Biophysical Characterization of the Short QT Mutation hERG-N588K Reveals a Mixed Gain-and Loss-of-Function

Morten Grunnet^{1,2}, Thomas Goldin Diness^{1,2}, Rie Schultz Hansen¹ and Søren-Peter Olesen²

¹NeuroSearch A/S, Pederstrupvej 93, 2750 Ballerup, and ²The Danish National Research Foundation Center for Cardiac Arrhythmia, The Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen N

Key Words

Ion channels • K-channel • Arrhythmic mechanisms • Ventricular arrhythmia • N588K

might also have impact on the pathological picture of the hERG-N588K channels ability to trigger arrhythmic events.

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Abstract

The short QT syndrome is a newly discovered pro-arrhythmic condition, which may cause ventricular fibrillation and sudden death. Short QT can originate from the apparent gain-of-function mutation N588K in the hERG potassium channel that conducts repolarising I_{Kr} current. The present study describes a profound biophysical characterization of hERG-N588K revealing both loss-of-function and gain-of-function properties of the mutant. Experiments were conducted after heterologous expression in both *Xenopus laevis* oocytes and mammalian cells and at both room temperature and at 37 °C. Also the impact of the β -subunits KCNE2 was investigated. The most prominent loss-of-function property of hERG-N588K was reduced tail currents but also the activation properties was compromised. Based on these biophysical results we suggest that the general view of hERG-N588K being a gain-of-function is modified to a mixed gain- and loss-of-function mutation. This

Introduction

Structural abnormalities account for the majority of sudden cardiac deaths. However, about 10-20 % of sudden cardiac death cases are observed in hearts showing no obvious morphologically changes [1, 2]. With the increased impact of clinical molecular genetics it has become clear that channelopathies, *i.e.* diseases caused by mutations in ion channels, can explain a number of the idiopathic sudden cardiac death syndromes. Examples of such cardiac electrical diseases are the Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia, the long QT syndrome and the short QT syndrome (SQTS) [3-5]. SQTS is a new clinical entity first described in 2000 [6]. This case report demonstrated that short QT intervals could be inherited and likely associated with both atrial and ventricular arrhythmias. Even though SQTS is a very rare disease it displays some common clinical characteristics. QT interval is undoubt-

edly shortened. So far, patients with corrected QT intervals up to 320 ms have been reported as SQTs even though no firm limit has been defined. Another manifestation of SQTs seen on the electrocardiogram is tall, narrow and symmetrically T waves in pre-cordial leads. One exception is the recently described family by Priori et al. demonstrating asymmetrical T waves in lead V3 [7, 8]. The effective refractory period is abbreviated in both atria and ventricles, and ventricular tachycardia can be triggered by extrasystoles. Approximately 50 % of patients have mild arrhythmic events such as palpitations and atrial arrhythmias, while major events such as syncope and cardiac arrest are less common [9]. First line treatment of SQTs is implantable cardioverter-defibrillators (ICD's) [10-12]. Until now drug treatment is restricted to the class Ia antiarrhythmic compounds quinidine and disopyramide [13, 14] while atrial fibrillation in SQTs patients can be prevented by propafenone [10]. A link between SQTs and sudden cardiac death was provided in 2003 [15]. Here two independent families with profound family histories of sudden cardiac death present in 3 and 4 generations were described. Examined individuals were characterized by Bazett corrected QT intervals below 300 ms and inducible ventricular fibrillations. SQTs as a channelopathy was demonstrated in 2004 in a case report where 2 out of 3 families with hereditary SQTs had missense mutations in the human ether a-go-go related gene (hERG) potassium channel. Recently, two other genes involved in cardiac repolarization have been linked to SQTs. These are the potassium channels KCNQ1 (Kv7.1) [16] and KCNJ2 (Kir2.1) [7] conducting the I_{Ks} and I_{K1} , respectively. Of the 3 genes so far linked to SQTs, the *KCNH2* (hERG or Kv11.1) channel has been characterized in greatest detail. In 2 independent families with SQTs the same missense mutation in hERG has been found, namely a substitution of asparagine in position 588 with a positively charged lysine [17].

hERG-WT channels conduct the cardiac repolarizing I_{Kr} current [18, 19]. The channel is characterized by complex gating. In short, upon activation by depolarisation fast and profound inactivation is observed. Inactivation is released during repolarization. This implies that hERG channels will be active at phase 3 in the cardiac action potential thereby explaining the importance of this channel as a dominant part of the repolarizing reserve in humans [20]. Biophysical characterization of hERG-N588K has revealed a channel with severely compromised inactivation abilities [14, 17, 21]. As a result hERG-N588K conducts large current during the plateau phase (phase 2) of the action potential, resulting in abbreviated action

potentials, and has been reported as a gain-of function mutation. In the present paper we demonstrate how hERG-N588K in addition to having reduced inactivation also exhibits slower activation and faster deactivation kinetics. It must therefore be expected that the hERG-N588K mutant will have reduced ability to conduct current at the end of repolarisation. Further, this mutant will also have a diminished open probability in the diastolic interval. We therefore stress that in patients carrying hERG-N588K the loss-of-function of repolarization current and diastolic hERG current may be at least as proarrhythmic as the gain-of-function of plateau current.

Materials and Methods

Molecular biology

cDNA encoding hERG1 were introduced into the custom-made vector pXOOM, which is optimized for expression in both *Xenopus laevis* oocytes and mammalian cells [22]. pGEM_KCNE2 was a kind gift from Dr. Nicole Schmitt. The N588K point mutation was introduced using the QuikChange® Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA).

cRNA preparation and capping were performed by *in vitro* transcription using the Ambion T7 m-Message Machine kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. mRNA was purified using the Ambion MEGAClear kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction and dissolved in TE buffer to approximate concentrations of 0.1 µg/µl. For proof of purity and integrity, mRNA was inspected by gel electrophoresis and concentrations were determined photometrically. mRNA was stored at -80 °C until injection.

Expression in Xenopus laevis oocytes

Xenopus laevis surgery and oocyte treatment were done as previously described [23]. Oocytes were collected under anaesthesia (Tricain 2 g/l, Sigma A-5040) at guidelines approved by the Danish National Committee for Animal Studies. Before injection of 50 nl mRNA (approximately 5 ng), oocytes were kept for 24 hours at 19 °C in Kulori medium consisting of (in mM) 90 NaCl, 1 KCl, 1 MgCl₂, 1 CaCl₂, 5 HEPES, pH 7.4 with NaOH. Injection of mRNA was accomplished using a Nanoject microinjector from Drummond (Drummond Scientific, Broomall, PA, USA). For oocytes expressing hERG-N588K and KCNE2 a molar 1:1 ration was applied. Oocytes were kept at 19 °C for 1-3 days before measurements were performed.

Expression in mammalian HEK-293 cells

HEK-293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (GibcoBRL, Life Technologies) at 37 °C in 5% CO₂. For stable expression, cells were incubated for 3-5 hour with a transfection mixture of cDNA, Lipofectamine and PLUS Reagent in Optimem media (Life Technologies). The stable

hERG-WT HEK cell line was made by picking single clones after selection with geneticin (0.5 mg/ml DMEM) for 3 weeks and subsequently testing for the expression of hERG-specific currents by patch-clamping. For expression of hERG-N588K transient transfections were performed with the pXOOM vector that was also used in oocytes as described above. The transiently transfected cells were used for experiments 24-48 hours after transfection.

Electrophysiological recordings in oocytes

Current through expressed WT-hERG channels, hERG-N588K or hERG-N588K/KCNE2 was monitored using a two-electrode voltage-clamp amplifier (Dagan CA-1B, Minneapolis, MN, USA). Electrodes were pulled from borosilicate glass capillaries on a horizontal patch electrode puller (DMZ universal puller, Zeitz instruments, München, Germany) and had tip resistance between 0.5 and 2.5 MΩ when filled with 2 M KCl. During the experiments oocytes were placed in a small chamber (volume: 200 μl) connected to a continuous flow system (flow: 3 ml/min.). hERG channels were activated by membrane depolarization and channel activity was measured in Kulori solution consisting of (in mM) 87 NaCl, 4 KCl, 1 MgCl₂, 1 CaCl₂, 5 HEPES, pH 7.4 with NaOH. The only exception being experiments designed to address the effect of hypo (2 mM K⁺) or hyperkalemic (10 mM K⁺) conditions. The composition of the Kulori solution was under these conditions (in mM) 89 NaCl, 2 KCl, 1 MgCl₂, 1 CaCl₂, 5 HEPES, pH 7.4 with NaOH and 81 NaCl, 10 KCl, 1 MgCl₂, 1 CaCl₂, 5 HEPES, pH 7.4 with NaOH.

The exact voltage protocols are indicated in the respective figures. All experiments were performed at room temperature. The condition of each single oocyte was controlled before measurements by recording membrane potentials. Only oocytes with membrane potentials more negative than -30 mV were used for current recordings. The hERG opener compound NS1643 was dissolved in DMSO and diluted 1000 times in Kulori medium. At this concentration, no effect of the applied vehicle was observed (data not shown).

Electrophysiological recordings in mammalian cells

The patch-clamp recordings were conducted with the EPC-9 amplifier (HEKA, Germany) and the acquisition software Pulse+PulseFit version 8.53, in the whole-cell mode on the hERG-expressing HEK cells. The cells were dialyzed with an intracellular solution (in mM: 119 K⁺-gluconate, 15 KCl, 5 EGTA, 5 K₂ATP, 3.2 MgCl₂, 5 HEPES, pH 7.2 with KOH) and superfused with an extracellular solution (in mM: 2 CaCl₂, 1 MgCl₂, 10 HEPES, 4 KCl, 140 NaCl, pH 7.4 with NaOH) at a rate of 2 ml/min. Sampling rate was 3-15 kHz depending on how detailed a resolution was needed. All recordings were performed at 36±1 °C.

Analysis of data

Data analysis and drawings were performed using IGOR software (WaveMetrics, Lake Oswego, OR, USA) or GraphPad Prism software (GraphPad Software, San Diego, CA, USA). All deviations of calculated mean averages are given as standard error of the mean (SEM) values. Significance was calculated as paired t-test or by ANOVA analysis. Activation data obtained from step protocols were fitted with Boltzmann equations

applying the equation: $I = I_{max} / (1 + \exp[(V_{50} - V_t)/k])$, where I is the current, V_{50} is the voltage required for half activation, V_t is the test membrane potential and k is the slope factor. Time constants for deactivation was calculated by fitting with the double exponential equation $I_{tail} = K_0 + K_{fast} * \exp[-(t/\tau_{fast})] + K_{slow} * \exp[-(t/\tau_{slow})]$, where t is the time in seconds and the fast and slow deactivation constants are given by τ_{fast} and τ_{slow} , respectively. Finally, the time constant for activation, obtained from tail envelope protocols, was calculated by fitting to the single exponential equation $I_{tail} = K_0 + K * \exp[-(t/\tau)]$, where t is the time in s and τ is the time constant for inactivation. Capacitive current was subtracted before fitting.

Results

For an initial comparison of hERG-WT, hERG-N588K and hERG-N588K/KCNE2 channels heterologous expression was performed in *Xenopus laevis* oocytes. From a holding potential of -80 mV channel activation was obtained by a step protocol from -80 mV to +110 mV in 10 mV increments. Tail current was recorded at -120 mV. Representative current traces for hERG-WT, hERG-N588K and hERG-N588K/KCNE2 channels are shown in Figure 1A, B and C, respectively. hERG-N588K has been reported as a gain-of-function mutation [14, 17, 21]. As expected, the steady state current originating from the step protocol increased as a function of the applied voltage at potentials more positive than approximately -40 mV, i.e. the channel is severely affected in its ability to inactivate. The summarized current-voltage (I/V) relationship for hERG-WT, hERG-N588K and hERG-N588K/KCNE2 recorded at steady state is depicted in Figure 1D. The bell shaped I/V curve reveals the inactivation of hERG-WT channels. Inactivation of hERG-N588K was observed at potentials more positive than +90 mV. KCNE2 seemed to restore partly the inactivation probabilities of hERG-N588K since inactivation for the hERG-N588K/KCNE2 combination was seen at potentials more positive than +60 mV. Current recordings of hERG-WT/KCNE2 channels were no different from recordings performed from hERG-WT expressed alone (data not shown). The gain-of-function property of N588K is however challenged if tail current is inspected. It is obvious from the traces shown in Figure 1A, B and C that the tail current amplitude is severely reduced. Summarized I/V curves for tail recordings are shown in Figure 1E. N588K revealed a substantial reduction in current amplitude compared to WT channels both in the presence and absence of KCNE2. Boltzmann fits to the tail currents also revealed a change in activation threshold for N588K. The voltage dependency of activation dem-

Fig. 1. Current-Voltage (I/V) relations for hERG-WT, hERG-N588K and hERG-N588K/KCNE2. Channels were activated by 1 s pulses to potentials ranging from -80 to +110 mV, and tail currents were recorded for 1 s at -120 mV. Between steps cells were clamped for 3 s at -80 mV. Representative current traces for hERG-WT are demonstrated in A, for hERG-N588K in B and for hERG-N588K/KCNE2 in C. I/V curves recorded from step potentials are depicted in D. Closed squares represent WT channels, open squares N588K channels and open triangles hERG-N588K/KCNE2 channels. Tail current measurements as a function of the previous step potential are shown in E. Closed circles represent WT channels, open circles N588K channels and closed triangles hERG-N588K/KCNE2. For all experiments $n = 7-11$.

onstrated by the $V_{0.5}$ was $-19.9 \pm V$ for hERG, 24.3 ± 3 mV for hERG-N588K and -1.2 ± 1 mV for hERG-N588K/KCNE2.

From a biophysical point of view the reduced tail current recorded from N588K could be due to a faster deactivation or a slower activation. A slower activation would be expected to leave fewer channels available to conduct current when negative potentials are applied. To address those possibilities we determined time constants for channel deactivation and activation.

Channel deactivation was addressed by a 1 s step to +20 mV followed by a step protocol from 0 to -120 mV in -10 mV increments. Representative traces for hERG-WT, hERG-N588K and hERG-N588K/KCNE2 are shown in Figure 2 A, B and C, respectively. Deactivation constants were calculated from a two-exponential decay equation. Reciprocal tau values for the slow component are depicted in Figure 2 D and for the fast component in Figure 2 E (reciprocal values reveal differences better). No significant difference was found between τ_{slow} for WT and N588K channels. In contrast, the slow component of deactivation for hERG-N588K was faster in the presence of KCNE2. τ_{fast} was faster for N588K compared to WT channels at potentials more hyperpolarized than -60 mV. In the presence of KCNE2 τ_{fast} was significantly faster for hERG-N588K at all

potentials tested. I/V relationships for current recordings from peak tail currents are shown in Figure 2 F. As can be seen, WT channels revealed larger current amplitudes at potentials from -120 mV to -40 mV where hERG channels at physiological conditions would be expected to be active. It should be noted that the reversal potential was changed for hERG-N588K and hERG-N588K/KCNE2 channels compared to hERG-WT. Calculated values for hERG-WT was -76 ± 4 mV, for hERG-N588K -65 ± 4 mV and for hERG-N588K/KCNE2 -64 ± 6 mV. Finally, the ratio between the fast and slow component of deactivation was determined. As revealed from Figure 2 G the fast component of deactivation is dominating when hERG-N588K was expressed together with KCNE2. Current recordings of hERG-WT/KCNE2 channels were no different from recordings performed from hERG-WT expressed alone (data not shown). To secure that observed biophysical properties were unaffected by expression level, these results are summarized from batches of oocytes with low expressing levels. The same studies were also performed on oocytes with high expression levels, and biophysical properties were unaffected by the amount of ion channels present (data not shown).

The inactivation properties for hERG-N588K and hERG-N588K/KCNE2 were clearly affected compared to hERG-WT channels as demonstrated in Figure 1.

Fig. 2. Deactivation measurements. Deactivation was addressed by an initial 1 s step to +20 mV followed by steps from 0 to -120 mV lasting 3 s. Cells were kept for 3 s at -80 mV between steps. Representative traces for WT channels are shown in A, for N588K in B and for hERG-N588K/KCNE2 in C. Deactivation constants are depicted as $1/\tau_{\text{slow}}$ in D and $1/\tau_{\text{fast}}$ in E. Please notice that τ values are shown as reciprocal values to better reveal observed differences in τ_{fast} for deactivation at negative potentials. The I/V relation for peak tail current is shown in F and the ratio between the fast and slow component of deactivation in G. Closed squares represent WT channels, open squares N588K channels and open triangles hERG-N588K/KCNE2. For all experiments $n = 8-10$.

Fig. 3. Inactivation properties for hERG-WT, hERG-N588K and hERG-N588K/KCNE2. Voltage dependent release from inactivation was addressed by a three step protocol. Summarized data demonstrates how half-point values for voltage dependent release from inactivation are right-ward shifted by more than 100 mV for hERG-N588K channels compared to hERG-WT channels. $V_{0.5}$ values are -78.9 ± 2 mV for hERG-WT channels, 50.3 ± 5 mV for hERG-N588K channels and 33.3 ± 6 mV for hERG-N588K/KCNE2 channels. For all experiments $n = 9$.

To investigate this in more detail a three step protocol was applied. Channels were fully activated and inactivated by a 1 s depolarising voltage step of +40 mV for hERG-WT and +120 mV for hERG-N588K and hERG-N588K/KCNE2. Release of inactivation without con-

comitant deactivation was obtained by short (10ms) steps to more hyperpolarized potentials. These were +20 mV to -150 mV for hERG-WT and +120 mV to -70 mV for hERG-N588K and hERG-N588K/KCNE2. The protocol was finalized by a step back to +40 mV for hERG-

Fig. 4. Activation time constants for hERG-WT and hERG-N588K. Time constants for activation were addressed by tail envelope protocols as indicated in the insert. From a holding potential of -80 mV channels were activated by steps to +20 mV. Time intervals at +20 mV were progressively increased before tail current was recorded at -120 mV. Representative traces for WT channels are depicted in A and for N588K in B. Activation time constants were calculated by plotting peak current amplitude at -120 mV as demonstrated in C for WT channels and in D for N588K channels. Summarized values for time constants measured using depolarizing pulses at 0, +20 and +40 mV are shown in E.

WT channels and to +120 mV for hERG-N588K and hERG-N588K/KCNE2 channels. Using this protocol it is possible to address the voltage dependent release from inactivation by plotting the current amplitude observed at the last depolarising step as a function of the previous voltage step. Data are summarized in Figure 3. When fitted to Boltzmann equations $V_{0.5}$ for hERG-WT channels was -78.9 ± 2 mV, for hERG-N588K 50.3 ± 5 mV and for hERG-N588K/KCNE2 33.3 ± 6 mV. Current recordings of hERG-WT/KCNE2 channels were no different from recordings performed from hERG-WT expressed alone (data not shown).

Activation of hERG-WT channels cannot be addressed by conventional step protocols since hERG activation is considerably slower than inactivation. To circumvent this intrinsic property of the channel we

applied a tail envelope protocol. From a holding potential of -80 mV channels were activated and inactivated by a depolarising voltage step with variable duration. Inactivation was subsequently released by a hyperpolarizing step to -120 mV that will almost instantly release inactivation. The peak tail current can therefore be seen as illustration of open channels obtained by the previous depolarized potential. Examples of recordings after depolarising steps to +20 mV from hERG-WT, hERG-N588K and hERG-N588K/KCNE2 channels are shown in Figures 4 A, B and C, respectively. Summarized peak tail currents for channels recorded under these conditions are shown in Figures 4D, E and F. Please notice the difference in time scale. These experiments were repeated with depolarizing pulses to 0 mV and to +40 mV. Calculated activation time constants are summarized in

Fig. 5. hERG-WT and hERG-N588K response to simulated action potentials. Channels were activated by voltage protocols resembling endocardial (left part of the figure) or epicardial (right part of the figure) action potentials. Recordings depicted were obtained at different concentrations of extracellular potassium being 2 mM in A, 4 mM in B and 10 mM in C. Black traces represent control recordings and red traces the corresponding measurement of channel activity on the presence of 30 μ M of the hERG channel activator NS1643. $n = 6-8$.

Figure 4G. As seen, τ -values were significantly smaller for WT channels compared to mutated N588K channels. When hERG-N588K was expressed together with KCNE2 the activation time constants were partly reverted towards values obtained for hERG-WT channels. Exact numbers for WT channels recorded at +40 mV, +20 mV and 0 mV were 37.2 ± 3 ms, 64.4 ± 5 ms and 189.1 ± 16 ms. The corresponding values for N588K were 268.4 ± 63 ms, 438.6 ± 127 and 1212.1 ± 163 ms, i.e. 6-7 fold slower than WT and for hERG-N588K/KCNE2 87.2 ± 15 ms, 287.9 ± 51 ms and 521.0 ± 98 ms, respectively. Current recordings of hERG-WT/KCNE2 channels were no different from recordings performed from hERG-WT expressed alone (data not shown).

Having addressed biophysical properties of hERG-N588K that could point at a loss-of-function phenotype of N588K we investigated how this channel would respond to voltage protocols resembling cardiac action potentials. Since hERG channel activity is increased by raising extracellular K^+ , these experiments were conducted in the presence of 2, 4 and 10 mM extracellular potassium. WT channels were used in comparison. For both types of channels it was further determined if channel activity could be increased by application of one of the newly described hERG channel activators called NS1643 [24]. Representative recordings are depicted in Figure 5. Black traces represent control recordings and red traces the corresponding measurement of channel

Fig. 6. Frequency dependent modulation of hERG-WT and hERG-N588K. Channel activation was obtained by squared voltage steps from -120 mV to +20 mV. A simulated heart rate of 60, 120 and 180 beats per minute (bpm) are demonstrated in A, B and C, respectively. Black traces are WT currents while N588K currents are depicted with grey traces. Summarized peak currents at depolarized (+20 mV) and repolarized (-120 mV) potentials are shown in D. Black bars represent WT currents and grey bars N588K current. n = 8.

activity on the presence of 30 μM of the hERG channel activator NS1643. Channels were activated by voltage protocols resembling action potentials from either the endocardium or epicardium. For recordings performed in 2 mM and 4 mM extracellular K^+ , WT channels responded with an asymmetrical current peaking at the very end of the action potential (Figure 5A and B). Due to change in reversal potential the largest current amplitude in 10 mM extracellular K^+ was inward and observed after full repolarisation (Figure 5C). Application of a hERG channel activator (30 μM NS1643) gave a profound increase

in WT current when recorded in 2 and 4 mM K^+ , while the current augment was less noticeable in 10 mM K^+ . The current increase in the presence of NS1643 was quantified as the increase in the area under the curve in what is equivalent to both the diastole and the systole. In 2 mM extracellular K^+ 30 μM NS1643 increase hERG current by $85.7 \pm 14\%$ when channels were activated by a voltage protocol mimicking an endocardial action potential and by $71.0 \pm 19\%$ when an epicardial protocol was applied. At 2 mM extracellular K^+ no significant tail current could be recorded since the reversal potential was

Fig. 7. Current-Voltage (I/V) relations for hERG-WT and hERG-N588K expressed in mammalian cells and recorded at physiological temperature. Channels were activated by 1 s pulses to potentials ranging from -80 to +40 (WT) or +110 mV (N588K), and tail currents were recorded for 1 s at -50 mV. Between steps cells were clamped for 3 s at -80 mV. Representative current traces for hERG-WT are demonstrated in A and in B for hERG-N588K. I/V curves recorded from step potentials are depicted in C and D, respectively. Open symbols represent WT channels and black symbols N588K channels. For hERG-WT experiments $n = 7$ and for hERG-N588K experiments $n = 3$.

to close to the -90 mV that was chosen as the resting potential. At 4 mM extracellular K^+ the current increase after NS1643 application was for endocardial protocols $35.2 \pm 9\%$ in the systole and $53.9 \pm 14\%$ in the diastole. The corresponding values for epicardial protocols were $41.0 \pm 9\%$ and $59 \pm 16\%$, respectively. Finally, in 10 mM extracellular K^+ NS1643 was only able to slightly increase the recorded hERG-WT current in the diastole to values of $12.1 \pm 5\%$ for endocardial protocols and $14.5 \pm 6\%$ for epicardial protocols. The mutated N588K channel responded completely different to application of NS1643. The current response followed the applied voltage protocol, and NS1643 was unable to further significantly increase the activity of hERG-N588K channels in any of the tested concentrations of extracellular K^+ .

Finally, we investigated how WT and N588K channels responded to different pacing frequencies (Figure 6). This was achieved by applying voltage protocols roughly mimicking cardiac action potentials with a heart rate of 60, 120 and 180 beats per minute (bpm).

Voltage steps were from -120 mV to +20 mV. The equilibrium potential for K^+ in the present experiments was -78 mV to -91 mV. The very negative step to -120 mV was therefore chosen to better reveal tail currents elicited by repolarisation. Time intervals at +20 mV and -120 mV were 200 ms and 800 ms at 60 bpm; 180 and 320 ms at 120 bpm; and 150 and 180 ms for 180 bpm, respectively. For all tested frequencies WT and N588K channels responded in an opposite manner. WT channels had small current amplitudes at depolarized potentials and large tail currents at repolarised potentials (black traces in Figure 6). In contrast, N588K responded with large current amplitudes after depolarization and reduced amplitudes after repolarisation (grey traces in Figure 6). Results are summarized in Figure 6 D.

Experiments performed after expression in oocytes points to the fact that hERG-N588K channels have both loss and gain-of-function. To confirm that these observations were not only related to the choice of expression system and temperature conditions experiments were

Fig. 8. Deactivation measurements in mammalian cells at physiological temperature. Deactivation was addressed by an initial 0.5 s step to +40 mV followed by steps from -10 to -110 mV lasting 1 s. Cells were kept for 3 s at -80 mV between steps. Representative traces for hERG-WT channels are shown in A and for hERG-N588K channels in B. To illustrate the fast deactivation at physiological temperatures enlargements of the deactivation curves are depicted in C and D, respectively. For hERG-WT experiments $n = 8$ and for hERG-N588K experiments $n = 4$.

repeated in a mammalian expression system at 36 ± 1 °C. HEK-293 cells expressing either hERG-WT or hERG-N588K were initially activated by step protocols from -80 to +40 (for WT) or to +110 mV (for N588K) in 10 mV increments. Tail currents were recorded at -50 mV. As depicted in Figure 7A and C, hERG-WT channels showed the expected bell shaped IV curved for steady-state recordings with peak current close to 0 mV. hERG-N588K channels had an affected ability to inactivate with peak current around +30 mV. Summarized IV curves for hERG-WT and hERG-N588K steady-state and tail currents are shown in Figure 7C and D, respectively. As can be observed from current recordings in Figure 7B deactivation seems extremely fast at physiological temperatures for hERG-N588K.

The fast deactivation was addressed in more detail as demonstrated in Figure 8. From a holding potential at +40 mV cells were clamped to potentials from -10 mV to -110 mV. Due to physiological temperatures applied in these experiments deactivation was faster than observed

in oocyte measurements. This can be seen in representative experiments in Figure 8A and B. Figure 8 C and D shows extracts of the first 25 ms period of the steps from -10 to -110 mV. The extremely fast deactivation for hERG-N588K observed in step protocols as in Figure 7 was confirmed in this experiment. Deactivation was almost instant at all tested potentials as seen in Figure 8B and in enlargement in Figure 8D. The extremely fast deactivation made it impossible to discriminate start of deactivation from capacitive currents and hence time constants were not determined.

The fast time constants for deactivation also made it impossible to determine reliable time constants for activation and inactivation. We did however make an attempt to measure voltage dependent release from inactivation by a 3-step protocol as demonstrated in Figure 3. In this experiment hERG-WT channels was clamped at +40 mV between steps, while voltage dependent release from inactivation was determined at potentials from -150 mV to +40 mV in 10 mV decrements.

Fig. 9. Inactivation properties for hERG-WT and hERG-N588K recorded in mammalian cells at physiological temperature. Voltage dependent release from inactivation was addressed by a three step protocol. Summarized data demonstrates how half-point values for voltage dependent release from inactivation are right-ward shifted by approximately -80 mV for hERG-N588K channels compared to hERG-WT channels. $V_{0.5}$ values are -79.5 ± 1.4 mV for hERG-WT ($n = 6$) channels and -1.2 ± 2.0 mV ($n = 5$) for hERG-N588K channels.

Each step lasted from 0.6 to 0.9 ms and was adjusted so significant deactivation was not observed during the step. hERG-N588K channels were clamped to +100 mV and stepped in potentials from -90 to +100 mV in 10 mV decrements. Each step lasted for 0.6 to 1.1 ms. As a consequence of the fast deactivation it was not possible to obtain perfect plateaus in the normalized IV curve for the hERG-N588K channels. Boltzman fits were therefore performed by pre-identifying minimum and maximum normalized current level to 0.0 and 1.0 within the tested voltage potentials. This will give a slight underestimation of the right-ward shifting of the hERG-N588K IV curved compared to hERG-WT controls. Results are presented in Figure 9. When expressed in mammalian cells and recorded at physiological temperatures the V_{50} values were calculated to -79.5 ± 1.4 mV ($n = 6$) for hERG-WT and to -1.2 ± 2.0 mV ($n = 5$) for hERG-N588K.

Discussion

The inactivation ability of hERG channels is severely affected by the N588K mutation. As a consequence, this mutated channel responds to depolarizing voltage steps by a progressive increase in current amplitude. This is in contrast to hERG-WT channels in which inactivation will result in a bell shaped current/voltage relationship when depolarizing voltage steps are applied. Based upon these biophysical characteristics there has been a proclivity to define hERG-N588K as a gain-of-function mutation. This makes sense when it comes to lengths of action potentials since all patients carrying the N588K mutant have reduced QT intervals. In the present paper we do however describe how the biophysical nature of hERG-N588K is actually negatively affected in several aspects. Foremost the tail current is reduced in ampli-

tude in hERG-N588K channels compared to hERG-WT channels. The analysis of the deactivation kinetics demonstrated that the fast component of deactivation was increased as seen by the reduction in τ_{fast} values (depicted as increased τ_{fast}^{-1} values in Figure 2). It is also interesting to notice how the reversal potential is shifted to more positive potentials in N588K compared to WT channels. This is consistent with earlier findings and *in silico* simulations have demonstrated how such a change in selectivity increase the chance of evoking early after depolarisations (EAD's) [14, 21, 25]. Since it is still an open question whether the β -subunit KCNE2 is a part of native I_{Kr} current co-expression experiments with hERG-N588K and KCNE2 were also performed. In the presence of KCNE2 tail current of hERG-N588K was even further compromised. The increase in deactivation velocity for hERG-N588K was confirmed in mammalian cells at physiological temperatures. To summarize tail current properties, hERG-N588K channels both have changed selectivity, reduced amplitude and closes faster than hERG-WT channels. The reduced tail current amplitude and selectivity changes of hERG-N588K are in agreement with earlier studies by Corderio et al. even though time constants for deactivation was not reported in this study [21]. Another biophysical parameter, that to our knowledge has not been addressed previously, is the activation properties of hERG-N588K. For hERG-WT channels channel inactivation is much faster than activation. Activation kinetics can therefore not be addressed by ordinary step protocols. To manage the challenge of fast inactivation, a so-called tail envelope protocol can be applied [26]. Using this approach activation time constants were calculated for hERG-WT, for hERG-N588K and for hERG-N588K/KCNE2 channels at three different potentials. A significant 6-7 fold slowing of activation was demonstrated for hERG-N588K for all tested

potentials compared to hERG-WT channels. Activation was not slowed to the same degree when KCNE2 was co-expressed with hERG-N588K, but was still significantly slower compared to WT channels. At any given time point less hERG-N588K channels will consequently be available upon repolarization compared to hERG-WT channels. It can be argued that a tail envelope protocol is superfluous for a channel like hERG-N588K since inactivation properties are seriously affected. We did however choose to use this approach to be able to compare directly with hERG-WT channels and to compensate for any slight inactivation that might be present in the hERG-N588K channels.

From these biophysical results it is obvious that hERG-N588K has some characteristics that can be defined as loss-of-function. From a physiological point of view it is interesting whether these properties will result in a reduction in cardiac repolarising conductance. To answer this question we recorded channel activation after stimulation with voltage protocols resembling endo- and epicardial action potentials. As demonstrated in Figure 5 it was found that hERG-WT channels responded by peak potassium conductance at the end of the action potential protocol. In addition, large tail currents lasting for longer than the applied action potential protocol were observed. This is to be expected due to the inactivation and slow deactivation properties of this channel. When the action potential reach phase 3 and repolarisation is initiated, hERG channels are released from inactivation and will start to contribute to the repolarising potassium conductance. Increasing extracellular potassium is known to interfere with the channels ability to inactivate. This is observed in Figure 5 where hERG-WT current follows the shape of the simulated action potential to a larger extent in 10 mM K⁺ compared to 2 mM K⁺. It could also be seen that the hERG channel activator NS1643 was more effective in increasing hERG-WT current in low (2 mM) extracellular potassium compared to higher concentrations of extracellular potassium (10 mM). This is a likely result since we have earlier demonstrated that NS1643 primarily increases hERG-WT current by affecting the inactivation properties of the channel [24]. When similar experiments were conducted with hERG-N588K channels two features caught attention. First, the current amplitude of the mutated channel completely followed the shape of the applied action potential protocol. This also means a lack of potassium conductance in what would be equal to the diastolic interval. Second, the hERG channel activator NS1643 had only minor effect on hERG-N588K

channels in agreement with the notion that NS1643 increases hERG-WT current by affecting the inactivation properties of the channel. Finally, we also investigated whether the biophysical properties of hERG-N588K were affected by stimulation frequencies. With protocols simulating heart rates of 60, 120 and 180 beats per minute the current contribution of hERG-WT and hERG-N588K channels to the plateau phase (phase 2) and the repolarising phase (phase 3) of the action potential was addressed. As demonstrated in Figure 6, hERG-WT channels contributed only minor to phase 2 current while a major contribution was observed during the repolarizing phase 3. hERG-N588K responded to these simulated action potential protocols in a completely opposite fashion. Current contribution was large during phase 2 and minor during phase 3 leaving no contribution from this mutated channel to the repolarization reserve. Due to the fast deactivation of N588K hERG channels the number channels able to counteract depolarizing events in the post-repolarization period is significantly reduced and thereby the ability of the myocytes to counteract triggered activity. When experiments were performed at physiological temperatures the deactivation of hERG-N588K was so fast that no I_{Kr} current can be expected in the diastolic interval. From these results, we find it reasonable to conclude that hERG-N588K should be considered as a loss-of-function mutation in several contexts.

Having the biophysical characterization of hERG-N588K in mind, some of the arrhythmic events observed in these short QT patients could be explained by 1) increased tendency to respond to triggered activity in the post-repolarization period, and 2) and reduction in the repolarising reserve leading to triangulation and early after-depolarizations. These potentially pro-arrhythmic mechanisms should be considered along with the short cycle times and tendency to amplified transmural dispersion in short QT patients [21, 27, 28]. Whether any of these parameters have a more prominent pro-arrhythmic effect is an open question that is best solved by *in silico* studies. It is also highly likely that the combination of the described parameters together constitute a total increase in the likelihood for developing arrhythmias.

hERG channel activation, resulting in a shorting of the QT interval, has recently been suggested as a new anti-arrhythmic approach [24, 29-31]. This would evidently not present an attractive mechanism if the compounds mediated biophysical effects similar to those of the hERG-N588K short QT mutation. Since the described hERG channel activators functionally result in a pure gain

of hERG channel function their effects are quite different. An increased repolarization reserve has been demonstrated for these compounds and the relative shape of the action potential remains unchanged indicating no action potential triangulation [24, 29]. In addition, hERG activators have been described to increase hERG current independently of pacing frequency and also to decrease transmural dispersion [31, 32]. We therefore state that the short QT mutation hERG-N588K has a completely different mode of action compared to increased hERG current obtained by hERG channel activators.

In conclusion, we have demonstrated that the short QT hERG-N588K from a physiological point of view can be conceived as a mixed gain-of-function and loss-of-function mutation changing hERG channel kinetics in

several ways. This channel display severely compromised inactivation, slower activation and faster deactivation compared to hERG-WT channels. As a consequence, patients carrying the hERG-N588K mutant will have a reduction in their repolarising reserve and a significantly reduced number of open hERG channels during early diastole. This might contribute to the increased risk of arrhythmic events in these patients.

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