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Regulation of Kv4.3 and hERG potassium channels by KChIP2 isoforms and DPP6 and response to the dual K⁺ channel activator NS3623

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ABSTRACT
Transient outward potassium current (Iₒₒ) contributes to early repolarization of many mammalian cardiac action potentials, including human, whilst the rapid delayed rectifier K⁺ current (Iₒᵣ) contributes to later repolarization. Fast Iₒₒ channels can be produced from the Shl family KCNDE gene product Kv4.3s, although accessory subunits including KChIP2.x and DPP6 are also needed to produce a near physiological Iₒₒ. In this study, the effect of KChIP2.1 & KChIP2.2 (also known as KChIP2b and KChIP2e respectively), alone or in conjunction with the accessory subunit DPP6, on both Kv4.3 and hERG were evaluated. A dual Iₒₒ and Iₒᵣ activator, NS3623, has been recently proposed to be beneficial in heart failure and the action of NS3623 on the two channels was also investigated. Whole-cell patch-clamp experiments were performed at 33 ± 1 °C on HEK293 cells expressing Kv4.3 or hERG in the absence or presence of these accessory subunits. Kv4.3 current magnitude was augmented by co-expression with either KChIP2.2 or KChIP2.1 and KChIP2/DPP6 with KChIP2.1 producing a greater effect than KChIP2.2. Adding DPP6 removed the difference in Kv4.3 augmentation between KChIP2.1 and KChIP2.2. The inactivation rate and recovery from inactivation were also altered by KChIP2 isoform co-expression. In contrast, hERG (Kv11.1) current was not altered by co-expression with KChIP2.1, KChIP2.2 or DPP6. NS3623 increased Kv4.3 amplitude to a similar extent with and without accessory subunit co-expression, however KChIP2 isoforms modulated the compound’s effect on inactivation time course. The agonist effect of NS3623 on hERG channels was not affected by KChIP2.1, KChIP2.2 or DPP6 co-expression.

1. Introduction
About 10 distinct potassium (K⁺) channels participate in the repolarization of cardiac action potentials (APs) [1]. However, how they map into the net AP repolarizing current is complicated; in the ventricles, the rapid and slow delayed rectifier K⁺ currents (Iₒᵣ and Iₒᵢ) influence AP repolarization over plateau voltages, whilst the inward rectifier K⁺ current (Iᵢᵢ) is involved in both setting the resting potential and mediating the final repolarization phase of the AP [1,2]. The transient outward K⁺ current, Iₒₒ, contributes to phase 1 repolarization but will also affect later repolarization phases of the AP by modifying the time- and voltage-dependent recruitment of other K⁺ currents (such as Iₒᵣ, Iᵢᵢ) as well as L-type Ca²⁺ current (IₐCa,L) [2]. In addition, Iₒᵢ will affect NCX current via effects on IₐCa,L-dependent Ca²⁺ release as well as via Ca-dependent inactivation of IₐCa,L [3–5]. Native Iₒᵢ has components with fast and slow recovery kinetics (Iₒᵢ,f and Iₒᵢ,s respectively) and KCND2 (Kv4.2) and KCND3 (Kv4.3) underlie Iₒᵢ,s while KCNA4 (Kv1.4) is responsible for Iₒᵢ,f [2,6].

The normal physiological behaviour of many cardiac K⁺ channels appears to require both pore-forming (α) and accessory (β) subunits to be co-expressed and associated [7]. Native Iₒₒ,f channels require interactions between α-subunits and K⁺ Channel interacting Protein 2 (KChIP2) β-subunits, but other proteins such as DPP6 and members of the KCNE family may also modulate the current [6,8–11]. Two splice variants of KChIP2 (called KChIP2L and KChIP2S) were discovered by RT-PCR cloning [12] and the shorter form KChIP2S (also called KChIP2 isoform-1 or KChIP2.1) was identified as the predominant isoform in human heart [13]. Additional expression cloning from human revealed another splice variant of KChIP2, KChIP 2.2 which was 32 amino acids shorter than KChIP2.1 and which, like KChIP 2.1, also increased Kv4.2 channel cell-surface expression and slowed inactivation [14] (for a review of KChIP isoforms and nomenclature see Table 1 in [15]). KChIP2.1 and KChIP2.2 are produced by alternative splicing from the KCNDE gene removing exons 3 and 2+3 to produce isoforms coding of 252 and 220 amino acids respectively [16].

The AP depolarization also activates Iₒᵣ, which plays a key role in determining action potential duration [17]. Recordings at physiological temperature from recombinant hERG channels (Kv11.1) expressed in...
mammalian cells closely approximate native $I_{to}$ [18]. The accessory subunits of native $I_{to}$ channels have been a matter of some debate as hERG can co-assemble with both KCNE1 and KCNE2 and clinically observed mutations in these subunits can influence hERG current and the channel’s pharmacological sensitivity [19–21]. The potassium channel regulatory protein KCRI has also been shown experimentally to influence drug sensitivity of hERG channels [22] and the possible interaction with other $K^{+}$ channel regulatory units is uncertain. Recent data suggest that hERG channel current magnitude is influenced by Kv4.3 co-expression [23], but no such information exists for $I_{to}$ beta subunits. KChIP2 has recently been identified to act as a core transcriptional regulator of cardiac excitability [24]. That KChIP may also influence hERG current and the possible role of KChIP2/DPP6 in the aforementioned oocyte experiments [35] or that the augmentation of $I_{to}$ [29] might be due to some other mechanism has not been examined experimentally.

The present study had three aims: first, to compare the gating properties of Kv4.3 co-expressed with either KChIP2.1 or KChIP2.2 with and without DPP6. Second, to examine the effects of NS3623 on Kv4.3 and hERG, in both the presence and absence of KChIP2 and DPP6 in a mammalian cell expression system. Finally, to examine the possibility that KChIP2.1, KChIP2.2 and DPP6 expression may affect Kv11.1 (hERG) by transfecting each $\beta$-subunit (along with GFP protein as a reporter) in a stably transfected mammalian (HEK 293) cell line which expresses hERG channels.

### 2. Methods

#### 2.1. DNA constructs

The cDNA constructs coding for human short Kv4.3 isoform 1 precursor (KCND3 gene cloned in pCDNA3, NCBI reference sequence NM_172198.2) [36] and KChIP2 variant 2 (KCNIP2 gene cloned in pCDNA3, NCBI reference sequence NM_173195.2) [14], herein designated as KChIP2.2 (220 amino acid long) were kindly provided by

| Comparison of biophysical parameters for HEK 293 cells expressing recombinant Kv4.3 alone or in the presence of KChIP2 isoforms and/or DPP6. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Kv4.3           | KChIP2.2        | KChIP2.1        | KChIP2.2/DPP6   | KChIP2.1/DPP6   |
| $I_{A,t,0}$ @ +40 mV$^*$ | 453 ± 62 (n = 33) | 780 ± 87 (n = 16)$^1$ | 1044 ± 88 (n = 13)$^2$ | 873 ± 79 (n = 14)$^3$ | 828 ± 107 (n = 15)$^3$ |
| Recovery $\tau_{R}$ (ms)$^4$ | 46.10 ± 6.19 (n = 18) | 5.88 ± 0.55 (n = 12)$^5$ | 11.99 ± 1.8 (n = 13)$^6$ | 5.30 ± 0.47 (n = 13)$^7$ | 8.21 ± 1.03 (n = 14)$^7$ |
| Decay $\tau_{C}$ (ms)$^8$ | 11.16 ± 0.89 (n = 23)$^9$ | 13.82 ± 0.74 (n = 12)$^9$ | 21.24 ± 2.88 (n = 12)$^9$ | 9.17 ± 0.59 (n = 12)$^9$ | 10.52 ± 1.10 (n = 13)$^9$ |
| Decay $\tau_{C}$ (ms)$^8$ | 84.7 ± 4.9 (n = 23) | 132.8 ± 17.3 (n = 12)$^9$ | 146.4 ± 23.9 (n = 12)$^9$ | 82.6 ± 4.1 (n = 12)$^9$ | 71.2 ± 6.9 (n = 13)$^9$ |
| $I_{A,t,0}$ + NS3623 @ +40 mV$^*$ | 567 ± 87 (n = 16) | 796 ± 145 (n = 9) | 1142 ± 124 (n = 10)$^1$ | 948 ± 90 (n = 12)$^1$ | 917 ± 95 (n = 10)$^1$ |
| Recovery $\tau_{R}$ (ms) + NS3623$^*$ | 146.2 ± 18.1 (n = 14) | 21.3 ± 3.8 (n = 9)$^1$ | 30.9 ± 3.4 (n = 10)$^1$ | 19.4 ± 5.1 (n = 8)$^1$ | 31.4 ± 5.4 (n = 9)$^1$ |
| Decay $\tau_{C}$ (ms) + NS3623$^*$ | 9.85 ± 0.69 (n = 14) | 19.37 ± 1.03 (n = 9)$^1$ | 26.8 ± 3.42 (n = 10)$^1$ | 14.54 ± 0.97 (n = 12)$^1$ | 14.83 ± 1.13 (n = 11)$^1$ |
| Decay $\tau_{C}$ (ms) + NS3623$^*$ | 65.1 ± 2.53 (n = 14) | 71.3 ± 7.1 (n = 9) | 86.8 ± 9.6 (n = 10)$^1$ | 60.3 ± 4.2 (n = 12)$^1$ | 67.4 ± 4.7 (n = 11) |
| % Change (IP$_{A,t}$) $^*$ | 144 ± 5% | 131 ± 2% | 132 ± 2% | 141 ± 3% | 129 ± 3% |
| P = .0001 | P = .0001 | P = .0002 | P = .0002 | P = .0002 |
| Fold change (P) | 3.83 ± 0.57 | 4.68 ± 0.39 | 3.84 ± 0.47 | 3.04 ± 0.65 | 3.72 ± 0.40 |
| $\tau_{C}$ ($\tau$) | 92 ± 5% | 132 ± 6% | 159 ± 6% | 156 ± 15% | 156 ± 15% |
| % Change (P) | 77 ± 3% | 51 ± 8% | 69 ± 12% | 73 ± 4% | 98 ± 9% |
| P = .0001 | P = .0001 | P = .0005 | P = .001 | P = .577 |
| Fold change (AUC) $^{**}$ | 1.27 ± 0.06 | 1.50 ± 0.07 | 1.45 ± 0.11 | 2.09 ± 0.22 | 2.26 ± 0.18 |
| P = .769 | P = .865 | P = .0005 | P < .0001 |

$^*$ One-way ANOVA.

$^*$ Wilcoxon matched-paired signed rank test.

$^1$ Compared to Kv4.3.

$^2$ Compared to KChIP2.2.

$^3$ Compared to +KChIP2.1.

$^4$ Values shown are against the extent of increase in current integral (AUC) due to NS3623 compared to Kv4.3 alone.
Professor Robert Bähring (Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany). The cDNA constructs coding for human DPP6 variant 1 (DPP6) gene cloned in pcDNA3.1 + , NCBI reference sequence NM_130797.3) and KCNIP2.1 (KCNIP2 gene cloned in pCMV-3Tag-1a, NCBI reference sequence NM_173192.2) were synthesized and sequenced by GenScript (GenScript, Piscataway, New Jersey, USA).

2.2. Cell culture and transfection

HEK 293 cells (European Collection of Cell Cultures, Porton Down, UK) transfected with Kv4.3 or a stable HEK 293 cell line expressing wild-type (WT) hERG (Kv11.1) channels (kindly provided by Professor Craig January) [37] were used. Additional K⁺ channel β-subunits were also transfected into the cells (as described below). Cells were maintained at 37 °C in a humidity controlled incubator with 5% CO₂ atmosphere and cultured in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific, Life technologies division, Paisley, UK) supplemented with 10% (v/v) heat inactivated fetal bovine serum (Thermo Fisher Scientific, Life Technologies division, Paisley, UK) and 50 μg/mL Gentamicin (Merck KGaA, Darmstadt, Germany). In the case of hERG-expressing HEK 293 cells, the medium was further supplemented with 400 μg/ml G418 selection antibiotic (Thermo Fisher Scientific, Life Technologies division, Paisley, UK). Prior to transfection, cells were plated for 48 h onto 12-well plates using a non-enzymatic agent (Merck KGaA, Darmstadt, Germany) before transfection. Transfection reactions were prepared using OPTI-MEM® 1 (Thermo Fisher Scientific, Life Technologies division, Paisley, UK). For Kv4.3 experiments 0.3 μg of DNA was transfected along with either 1 μg of a GFP construct (control) or KCNIP2.1/2.2. In experiments where a KCNIP2 isoform and human DPP6 were co-transfected, 0.8 μg of each DNA was transfected and compared to a “control” condition, in which 1.6 μg of GFP alone was transfected. For hERG experiments, a matching concentration of GFP DNA was used, in order to have an equivalent DNA concentration across all conditions. Lipofectamine™ 2000 transfection agent (Thermo Fisher Scientific, Life Technologies division, Paisley, UK) was used at a 2:1 ratio with DNA. The culture medium was replaced 5 h after transfection. After 24 h, cells were collected and replated onto 13 mm round glass coverslips, pre-treated for 4 h with 200 μg/mL Poly-D-Lysine (Merck KGaA, Darmstadt, Germany). Patch-Clamp recordings started 3 h after plating.

2.3. Reagents and solutions

The compound NS3623 was purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA) and dissolved in DMSO to a final concentration of 10 μmol/L. Individual aliquots were frozen at −20 °C and thawed for use on the day of the experiment. The DMSO stock (Merck KGaA, Darmstadt, Germany) was diluted to a final concentration of 0.1% in extracellular solution to give an 10 μmol/L NS3623 solution, as used previously [29]. All reagents to prepare solutions were purchased from Merck KGaA (Darmstadt, Germany) or Sigma-Aldrich (Poole, Dorset, UK). The extracellular solution contained (in mmol/L): 138 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 10 Glucose and 0.33 NaH₂PO₄ (pH adjusted to 7.4 with concentrated NaOH). The intracellular solution used for Kv4.3 (Iₒ current) recordings was based on that from previous studies of Kv4.3 [32]. The patch pipette solution contained in mmol/L: 90 KAspartate, 30 KCl, 10 NaCl, 1 MgCl₂, 5 EGTA, 5 MgATP, 10 HEPES, 5.5 Glucose (pH adjusted to 7.3 with KOH). The intracellular solution for hERG (Iₒ current) recordings was similar to that used in prior studies from our laboratory [38–40], containing in mmol/L: 130 KCl, 1 MgCl₂, 5 EGTA, 5 MgATP, 10 HEPES (pH adjusted to 7.3 with KOH).

2.4. Electrophysiology and data analysis

Patch pipettes (2.5–3 MΩ resistance) were fabricated from borosilicate glass capillaries (Friedrich & Dimonock, Millville, New Jersey, USA) using a P-87 puller (Sutter Instruments, Novato, California, USA). Data were acquired and recorded with Clampex 10.3 software using an Axopatch 200 amplifier and Axon Digidata® 1322A (Molecular Devices, Sunnyvale, California, USA). Data were digitized at 20 kHz during all voltage protocols and a bandwidth of 2 kHz was set on the amplifier. For whole-cell recordings, cells were continuously perfused with extracellular solution at 33 ± 1 °C. Access resistance (Rₐ) was always below 5 MΩ and series resistance was typically compensated by ∼40%. Voltage protocols are described in the “Results” section. Patch-Clamp recordings were analysed using Clampfit 10.3 (Molecular devices, Sunnyvale, USA). Statistics and graphs were prepared using Excel Professional Plus 2013 (Microsoft Corporation, Redmond, Washington, USA) and Prism 7 for Windows (GraphPad Software, La Jolla, California, USA).

2.5. Statistics

Statistical significance was assessed by applying a non-parametric test for comparing two different conditions within a group (Wilcoxon matched-paired signed rank test) and analysis of variance or one-way ANOVA (Tukey post-hoc analysis) when comparing three or more groups. Two-way ANOVA test (Sidak post-hoc test) was used when multiple comparison between different groups was necessary. In all cases, a p value of less than .05 was required for statistical confidence. Values are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Effect of KCNIP2 expression on Kv4.3 biophysical properties

Initial experiments examined the effect of both KCNIP2.2 and KCNIP2.1 on Kv4.3. The voltage protocol involved square voltage steps from −60 to +40 mV (Vₒ) in 10 mV increments from a holding voltage (Vₜₐₜ) of −80 mV, as illustrated in the inset to the top panel of Fig. 1A. Co-expression of Kv4.3 with either KCNIP2 isoform produced a robust increase in outward currents when compared to Kv4.3 alone (Fig. 1A and B). For Kv4.3, current density at +40 mV was 490 ± 108 pA/pF (n = 7), which increased to 833 ± 107 pA/pF (n = 12) and 1043 ± 88 pA/pF (n = 13) for cells expressing KCNIP2.2 and KCNIP2.1, respectively (Fig. 1B and Table 1). In addition, we evaluated the effect of expressing both KCNIP2 isoforms with human DPP6 as the presence of both accessory subunits may be necessary to recapitulate native Iₒ [9]. The presence of DPP6 reduced the augmentation of Kv4.3 current produced by KCNIP2.1, resulting in both KCNIP isoforms having (essentially) the same agonistic effect on Kv4.3 in the presence of DPP6 as shown in Fig. 1C and detailed in Table 1.

Fitting a bi-exponential decay function to the Iₒ inactivation time course showed that expression of KCNIP2.1 consistently increased both the fast and slow time constants compared to Kv4.3 alone (Fig. 1D, Table 1). Values for tau fast were 11.16 ± 0.89 ms (Kv4.3; n = 23) vs 21.24 ± 2.88 ms (KCNIP2.1; n = 12) (One-way ANOVA; p < .001), while slow time constant values were 84.7 ± 4.9 ms (n = 23) vs 146.4 ± 23.9 ms (n = 12) (One-way ANOVA; p < .001). Interestingly, co-expression of KCNIP2.2 increased the Kv4.3 slow time constant to 132.8 ± 17.3 ms (n = 12; One-way ANOVA p < .005), but not the fast component (Fig. 1D, Table 1). Finally, addition of DPP6 with KCNIP2 isoforms opposed the changes in Kv4.3 inactivation due to KCNIP2 expression alone (Fig. 1D, Table 1).

The time-course of recovery of Iₒ from inactivation was measured using a protocol consisting of two square voltage pulses (500 ms and 200 ms long, respectively) to +40 mV with varying interpulse intervals, Δt (10–2000 ms) as illustrated in the inset to Fig. 1E. A plot of the
fraction of recovered current against inter-pulse interval was generated and fitted with a mono-exponential function whose time constant \((\tau_{\text{rec}})\) characterized the rate of recovery. The expression of KChIP2 isoforms decreased Kv4.3 \(\tau_{\text{rec}}\) from 46.10 ± 6.19 ms (n = 18) to 5.88 ± 0.55 ms (p < .0001, n = 12) and 11.99 ± 1.87 ms (p < .0001, n = 13) in the presence of KChIP2.2 and KChIP2.1, respectively (Fig. 1E). Co-expression of DPP6 with KChIP2.1/2.2 isoforms had only small effects on \(\tau_{\text{rec}}\) compared to KChIP alone (Fig. 1F, Table 1).

### 3.2. NS3623 modulates the gating properties of the Kv4.3/KChIP2/DPP6 protein complex

Recently, 5 μmol/L NS3623 has been reported to increase native \(I_{\text{o}}\) in canine ventricular myocytes at membrane potentials above +10 mV [29]. In addition, recovery from inactivation was shown to be slightly faster in the presence of NS3623. We therefore examined the effect of 10 μmol/L NS3623 on Kv4.3 currents at +30 mV alone, in the presence of KChIP2 isoforms with and without DPP6. Representative current traces are shown in Fig. 2A. In all cases, application of NS3623 resulted in an increase in current magnitude, but to an extent that depended on isoform co-expression. The greatest increase in current was seen in cells expressing Kv4.3 alone (x1.44 ± 0.05, p = .0001 n = 16) and co-expression with KChIP2.1/DPP6 resulted in a significantly reduced agonism (Table 1 and Fig. 2B). Application of NS3623 in the presence of either KChIP2.2 or KChIP2.1 led to a ~35% increase in the Kv4.3 current fast inactivation time constant (Fig. 2C, Table 1). Without these subunits, the time constant was not detectably altered by NS3623 (p = .15, Table 1). When DPP6 was also expressed with KChIP2.1/2.2, the Kv4.3 current fast inactivation time constant was further increased (p < .0005 and p = .001 in KChIP2.2 and KChIP2.1, respectively). The opposite effect was observed for slow time constants (Table 1). The KChIP2-mediated increase in time constants was generally reversed by 10 μmol/L NS3623 application (Table 1). Furthermore, values from all conditions (including Kv4.3 alone), with the exception of KChIP2.1 (with and without DPP6 co-expression), showed a significant acceleration of the slow component of inactivation (paired t-test; p < .05, Table 1). Nevertheless, the overall effect of NS32523 was greatest in the presence KChIP2.x co-expressed with DPP6, as shown by the change in current integral (AUC Table 1).

Recovery of \(I_{\text{o}}\) from inactivation was slowed by NS3623 in all our Kv4.3 expression conditions (i.e. KChIP2.1/2.2 +/− DPP6) by a factor of 3–4 (see Fig. 2D–F; Table 1). However, the current from cells expressing accessory subunits still recovered much faster than from cells expressing Kv4.3 alone (Fig. 2D and E; Table 1).

### 3.3. KChIP2.1/2.2 and DPP6 do not affect hERG current density or voltage-dependence

The possible effects of KChIP2.1/2.2 and DPP6 on hERG current (\(I_{\text{hERG}}\)) were evaluated with a standard hERG voltage clamp protocol (i.e. a voltage step from a \(V_{\text{h}}\) of −80 to +20 mV to activate the current followed by a repolarizing step to −40 mV – see Fig. 3A). Both end-pulse and tail currents showed the expected electrophysiological characteristics, (Fig. 3B and C). Current densities in the absence of any accessory subunit were 51.6 ± 4.5 pA/pF (n = 32) and 100.4 ± 7.5 (n = 32) for \(I_{\text{end pulse}}\) and \(I_{\text{tail}}\), respectively and current magnitudes were not changed by co-expression with KChIP2.1/2.2 or DPP6 (Table 2). We also examined the normalised voltage dependence of \(I_{\text{hERG}}\) when applying a 2-s-long voltage steps from a \(V_{\text{h}}\) of −80 mV to potentials between −40 and +60 mV. Each test pulse was followed by a repolarization step to −40 mV (Fig. 3D and Table 2). As is typical for \(I_{\text{hERG}}\), current increased with progressive depolarization up to 0 mV (e.g. [37]). Further depolarization to test potentials above +10 resulted in current decline, as indicated by the region of negative slope on the end-pulse I-V relation (Fig. 3E). Tail current activation upon
repolarization to −40 mV followed a sigmoidal activation pattern which could be fitted by a Boltzmann function (Fig. 3F). Examination of the Boltzmann half-activation voltage \( (V_{1/2}) \) and slope parameters showed essentially no change with KChIP2.1/2.2 and DPP6 (Table 2).

### 3.4. Effect of KChIP2.1/2.2 and DPP6 on \( I_{hERG} \) rectification and deactivation time course

The effect of KChIP2.1/2.2 and DPP6 co-expression on \( I_{hERG} \) rectification properties and deactivation time course was examined using...
the voltage protocol shown in Fig. 4Ai and test pulses were applied at 12 s intervals to ensure full recovery between pulses. Fig. 4Aii shows representative currents elicited by this protocol and Fig. 4B shows the resulting I-V relation. Ih\textsubscript{ERG} exhibited a voltage-dependence that was very similar to previous studies (e.g., [37,41]). Accessory subunit expression had no measurable effect on the resulting currents (Fig. 4B). In all cases, the fully activated I-V relation was maximal with a repolarization step to \(\sim 30\) mV. Likewise, all groups showed a similar reversal

<table>
<thead>
<tr>
<th></th>
<th>hERG</th>
<th>hERG/KChIP2.2</th>
<th>hERG/KChIP2.1</th>
<th>hERG/DPP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>I\textsubscript{End Pulse} (pA/pF) (^a)</td>
<td>51.5 ± 4.5 (n = 32)</td>
<td>55.9 ± 6.7 (n = 21)</td>
<td>47.3 ± 4.5 (n = 28)</td>
<td>41.1 ± 3.3 (n = 24)</td>
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<tr>
<td>I\textsubscript{Tail} (pA/pF) (^a)</td>
<td>100 ± 7 (n = 32)</td>
<td>118 ± 13 (n = 21)</td>
<td>90 ± 8 (n = 27)</td>
<td>94 ± 9 (n = 26)</td>
</tr>
<tr>
<td>I-V relation tail (V_{1/2}) (mV) (^e)</td>
<td>(-21.9 ± 1.8) (n = 13)</td>
<td>(-23.6 ± 2.5) (n = 6)</td>
<td>(-22.9 ± 6.3) (n = 9)</td>
<td>(-21.0 ± 2.3) (n = 15)</td>
</tr>
<tr>
<td>I-V relation tail (\kappa) (mV) (^e)</td>
<td>6.13 ± 0.25 (n = 13)</td>
<td>5.99 ± 0.49 (n = 6)</td>
<td>5.94 ± 0.60 (n = 9)</td>
<td>5.9 ± 0.2 (n = 15)</td>
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<tr>
<td>Activation (\tau_{act}) (ms) (^e)</td>
<td>64.1 ± 7.6 (n = 12)</td>
<td>41.1 ± 5.3 (n = 6)</td>
<td>66.8 ± 11.2 (n = 10)</td>
<td>68.9 ± 20.9 (n = 8)</td>
</tr>
<tr>
<td>Reversal (E_{rev}) (mV) (^e)</td>
<td>(-84.0 ± 0.9) (n = 9)</td>
<td>(-84.7 ± 0.5) (n = 6)</td>
<td>(-84.4 ± 0.6) (n = 9)</td>
<td>(-85.5 ± 0.7) (n = 7)</td>
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<td>Recovery (\tau_{rec}) (ms) (^e)</td>
<td>4.29 ± 0.43 (n = 12)</td>
<td>3.48 ± 0.22 (n = 10)</td>
<td>5.22 ± 0.38 (n = 13)</td>
<td>4.29 ± 0.47 (n = 10)</td>
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<tr>
<td>Deactivation (\tau_{fast}) (ms) (^e)</td>
<td>126 ± 11 (n = 9)</td>
<td>140 ± 21 (n = 5)</td>
<td>151 ± 23 (n = 6)</td>
<td>148 ± 13 (n = 6)</td>
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<tr>
<td>Deactivation (\tau_{slow}) (ms) (^e)</td>
<td>692 ± 37 (n = 9)</td>
<td>852 ± 162 (n = 5)</td>
<td>764 ± 91 (n = 6)</td>
<td>792 ± 74 (n = 6)</td>
</tr>
<tr>
<td>I\textsubscript{End Pulse} (pA/pF) Control (^a)</td>
<td>43.9 ± 6.8 (n = 12)</td>
<td>39.6 ± 5.8 (n = 11)</td>
<td>36.0 ± 6.8 (n = 11)</td>
<td>48.1 ± 10.8 (n = 7)</td>
</tr>
<tr>
<td>I\textsubscript{End Pulse} (pA/pF) +NS3623 (^a)</td>
<td>60.8 ± 10.8 (n = 12)</td>
<td>52.9 ± 8.9 (n = 11)</td>
<td>53.0 ± 11.5 (n = 11)</td>
<td>62.7 ± 13.4 (n = 7)</td>
</tr>
<tr>
<td>% Change I\textsubscript{End Pulse} (pA/pF) (^a)</td>
<td>135 ± 4% (n = 12)</td>
<td>130 ± 3% (n = 11)</td>
<td>145 ± 6% (n = 11)</td>
<td>131 ± 6% (n = 7)</td>
</tr>
<tr>
<td>I\textsubscript{End Pulse} (pA/pF) Control (^a)</td>
<td>P = .0005</td>
<td>P = .001</td>
<td>P = .001</td>
<td>P = .015</td>
</tr>
<tr>
<td>I\textsubscript{End Pulse} (pA/pF) +NS3623 (^a)</td>
<td>99.5 ± 13.5 (n = 12)</td>
<td>100.3 ± 11.9 (n = 11)</td>
<td>83.3 ± 9.1 (n = 11)</td>
<td>112.5 ± 22.5 (n = 7)</td>
</tr>
<tr>
<td>% Change I\textsubscript{End Pulse} (pA/pF) (^a)</td>
<td>122 ± 3% (n = 12)</td>
<td>119 ± 2.3% (n = 11)</td>
<td>130 ± 3% (n = 11)</td>
<td>124 ± 4% (n = 7)</td>
</tr>
</tbody>
</table>

\(^a\) Compared to +KChIP2.2.

\(^e\) One-way ANOVA.

\(^a\) Wilcoxon matched-paired signed rank test.

\(^e\) Compared to hERG/KChIP2.2.
Itail amplitudes were then fitted using a mono-exponential function, giving fast (τfast) and slow (τslow) components (Fig. 4D, left and right panels and Table 2). Similar to the lack of effect of subunit co-expression on the fully activated I-V relation, both components of deactivation were unaffected by the addition of KChIP2.1/2.2 or DPP6.

3.5. KChIP2.1/2.2 and DPP6 effects on IhERG activation and inactivation recovery

Activation time course of IhERG was evaluated using an “envelope of tails” protocol [37,42,43]. The protocol consisted of a variable duration pre-pulse to +20 mV (from 10 to 3000 ms) followed by a repolarizing step to −40 mV to de-activate IhERG and produce a tail current (Itail). Itail amplitudes were then fitted by a monoexponential function to give time constants for activation which were compared across different expression conditions (Fig. 5A and Table 2). Neither co-expression with KChIP2.1 nor DPP6 altered the activation time-course (p = .99 for both groups, One-way ANOVA). However, KChIP2.2 co-expression resulted in a ∼35% increase in the rate of IhERG activation (p = .049, One-way ANOVA) (Fig. 5B and Table 2).

To examine the time dependence of recovery from inactivation we used a 2 s long depolarizing step to +40 mV to activate IhERG current (during which a proportion of hERG channels inactivate), followed by a variable length repolarization step to −40 mV (between 2 and 20 ms) to allow the channels to recover from inactivation. This was followed by a 20 ms test step to +40 mV to probe the extent of recovery from inactivation. Currents were normalized to the maximal current (I_{max}) at each repolarization potential for each cell by fitting a bi-exponential function, giving fast (τfast) and slow (τslow) components.

The deactivation time-course was calculated from the tail currents (Itails) at each repolarization potential for each cell by

\[ I_{max} = I_{peak} \times e^{-\frac{t}{\tau_{fast}}} + I_{steady} \times e^{-\frac{t}{\tau_{slow}}}. \]

As in the activation protocol, no significant differences were observed (p > .99 for both groups, One-way ANOVA). However, KChIP2.2 co-expression resulted in a significantly faster recovery time-constant compared to KChIP2.1 (Fig. 5D and Table 2. One-Way ANOVA, p = .016).

3.6. Effect of KChIP2 isof orm and DPP6 expression on IhERG activation during the ventricular AP

Although KChIP2.1/2.2/DPP6 co-expression had only small effects on the individual kinetic parameters of IhERG, the acceleration of activation by (for example) KChIP2.2 and other kinetic interactions during the dynamic AP might produce some summative effect. We therefore examined potential modulation of IhERG by KChIP2.1/2.2/DPP6 accessory subunits under AP clamp as the most physiological stimulus, using a digitised human ventricular AP waveform as the voltage command [38,40,44]. The AP waveform as well as an exemplar IhERG record from a cell expressing only hERG are shown in Fig. 6A and Table 2. Consistent with prior studies, IhERG current slowly increased during the plateau phase before quickly increasing in amplitude once the repolarization phase of the action potential started, with a maximal peak at ∼−40 mV [38,44]. Current dramatically declined during terminal repolarization. As expected from the above data, only small differences on the activation profile are seen in the normalized instantaneous I-V relation for all conditions (Fig. 6B cf. Table 2), specifically at voltages more depolarized than −40 mV. Maximal currents occurred late in repolarization in all conditions, with a mean membrane potential of −35.58 ± 0.94 mV (n = 11) in hERG-only expressing cells. The voltages in the other groups were: −35.91 ± 1.11 mV (hERG/KChIP2.1; n = 7), −35.63 ± 2.68 mV (hERG/DPP6; n = 8) and −35.91 ± 1.11 mV (hERG/KChIP2.2; n = 7) and −35.63 ± 2.68 mV (hERG/KChIP2.1; n = 7) and −35.91 ± 1.11 mV (hERG/DPP6; n = 8). No significant changes due to KChIP2.1/2.2/DPP6 co-expression were detected (One-Way ANOVA, p = .27).

3.7. Effect of KChIP2 and DPP6 in the NS3623-mediated IhERG activation

In addition to the effect of NS3623 on Ih in ventricular cardiomyocytes, this compound also activates hERG ion channels in ex vivo preparations [29,35,45]. To confirm the activity of NS3623 against IhERG and to explore the involvement of either KChIP2.1/2.2 isoforms and DPP6 in the compound-mediated response, we used our “standard” IhERG Voltage step protocol (Fig. 3A) before and after adding 10 μmol/L NS3623. Representative IhERG traces in control (black line) and with
10 μmol/L NS3623 (dotted line) are shown in Fig. 7A and a plot of $I_{hERG}$ tail amplitudes (at $-40$ mV) over time is shown in Fig. 7B. The results show that $I_{hERG}$ is quickly, and reversibly, activated by NS3623. An increase in $I_{hERG}$ in response to NS3623 was always observed when KChIP2.1/2.2 and DPP6 were co-expressed and the degree enhancement of $I_{hERG}$ was independent of KChIP2.x and DPP6 co-expression (see Fig. 7C and D, Table 2).

4. Discussion

To our knowledge, this is the first study to investigate a potential modulatory role of the accessory subunits KChIP2.1, KChIP2.2 and DPP6 on recombinant hERG channels and to evaluate the dual potassium channel opener NS3623 on recombinant Kv4.3 channels. In addition, we have examined the effect of the 220 amino acid long isoform KChIP2.2 as well as KChIP2.1 on Kv4.3 and its response to NS3623, both of which are expressed in human cardiac tissue [14,46]. It is important to point out that we have used expression of GFP protein as a reporter to identify cells expressing the ancillary subunits of interest. Although this approach does not guarantee that all constructs are expressed within the same cells, it was reassuring that the current density-voltage (I-V) plots in Fig. 1B and C showed distinct patterns depending on whether KChIP2.1 or KChIP2.2 was co-transfected with Kv4.3 or with the presence/absence of DPP6. Furthermore, for Kv4.3 experiments, we have used substantial replicate ‘n’ numbers (see Figs. 1 and 2), so we are confident that our measurements should reflect successful transfection.

4.1. KChIP2.1, KChIP2.2 and DPP6 effects on Kv4.3 electrophysiology

Expression of both KChIP2.1 and KChIP2.2 resulted in a larger Kv4.3-mediated outward current (giving a $\sim 2.3$ and $\sim 1.7$-fold larger current at +40 mV respectively) than Kv4.3 alone. Qualitatively, our results are in reasonable accord with previous studies assessing KChIP2.1 and Kv4.3 expressed in HEK 293 cells [46], CHO cells [47] and Xenopus oocytes [48,49], although our data show that KChIP2.2 increases current magnitude more weakly than KChIP2.1 ($p = .0033$ at +40 mV, Two-way ANOVA). Importantly, addition of DPP6 reduced the difference in current density produced by the KChIP2.1/2.2 isoforms by reducing the current density which was increased by KChIP2.1.
co-expression while leaving KChIP2.2 current density largely unaltered (p = .915 at +40 mV, Two-way ANOVA). The latter result is similar to the finding that co-expression of DPP6 with KChIP2.1 had no significant effect on current density compared to KChIP2.1 alone [50]. This suggests that DPP6 is not only a chaperone for Kv4.3 expression levels [50] but can also stabilize the properties of the Kv4.3/KChIP2.x complex across different KChIP2.x isoforms (Table 1).

It is unclear whether \( I_{to} \) inactivation follows a mono- or a bi-exponential time course [15,48,51] but in our experiments, mono-exponential fits did not properly describe Kv4.3 inactivation. The rate of Kv4.3 current inactivation was slowed by accessory subunit co-expression in our experiments and a larger effect was observed in cells co-expressing Kv4.3/KChIP2.1 compared to Kv4.3/KChIP2.2. Whilst KChIP2.1 slowed inactivation (increasing both the fast and slow inactivation time constants by \( \sim 90\% \) and \( \sim 70\% \) respectively), in cells expressing KChIP2.2 the increase was smaller and the change in fast component change was no longer statistically significant. Our fast time constant values are slightly slower than reported by Lundby et al. [32] for experiments in a CHO expression system at 37 °C but this may be explained by the temperature difference between our studies [52]. Our data differs from that reported in Xenopus oocytes [15], suggesting that both the cell system and experimental temperatures may differentially affect biophysical properties.

Co-expression of either KChIP2 isoform with DPP6 did not significantly change inactivation time constants compared to Kv4.3 alone, suggesting DPP6 opposes the kinetic increase mediated by KChIP2.x. A similar result has been previously reported for the KChIP2.1 isoform in oocytes [32]. However, these results differ from an earlier study in CHO cells where expression of KChIP2.2 and KChIP2.2 + DPP6 showed similar time constants [9]. The recovery from inactivation showed the expected acceleration following expression of either KChIP2 isoforms or KChIP2/DPP6 co-expression, although our time constants are faster than reported in previous studies (albeit under somewhat different conditions). While our results show no significant difference between KChIP2.2 and KChIP2.2 + DPP6 (~7 ms), Radicke et al. [52] reported an accelerated rate of recovery when DPP6 is co-expressed with KChIP2.2. However, their rate in CHO cells is considerably slower (~14 ms at 37 °C) than we observed in HEK293 cells and this difference is not explainable by the temperature difference between our studies because the Q10 is \( \sim 2 \) [52].

4.2. Effect of KChIP2.2, KChIP2.1 and DPP6 on recombinant hERG channels

In contrast to a recent report demonstrating that interactions between recombinant Kv4.3 and hERG channels can result in an increase in \( I_{hERG} \) density [23], co-expression of KChIP2.2, KChIP2.1 or DPP6 along with recombinant hERG channels resulted in no change in the magnitude of \( I_{hERG} \). The voltage and time-dependent properties of \( I_{hERG} \) were generally unaltered although KChIP2.2 co-expression appeared to accelerate hERG activation (Table 2). Furthermore, direct examination of \( I_{hERG} \) profile under AP voltage clamp showed no significant difference in the profile of current activation with and without \( I_{to} \) accessory unit co-expression. Some recent studies have also investigated the effects of KChIP2 in guinea pig ventricular myocytes, which lack a functional \( I_{to} \) but still expresses KChIP2 [25,53]. Knocking-down KChIP2 expression in guinea pig ventricular myocytes leads to AP prolongation which is not due to changes in \( I_{to} \) and \( I_{to} \) but an increase in Cav1.2 calcium channel expression [25]. Thus although KChIP2.x may be considered a “master regulator of cardiac repolarization” [54] \( I_{hERG} \) appears to escape this regulation.

4.3. Effect of NS3623 on recombinant Kv4.3 and hERG channels

Hansen and colleagues characterized the effect of NS3623 on \( I_{hERG} \) in Xenopus oocytes, reporting: (1) an increase in \( I_{hERG} \) amplitude; (2) a rightward shift in the voltage-dependence of inactivation and (3) a slower onset of inactivation [35]. Finally, evaluation of a mutant lacking inactivation (hERG-S620T) showed that the compound did not further augment \( I_{hERG} \). Since no accessory subunits were co-expressed with hERG in that study, their results support the idea of a direct interaction between NS3623 and hERG protein. Further studies in guinea pig (lacking \( I_{to} \)) showed NS3623 shortens the AP and decreases the appearance of extrastyles in perfused hearts as well as reversing drug-induced QT prolongation, supporting the idea of some therapeutic potential for this compound [45]. Our results show that \( I_{to} \), modulating \( \beta \)-subunits do not influence the agonism of \( I_{hERG} \) by NS3623, consistent with both the lack of modulatory effects of KChIP2/DPP6 on the channel and a direct \( \alpha \)-subunit effect.

In relation to the effect of NS3623 on Kv4.3, our results show that the compound augments current amplitude without accessory subunit co-expression in contrast to findings by Hansen and colleagues using Xenopus oocytes, who failed to detect Kv4.3 activation [35]. The reason for this discrepancy is not clear, but may be related to the different expression systems and recording conditions (i.e. a mammalian cell line at 33 °C in the present study versus Xenopus oocytes at room temperature). The concordance of our findings with the recent report by Calloe et al. [29] using canine heart preparations (which showed an agonist effect of NS3623 on \( I_{to} \)) highlights the importance of using mammalian cell expression systems for Kv4.3 studies. The rapid response to NS3623 which augments Kv4.3 current is consistent with acute effects of NS3623 on \( I_{to} \) leading to an increased epicardial action potential notch seen in canine left ventricular wedge preparations [29]. Similarly, effects on current kinetics are also consistent with the primary modulatory effects of NS3623 being ion channel function rather than trafficking/expression. Integrated charge transfer during current activation at +40 mV in the presence of NS3623 was increased by ~50% by KChIP 2.1/2.2 expression and this was further increased by DPP6 co-expression to ~100% (AUC in Table 1). Thus the addition of these subunits significantly augments the effect of NS3623 and this raises the possibilities of both multiple interaction sites for NS3623 and that these effects may be different in different regions of the heart depending of the level/composition of subunit expression. This notion is consistent with a prior study of the effects of the related compound NS5806 on canine \( I_{to} \) [47]; these were greatest in epi- and mid-myocardial cells, which had the highest levels of KChIP2. Whether such differential expression and response could be beneficial for treatment of disease should be considered in future studies. Of course, as is the case for all expression studies, it is not possible to rule out the possibility that the NS3623 response may be altered by differing relative expression levels of Kv4.3 and KChIP2/DPP6 or that other accessory subunits not studied here may contribute to the Kv4.3 response in native tissue.

4.4. Conclusions

Despite structural similarities, our data show that NS3623 binding sites must, in some ways, be different from the binding site(s) for NS5806 since stimulation of Kv4.3 current by NS5806 requires the presence of KChIP2 [47]. Our results show that, although KChIP2 isoforms and DPP6 proteins are not required for Kv4.3 activation by NS3623, their presence influences the compound’s effects on the rate of current decay, dominating the overall effect as reflected by total charge transfer (AUC). In addition, the change in kinetics depends on \( \beta \)-subunit combinations, with no change in the slow inactivation component when only KChIP2.1 is expressed. This observation suggests KChIP2 influences the gating effect of NS3623 on Kv4.3. Whether this effect is the result of the drug having multiple binding sites on Kv4.3 and/or accessory subunits is unclear. Experiments with the KChIP3 isoform have shown NS5806 can bind at a hydrophobic site within the C terminus, modulating the interaction between KChIP3 and Kv4.3 [55]. NS5806 has an additional trfluoromethyl group and bromine compared to NS3523. It is remarkable how such a small molecular difference can...
lead to such different mechanisms of action. Further studies are therefore needed to identify the interaction sites between Kv4.3 (and KChIP2) and where the site(s) that mediate differential drug effects on current amplitude and kinetics are located. Elucidation of the underlying molecular basis of the differences between NS2623 and NS5806 could lead to new therapeutic developments being derived from these prototype drugs. At this juncture, we can only suggest that NS3623 either binds to both Kv4.3 and KChIP2 and/or that there are two binding sites on Kv4.3 where one of them inhibits KChIP2 binding.

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Conflicts of interest

None.

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