0.01 respectively for \tau_f and \tau_s versus WT). However, the relative proportion of fast and slow deactivation did not differ between WT and T618I I_{hERG} (quantified in Figure 2C as proportion of total deactivating current described by \tau_f).

Effects of the T618I hERG Mutation on I_{hERG} Inactivation

In order to characterize the effect of the T618I mutation on the voltage-dependence of I_{hERG} inactivation, voltage dependent availability of I_{hERG} was determined for WT- and T618I-hERG by applying voltage protocols used in prior investigations from our laboratory to study effects on inactivation of gain-of-function hERG mutations [16,23]. These were comprised of an initial (500 ms) depolarizing step to activate and then fully inactivate I_{hERG} followed by brief (2 ms) repolarizing steps to a range of potentials to relieve inactivation to varying extents, followed by a third depolarization step that elicited a rapidly inactivating I_{hERG}. The magnitude of peak current elicited by the third step reflected the extent of availability induced by the (second) repolarizing step. Similar to prior studies of gain-of-function hERG mutations performed in our laboratory [16,23], in order to ensure complete inactivation of I_{hERG} during the initial step of the voltage protocol, for T618I I_{hERG} a depolarizing step to +80 mV was used, compared to +40 mV for WT I_{hERG}. The lower panels of Figures 3Ai and Aii show the portion of the protocol that incorporated the repolarizing step and subsequent depolarization phases, with the upper panels showing corresponding I_{hERG} records. Peak current amplitudes were obtained by fitting the declining phase of the transient I_{hERG} records with a mono-exponential function and extrapolation to the beginning of the third pulse [16,23]. The resulting values were normalized to the maximal current seen during the protocol and were plotted against repolarization step voltage. The availability/inactivation V_{0.5} value for WT I_{hERG} derived from a fit to the data with equation 2 was ~65.5±2.2 mV with a k value of 19.2±0.6 (n = 11 cells). For T618I I_{hERG}, the corresponding values were: V_{0.5} of ~40.7±5.1 mV and k of 26.9±1.9 mV (n = 6 cells; and, respectively, p<0.01 and 0.001 versus control). For the sake of completeness, the data were further analysed by correction for deactivation using the method of Smith et al. [31] then plotted against voltage and fitted with equation 2 (Figure 3B), which gave V_{0.5} values of ~67.2±2.0 and ~44.3±5.1 mV respectively for WT and T618I I_{hERG} (p<0.01) and respective k values of 21.0±0.6 and 29.8±1.7 mV (P<0.001). Thus, I_{hERG} inactivation V_{0.5} was positively shifted by ~+23 to +25 mV for T618I I_{hERG} compared to WT I_{hERG}, with an accompanying 7 to 9 mV increase in k value. In order to establish the overall effects of altered steady-state voltage-dependent kinetics of the T618I mutation, we calculated ‘window current’ for WT and T618I hERG, as the activation-inactivation variable product across a range of voltages between ~80 and +60 mV. Figure 3C shows that the I_{hERG} window was both positively shifted and significantly larger for T618I than WT I_{hERG}.

The time-course of development of inactivation for WT and T618I I_{hERG} was compared by mono-exponential fitting of the decline of transient currents elicited following the repolarizing step to ~40 mV. This yielded \tau values for WT and T618I I_{hERG} inactivation of 1.86±0.17 ms and 3.32±0.28 ms respectively (Figure 3D; n = 11 and 6 respectively; p<0.01). To compare the rate of recovery from inactivation between the two channels, we used a protocol used in prior I_{hERG} studies ([23,30] see also inset of Figure 3E): a 500-ms depolarisation to +40 mV was applied from a holding potential of ~80 mV to activate and inactivate I_{hERG}. Membrane potential was then repolarised to ~40 mV for an increasing periods of time (between 2 and 20 ms) to induce recovery from inactivation. Transient currents were then subsequently elicited by a 100-ms depolarisation to +40 mV.
Figure 3E shows plots of WT and T618I peak outward transient current magnitude against the duration of the repolarization step (with currents normalized to maximal current seen during the protocol). Fits to the data with a mono-exponential function gave $\tau$ values of $1.99 \pm 0.12$ ms for WT ($n = 7$) and of $1.93 \pm 0.30$ ms for T618I ($n = 9$) ($p > 0.05$).
Collectively, the results from these experiments indicate that the T618I mutation induced a positive shift in the voltage-dependence of IhERG inactivation, augmented the IhERG ‘window’, slowed the time-course of development of IhERG inactivation, but did not alter significantly the rate of recovery of IhERG from inactivation.

Figure 2. WT and T618I IhERG time-course of activation and deactivation. (A) Plots of time-course of IhERG activation obtained using an “envelope-of-tails” protocol—see inset and ‘Results’ text. For each cell, the peak current amplitudes at each time-point were normalized to the maximal current observed during the protocol. (Bi, Bii) Bar charts comparing $\tau_f$ (Bi) and $\tau_s$ (Bii) values for deactivation of WT ($n = 11$) and T618I ($n = 11$) hERG tail currents on repolarisation to $-40$ mV following a 2 s depolarisation from $-80$ mV to $+20$ mV. Currents were fitted with a standard bi-exponential equation (C) Bar-chart showing the proportion of fast deactivation on repolarisation to $-40$ mV for WT and T618I IhERG ($n = 11$ cells for each condition). Asterisks in Bi and Bii denote statistical significance: *p<0.05; **p<0.01.

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The Short QT Syndrome T618I hERG Mutation

**Ai**

WT

| Current amplitude (nA) | Membrane potential (mV) |
|-----------------------|-------------------------|
| 16                    | -140                    |
| 12                    | -120                    |
| 10                    | -100                    |
| 8                      | -80                     |
| 6                      | -60                     |
| 4                      | -40                     |
| 2                      | -20                     |
| 0                      | -10                     |

Time (ms) 520 530 540 550 560 570

**Aii**

T618I

| Current amplitude (nA) | Membrane potential (mV) |
|-----------------------|-------------------------|
| 10                    | -140                    |
| 8                      | -120                    |
| 6                      | -100                    |
| 4                      | -80                     |
| 2                      | -60                     |
| 1                      | -40                     |
| 0                      | -20                     |

Time (ms) 520 530 540 550 560 570

**B**

Normalized current (I/I_max) vs Membrane potential (mV)

**C**

Activation-inactivation parameter product vs Membrane potential (mV)

**D**

Tau inactivation (ms)

WT

T618I

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**E**

Normalized current (I/I_max) vs Time (ms)

WT

T618I
Effects of the T618I Mutation on \(h_{ERG}\) under Action Potential Voltage Clamp

Figure 4Ai shows a representative record of WT \(h_{ERG}\) elicited by a ventricular AP command (superimposed on the current trace in Figure 4Ai). As reported previously (e.g. [25,30,32]), the elicited current was comparatively small immediately after AP depolarisation, then increased progressively during the plateau phase of the AP, before declining during terminal repolarisation. Figure 4Aii shows similar recordings for T618I \(h_{ERG}\). The profile of current during the AP command differed from that for WT \(h_{ERG}\): current increased earlier during the AP command, peaking earlier during the AP plateau and then it declined during the latter part of the plateau phase. Figures 4Bi and Bii show representative normalized instantaneous current-voltage (\(I-V\)) relations for \(h_{ERG}\) during the repolarising phase of the AP command. Peak outward current was positively shifted by \(\sim +35\) mV for T618I \(h_{ERG}\) (from \(-30.7\pm1.2\) mV for WT, to \(+5.1\pm2.1\) mV for T618I \(h_{ERG}\); \(P<0.001\) versus WT). Example instantaneous conductance-voltage (\(G-V\)) relations (of [16,32,33]) for WT- and T618I-\(h_{ERG}\) are shown in Figures 4Ci and Cii, respectively. As described previously [16,32,33], the macroscopic conductance of WT-\(h_{ERG}\) increased throughout the AP repolarisation phase, being maximal late in repolarisation (Figure 4Ci). In contrast, for T618I \(h_{ERG}\) conductance increased steeply early in repolarisation (between \(-20\) and \(-20\) mV) and then progressively declined as the membrane potential followed the direction of membrane repolarisation. Figure 4D shows mean data for the maximal amplitudes of WT and T618I \(h_{ERG}\) during the applied ventricular AP command waveform, demonstrating a significantly greater (\(\sim 2.0\)-fold) maximal repolarising current when \(h_{ERG}\) channels incorporated the T618I \(h_{ERG}\) mutation. Considered collectively, the findings from our AP clamp experiments indicate that greater repolarising \(h_{ERG}\) tail current would be expected earlier during ventricular APs in the setting of T618I-linked SQT1.

Pharmacology of the T618I hERG Mutation

Figure 5A compares the response of WT and T618I \(h_{ERG}\) to 1 \(\mu M\) of the Class Ia antiarrhythmic drug quinidine, using conventional voltage-clamp. Figures 5Ai and Aii show representative \(I_{h_{ERG}}\) traces elicited by the voltage protocol shown in the lower panels (a standard depolarising step protocol used in previous studies of \(h_{ERG}\) pharmacology from our laboratory (e.g. [23,26,34])). Tail current magnitude was measured relative to instantaneous current at \(-40\) mV elicited by the brief (50 ms) step depolarising step that preceded the voltage command to \(+20\) mV in the absence and presence of the drug. 1 \(\mu M\) quinidine reduced WT \(h_{ERG}\) markedly, with \(53.9\pm2.1\%\) (\(n = 13\)) inhibition of the \(I_{h_{ERG}}\) tail current (compatible with prior reports of a submicromolar IC\(_{50}\) under similar recording conditions [20,35]). For T618I \(h_{ERG}\) the reduction in current was similar to that seen for the WT current, with 1 \(\mu M\) quinidine reducing T618I tail current magnitude by \(47.6\pm5.0\%\) (\(n = 8\); NS versus WT). A range of quinidine concentrations between 10 \(nM\) and 10 \(\mu M\) were tested and concentration response relations constructed as shown in Figure 5B. The derived IC\(_{50}\) for WT \(h_{ERG}\) was 0.64 \(\mu M\) (for C.I. and C.I. values see Table 1) whilst for T618I \(h_{ERG}\) the comparable value was 0.88 \(\mu M\). Thus, the IC\(_{50}\) for \(h_{ERG}\) tail block by quinidine for T618I \(h_{ERG}\) was \(\sim 1.4\)-fold that for WT \(h_{ERG}\) (see also Table 1).

The Class Ia antiarrhythmic drug disopyramide has been found to be effective against the N588K \(h_{ERG}\) SQT1 mutation [20,21], but its effects on T618I \(h_{ERG}\) are unknown. Therefore we tested the effects of disopyramide on the T618I \(h_{ERG}\) mutant (Figure 6). Figures 6Ai and Aii show representative \(I_{h_{ERG}}\) traces in the absence and presence of the drug, with the protocol shown in lower panels. As expected from previous studies [20,26,36], 10 \(\mu M\) disopyramide reduced WT \(h_{ERG}\) by 55.9\% (\(n = 13\)) whereas it reduced T618I \(h_{ERG}\) by 42.9\% (\(n = 5\); \(p<0.05\) versus WT). Three other disopyramide concentrations were tested on T618I mutant channels and concentration response relations constructed as shown in Figure 6B. For T618I \(h_{ERG}\) the disopyramide IC\(_{50}\) was 16.83 \(\mu M\) whilst for WT \(h_{ERG}\) inhibition the corresponding value was 7.68 \(\mu M\). Thus, the IC\(_{50}\) for T618I \(h_{ERG}\) tail inhibition by disopyramide was \(\sim 2.2\)-fold that for the WT channel.

Similar experiments were also conducted with the Class III antiarrhythmic drug D-sotalol and the Class Ic antiarrhythmic drug flecainide. Figures 6C and 6D show the concentration-response relations for inhibition of WT and T618I \(h_{ERG}\) by these drugs. For D-sotalol (Figure 6C) the derived IC\(_{50}\) for WT \(h_{ERG}\) was 112.2 \(\mu M\) whilst for T618I \(h_{ERG}\) the comparable value was 356.6 \(\mu M\) (\(\sim 3.2\)-fold that for WT \(h_{ERG}\)). For flecainide, the derived IC\(_{50}\) for WT \(h_{ERG}\) was 1.87 \(\mu M\) whilst that for T618I \(h_{ERG}\) was 4.67 \(\mu M\) (\(\sim 2.5\)-fold that for WT \(h_{ERG}\)). Results obtained under conventional voltage clamp for all four drugs are summarized in Table 1.

The limited data currently available on T618I \(h_{ERG}\) pharmacology appear to suggest some difference in the effect of the mutation on inhibition of pulse and tail currents by 1 \(\mu M\) quinidine and 500 \(\mu M\) sotalol, during conventional voltage clamp [24]. Ventricular APs involve dynamic changes in membrane potential that influence the profile of observed current; therefore we conducted additional experiments in which concentration-response relations for the 4 drugs examined under conventional voltage clamp were also determined from ventricular AP clamp experiments. For these, the percentage of inhibition of peak \(I_{h_{ERG}}\) during the AP waveform for three different drug concentrations was calculated for each drug. Concentration-response relations were then constructed as shown in Figure 7. Figures 7Ai and Aii
Figure 4. Action potential (AP) voltage clamp of WT and T618I I_{hERG}. (A) I_{hERG} (after p/4 subtraction) elicited by ventricular AP command for WT (Ai) and T618I (Aii) I_{hERG}. Currents are shown overlaid with the voltage protocol. (B) Instantaneous I–V relations for I_{hERG} elicited in A for WT (Bii) and T618I (Biii) I_{hERG}. Current during the repolarising phase of the AP are plotted. Arrows denote direction of repolarisation. (C) Instantaneous conductance-voltage (G–V) relations for I_{hERG} elicited in A for WT (Ci) and T618I (Cii) I_{hERG} during AP repolarisation. (D) Magnitude of peak repolarising current during AP voltage clamp, plotted for WT (n = 14) and T618I (n = 19) I_{hERG}. * denotes statistically significant difference from WT at p<0.05.

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show representative current traces for WT and T618I I_{hERG} in the presence and absence of quinidine; the ventricular AP command is superimposed over each set of traces. In this example, 1 mM quinidine reduced maximal I_{hERG} during repolarisation by 67% for WT I_{hERG} and 56% for T618I I_{hERG}. Figures 7B–E show concentration-response curves for inhibition of maximal I_{hERG} during repolarisation by quinidine, disopyramide, sotalol and flecainide (Figures 7B–E respectively), whilst Table 2 summarises numerical data for IC_{50} and nH values. The derived IC_{50} values for WT and T618I I_{hERG} inhibition by quinidine (Figures 7A,B) were, respectively, 0.55 mM and 1.09 mM (~2.0 fold the WT value). For disopyramide (Figure 7C), the WT I_{hERG} IC_{50} was 6.47 mM and that for T618I I_{hERG} was 10.65 mM (1.6-fold the WT value). For D-sotalol (Figure 7D) the IC_{50} for WT I_{hERG} was 109.5 mM, whilst that for T618I I_{hERG} was 189.2 mM (~1.7-fold that for WT I_{hERG}). Finally, we found flecainide (Figure 7E) to inhibit WT and T618I I_{hERG} with an IC_{50} of 1.96 mM and of 2.29 mM (1.2 fold the WT value) respectively. Thus, under AP clamp all four drugs exhibited comparatively modest attenuation of their inhibitory action with the T618I mutation.

Discussion

To our knowledge, the present study is the first to have established the effects of the T618I hERG mutation on the profile of I_{hERG} during physiological waveforms. Although limited (single concentration) in vitro data have been reported for racemic sotalol and quinidine [24], the present study is the first to provide concentration response data for any drug against T618I hERG and it is also the first to provide any in vitro data for T618I I_{hERG} inhibition by disopyramide, D-sotalol and flecainide.

Effects of the T618I Mutation on I_{hERG}

Although the recent study by Sun and colleagues is the first report of the occurrence of the T618I hERG mutation in a clinical context [24], one other investigation has utilised this mutation in the study of the role of a nearby S5 residue (H562) that is able to interact with the pore helix [37]. In that study, T618I I_{hERG} was shown to exhibit both increased currents at positive voltages and reduced tail currents compared to pulse currents following positive voltage commands [37]. These features are in accord with the subsequent report by Sun and colleagues and with our own data. However, Lees-Miller and colleagues reported a significant (>+30 mV) positive shift in I_{hERG} activation V_{0.5} for T618I hERG [37], whilst Sun et al. reported a small (~5 mV) negative shift in activation V_{0.5} compared to WT I_{hERG} [24]. Both studies were conducted using HEK cells for hERG channel expression, whilst Lees Miller et al. performed measurements at 36 °C [37] and the recording temperature for the study by Sun et al. was not given [24]. Thus, the reason for the apparently opposite observations in the two studies in respect of activation V_{0.5} is not clear. In our experiments, using a similar expression system and recording at 37 °C, there was a +15 mV shift in activation V_{0.5}, which is in good qualitative agreement with the findings of Lees Miller et al. [37], but differs from the negative activation shift of V_{0.5} reported by Sun and colleagues [24]. However, unlike Lees-Miller et al. [37] and in accord with Sun and colleagues [24], we saw a significant effect of the mutation on I_{hERG} deactivation time-course, with both τ_1 and τ_2 time constants of T618I I_{hERG} deactivation smaller than those for WT I_{hERG}. Also in accord with Sun and colleagues [24], we did not observe any significant alteration to the rate of I_{hERG} activation for the T618I hERG mutant. The shift in voltage dependence of I_{hERG} inactivation (availability) seen here (Figures 3A,B) is in qualitative agreement with positive shifted inactivation reported by Sun et al. [24], as is the positively shifted region of negative slope in the end pulse I–V relation (Figure 1C). However, the extent of positive shift in inactivation V_{0.5} (~+23–25 mV) seen here was smaller than that reported by Sun and colleagues (~+50 mV); the reason for this difference is at present not clear. Nevertheless, the shift steady-state inactivation seen here is demonstrably sufficient to lead to a significant functional impact: ‘window current’ calculations based on our derived activation and inactivation V_{0.5} and k values revealed significant augmentation, as well as positively shifted peak, of the I_{hERG} window for T618I hERG when compared with WT hERG (Figure 3C). Slowing of time-dependent development of inactivation of I_{hERG} (Figure 3D) may have a synergistic effect in permitting greater I_{hERG} to flow. A modulatory effect of the T618I mutation on I_{hERG} inactivation is not entirely unexpected, given that mutation of the nearby S620 residue (to S620T) has been established to abolish hERG channel inactivation (e.g. [38,39]), although in contrast to the S620T

Table 1. Pharmacology of the T618I hERG mutant studied with conventional voltage clamp.

| Drug        | WT I_{hERG} IC_{50} (μM) | WT I_{hERG} nH | T618I I_{hERG} IC_{50} (μM) | T618I I_{hERG} nH | Fold IC_{50} |
|-------------|---------------------------|----------------|------------------------------|-------------------|--------------|
| Quinidine   | 0.64 (CI 0.51–0.79)       | 0.65 (CI 0.55–0.75) | 0.88 (CI 0.41–1.88)         | 0.41 (CI 0.25–0.57) | 1.4 (0.8–2.4) |
| Disopyramide| 7.68 (CI 6.32–9.34)       | 0.87 (CI 0.66–1.07) | 16.83 (CI 8.56–33.09)       | 0.47 (CI 0.30–0.64) | 2.2 (1.4–3.5) |
| D-Sotalol   | 112.2 (CI 91.7–137.3)     | 0.74 (CI 0.57–0.91) | 356.6 (CI 305.6–416.1)      | 0.85 (CI 0.70–1.00) | 3.2 (3.0–3.3) |
| Flecainide  | 1.87 (CI 1.56–2.25)       | 0.81 (CI 0.68–0.94) | 4.67 (CI 3.06–7.13)         | 0.58 (CI 0.45–0.71) | 2.5 (2.0–3.2) |

IC_{50} and nH values shown are derived from fits to concentration-response relations in Figures 5 and 6, obtained from fractional inhibition of I_{hERG} using a voltage step protocol (shown in Figures 5A and 6A). Columns show mean values and 95% confidence intervals (CI). The right-hand column expresses the ratio of the T618I IC_{50} to the WT IC_{50} value, to one decimal place. The numbers in parentheses in the right hand column represent the range of ratio values for the ± CIs for derived T618I/WT IC_{50}.

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It is clear that the T618I mutation produces a more modest, partial attenuation of I\textsubscript{hERG} inactivation.

Pharmacology of T618I h\textsubscript{hERG}

It is well established that the inactivation process is important for binding to the h\textsubscript{hERG} channel of a range of drugs, but it is also the case that not all drugs are equally dependent upon inactivation for binding to the channel to occur (e.g. \cite{18,20,21,38–42}). The SQTL N588K mutation has been shown to lead to markedly elevated IC\textsubscript{50} values for I\textsubscript{hERG} blockade by methanesulphonanilide compounds including sotalol (20-fold WT I\textsubscript{hERG} IC\textsubscript{50} for D-sotalol) and E-4031 (~11.5-fold WT I\textsubscript{hERG} IC\textsubscript{50}), whilst those for quinidine (~3.5–6-fold WT I\textsubscript{hERG} IC\textsubscript{50}) and disopyramide (1.5-fold WT I\textsubscript{hERG} IC\textsubscript{50}) are comparatively little affected \cite{18,20}. In the present study, the T618I h\textsubscript{hERG} mutation elevated the IC\textsubscript{50} for D-sotalol by ~3 fold under conventional voltage clamp, which is substantially less than that seen for N588K h\textsubscript{hERG} \cite{18}. With a voltage protocol similar to that used in the present study, I\textsubscript{hERG} N588K h\textsubscript{hERG} availability was found to be positively shifted by ~+62 mV \cite{16}. In the present study, the shift in the voltage dependence of T618I I\textsubscript{hERG} inactivation was ~+23–25 mV. It therefore seems likely that the smaller effect of the T618I mutation in attenuating I\textsubscript{hERG} block by D-sotalol can be attributed to the ability of the T618I channel to inactivate to a greater extent than has been found to be the case for N588K h\textsubscript{hERG}. In this regard, it is noteworthy that the results of experiments in which different...
inactivation mutations have been combined in order to titrate hERG inactivation suggest that hERG blocking potency is not related to inactivation in a linear fashion. Thus, for both quinidine and disopyramide single mutations that reduced macroscopic hERG inactivation to $\sim20\%$ produced only modest (1.5 and 3.5 fold) reductions in the potency of pyridine and quinidine, whilst double-mutations that reduced inactivation to $<10\%$ led to elevations of IC50 by 6.5 and 7-fold respectively [21]. For T618I hERG in this study, the IC50 for quinidine under conventional voltage clamp was $\sim1.4$ that of the WT channel, whilst for disopyramide it was $\sim2.2$-fold that of the WT channel. The attenuation of hERG inactivation by the T618I mutation therefore appears to be insufficient to interfere dramatically with drug binding. At the same time, the greater effect of this mutation on disopyramide’s potency under conventional voltage clamp than that seen previously for the N588K mutation (which impairs inactivation to a greater extent than does T618I [15,16,21,24]) suggests that other effects of the mutation on channel conformation as well as upon inactivation per se may contribute to its overall effect on disopyramide binding. To our knowledge there are no prior data available on effects of inactivation-attenuating hERG mutants on hERG blocking potency of flecainide. A prior study from our laboratory has shown that the characteristics of flecainide inhibition of WT hERG are qualitatively similar to those of the Class Ia antiarrhythmic quinidine and of another Class Ic drug, propafenone [35]. Given that quinidine and propafenone have all been shown to exhibit comparatively little dependence on hERG channel inactivation to exert their inhibitory effects [20,21,36,42] and also that the T618I mutation produced only a modest effect on hERG blocking potency in this study, it seems reasonable to conclude that hERG channel inactivation is not a major determinant of flecainide potency against hERG. Further experiments on attenuated inactivation mutants are required to determine unequivocally whether or not this is the case.

We also compared between WT and T618I hERG the potency of hERG inhibition under AP clamp, for each of the drugs studied under conventional voltage clamp. It is known that drug inhibitory potency against hERG can vary depending on stimulus protocol [34,43,44]. In our experiments, both stimulus waveform (step versus AP command) and stimulus frequency (repetitive pulsing once every 12 s versus once every second -to apply APs at a physiological rate) differed between the protocols used to obtain the data in Figures 6 and 7. However, the IC50 values for WT hERG inhibition by any of the drugs studied did not differ greatly between conventional and AP clamp protocols (see Tables 1 and 2). In general, however, differences between IC50 values obtained with conventional and AP clamp protocols were greater for T618I hERG, although the C1 range for IC50 with the two protocols showed either some overlap (quinidine, disopyramide, D-sotalol) or little separation (flecainide). The range of T618I/WT IC50 ratio values was found to be somewhat smaller (1.2–2.0) under AP clamp than under conventional voltage clamp (1.4–3.2), with a marked reduction in this for D-sotalol. Contributory factors to this may be intrinsic voltage-dependence of inhibition [34–36] together with the occurrence (and hence measurement) of peak hERG at a comparatively positive voltage for T618I compared to WT hERG during the AP waveform [and compared to the measurement voltage ($\sim40$ mV) for T618I hERG tails under conventional voltage clamp], and a greater sensitivity of drug block to duty-cycle (rate) for the mutant. On the basis of our findings, future detailed investigation of effects of T618I hERG kinetics on channel block are likely to be instructive in this regard, though are beyond the intended scope of the present study.

One puzzling aspect of our pharmacology data is that for the Class I drugs studied, $n_H$ values derived from concentration-response relations obtained under conventional voltage clamp were substantially lower ($<0.5$ for quinidine and disopyramide) for T618I than for WT hERG, whilst this was not the case under AP clamp (compare Tables 1 and 2). The low $n_H$ values under conventional voltage clamp do not appear to be attributable to voltage-drop down uncompensated series resistance for T618I hERG recordings: estimated voltage drop was lower for quinidine ($2.91 \pm 0.37$ mV; $n = 16$) than for D-sotalol ($7.61 \pm 0.37$ mV; $n = 21$), although the $n_H$ value was higher for D-sotalol than for quinidine (Table 1). On the other hand, were the marked

### Table 2. Pharmacology of the T618I hERG mutant studied with action potential voltage clamp.

| Drug     | WT $\text{IC}_{50}$ ($\mu$M) | WT $n_H$ | T618I $\text{IC}_{50}$ ($\mu$M) | T618I $n_H$ | Fold $\text{IC}_{50}$ |
|----------|-----------------------------|---------|--------------------------------|-------------|-----------------------|
| Quinidine | 0.55 (C1 0.43–0.71)         | 0.91 (C1 0.65–1.18) | 1.09 (C1 0.82–1.46) | 0.92 (C1 0.59–1.26) | 2.0 (1.9–2.1) |
| Disopyramide | 6.47 (C1 3.76–11.12)     | 0.71 (C1 0.40–0.99) | 10.65 (C1 5.73–19.80) | 0.69 (C1 0.35–1.05) | 1.6 (1.5–1.8) |
| D-Sotalol   | 109.5 (C1 70.7–169.6)    | 0.92 (C1 0.47–1.37) | 189.2 (C1 133.3–268.6) | 0.90 (C1 0.56–1.25) | 1.7 (1.6–1.9) |
| Flecainide  | 1.96 (C1 1.49–2.59)      | 1.05 (C1 0.79–1.30) | 2.29 (C1 1.72–3.05) | 0.86 (C1 0.67–1.05) | 1.2 (1.1–1.2) |

$\text{IC}_{50}$ and $n_H$ values shown are derived from fits to concentration-response relations in Figure 7, obtained from fractional inhibition of hERG using a voltage step protocol (shown in Figure 7A). Columns show mean values and 95% confidence intervals (C1). The right-hand column expresses the ratio of the T618I IC50 to the WT IC50 value, to one decimal place. The numbers in parentheses in the right hand column represent the range of ratio values for the ± C1s for derived T618I/WT IC50s.

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Figure 7. Pharmacology of WT and T618I $I_{hERG}$ under AP voltage clamp. (A) Shows representative traces (after p/4 subtraction) of WT (Ai) and T618I (Aii) $I_{hERG}$ elicited by AP voltage clamp protocol (overlain) in control solution and after exposure to 1 μM quinidine. Note different current scales in Ai and Aii. AP commands were applied at 1 Hz. (B–D) Concentration response relations for inhibition of WT and T618I peak repolarising current observed during AP clamp by quinidine (B; 3 concentrations tested, n = 4 to 5 cells per concentration); disopyramide (C; 3 concentrations tested, n = 4 to 5 cells per concentration); D-sotalol (D; 3 concentrations tested, n = 4 to 5 cells per concentration); flecainide (E; 3 concentrations tested, n = 4 to 5 cells per concentration). doi:10.1371/journal.pone.0052451.g007
reduction in n_\text{H} for quinidine and disopyramide strongly reflective of altered drug-channel interaction due to the T618I mutation it might be anticipated also to occur for data from AP clamp experiments and this was not the case. The basis for the apparently low n_\text{H} for quinidine and disopyramide for T618I I_{\text{hERG}} remains unexplained at the present time. Arguably, the more (patho)physiologically relevant pharmacological data are those obtained under AP clamp at a physiologically relevant rate; the major conclusion from those data (Figure 6 and Table 2) is that under these conditions the T618I mutation did not produce a large attenuation of inhibitory potency for any drug studied.

Clinical Relevance

In this study we observed that, under AP clamp, T618I mutant I_{\text{hERG}} exhibited an altered current profile, peaking earlier during the AP plateau than was the case for WT I_{\text{hERG}}. Previous studies in which the SQT1 N588K hERG mutant has been studied under ventricular AP clamp have shown an inverted U or bow-shaped current profile peaking at ~−20 mV, consistent with the occurrence of little inactivation over physiologically relevant membrane potentials [13,15,16,33]. The N588K hERG mutation produces a greater attenuation of I_{\text{hERG}} inactivation than does T618I hERG and our AP clamp data are suggestive of an electrophysiological phenotype for T618I hERG during the ventricular AP that is intermediate between those of WT and N588K I_{\text{hERG}}. Accordingly, the effect of the T618I mutation in accelerating ventricular AP repolarisation can also be predicted to be less than that of N588K hERG. This is in agreement with the less extensive QTc interval shortening for SQT1 patients with the T618I mutation (mean in affected individuals of 316 ms) [24] than those with the N588K mutation (QTc of ≤300 ms in the first two SQT1 genotyped families [13] and a QT of 230 ms in the proband of a third family [14]). The normally slow deactivation of I_{\text{Kr}}/I_{\text{hERG}} can contribute to resting membrane conductance and protection from premature depolarisation immediately after completion of ventricular AP repolarisation; in pathological settings accelerated I_{\text{Kr}} deactivation may increase excitability early in diastole [30,45]. Whether or not the faster deactivation of T618I than WT hERG is able to contribute abbreviated refractory period and susceptibility to programmed stimulation (as clinically observed for SQT1 patients with the T618I mutation [24]) remains to be established, but warrants future in silico investigation [30,46].

A first line treatment for the SQTS is the use of implantable defibrillators (ICDs) to protect against sudden death, although ICD use itself carries the risk of inappropriate shocks [47]. A recent report of long term follow up of SQTS patients noted that 58% of patients with ICDs had device-related complications [48]. The same report [48] notes that the T618I SQT1 mutation has now been found in a second family in addition to that originally identified by Sun and colleagues [24]. Pharmacological therapy is therefore attractive both for patients in whom ICDs are not fitted and as an adjunct therapy to reduce arrhythmic events and restore QT intervals towards normal. Our findings extend those previously obtained at a single (>70% blocking) quinidine concentration [24]; collectively the full concentration response data obtained with both conventional voltage and AP clamp protocols indicate that quinidine largely retains its potency against T618I I_{\text{hERG}}. The available in vitro data therefore indicate that quinidine is likely to be beneficial in T618I-linked SQTS. Concordant with this, hydroquinidine has recently been reported to have a positive effect on QTc intervals in T618I hERG carriers [49]. However, whilst the available evidence from long-term follow up of SQTS patients is that (hydro)quinidine is also effective in arrhythmia prophylaxis [48], diminishing availability of quinidine [49] makes it attractive to find alternative pharmacological therapies for use in SQTS patients. Disopyramide is effective against the N588K mutation in vitro [20] and has shown benefits in SQT1 patients [19]. Our experiments indicate that although there is a modest reduction in disopyramide potency for T618I I_{\text{hERG}}, both under conventional and AP voltage protocols, our data indicate that some I_{\text{hERG}}/I_{\text{Kr}} blockade can nevertheless be expected to occur within the clinical concentration range (~6–8 µM; [50]). Thus, disopyramide may be worthy of investigation as a potential treatment for T618I-linked SQT1. Sun et al. have suggested that T618I hERG carriers may be less resistant to drugs like sotalol than had been previously found for N588K-linked SQT1 [24]. Our data with D-sotalol, particularly those obtained under AP voltage clamp, support this proposition; the reduction in I_{\text{hERG}} blocking potency of D-sotalol by T618I hERG appears to be substantially less than that produced by N588K hERG [18]. It is possible, therefore, that sotalol may be worthy of clinical investigation for T618I-linked SQT1. Future work may also be warranted to determine whether higher affinity methanesulphonanilide Class III drugs than sotalol that are in clinical use (ibutilide, dofetilide) are able to exert some inhibition of T618I at clinically relevant concentrations. Of particular note, flecainide was found to exert marked inhibition of both WT and T618I I_{\text{hERG}} at concentrations relevant to clinical serum levels (0.5 to 2.4 µM; [51]), with little difference between WT and T618I I_{\text{hERG}} IC_{50} under AP clamp. Flecainide has been tested previously in a group of SQT1 (N588K hERG) patients unresponsive to sotalol but responsive to hydroquinidine [52]. In that study it was found to produce a small prolongation of QT interval in some patients, which was largely attributable to QRS interval lengthening [52]. To our knowledge, comparable data are lacking in patients with the T618I hERG SQT1 mutation. However, on the basis of our findings flecainide may warrant investigation in this group. Sun and colleagues have also provided evidence that a >70% blocking concentration of quinidine reduces the inactivation shift for T618I I_{\text{hERG}} [24]. However, in our AP clamp experiments none of the drugs studied produced any consistent correction of T618I IhERG profile during a physiological waveform; this suggests that potential benefits of the drugs studied here for QT intervals in patients with the T618I hERG mutation are likely to be attributable to reduction in total repolarising I_{\text{hERG}}/I_{\text{Kr}} without restoration of the current’s normal time- and voltage-dependent profile during ventricular APs.

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Author Contributions

Conceived and designed the experiments: AEH JCH. Performed the experiments: AEH DM YZ. Analyzed the data: AEH DM YZ. Wrote the paper: AEH JCH. Involved in ventricular AP clamp approach used in the study and edited/commented on a completed form of the manuscript: HZ.

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