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Modulation of human cardiac transient outward potassium current by EGFR tyrosine kinase and Src-family kinases

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Aims: The human cardiac transient outward K^+ current I_{to} (encoded by Kv4.3 or *KCND3*) plays an important role in phase 1 rapid repolarization of cardiac action potentials in the heart. However, modulation of I_{to} by intracellular signal transduction is not fully understood. The present study was therefore designed to determine whether/how human atrial I_{to} and hKv4.3 channels stably expressed in HEK 293 cells are regulated by protein tyrosine kinases (PTKs).
Methods and results: Whole-cell patch voltage-clamp, immunoprecipitation, Western blotting, and site-directed mutagenesis approaches were employed in the present study. We found that human atrial I_{to} was inhibited by the broad-spectrum PTK inhibitor genistein, the selective EGFR (epidermal growth factor receptor) kinase inhibitor AG556, and the Src-family kinases inhibitor PP2. The inhibitory effect was countered by the protein tyrosine phosphatase inhibitor orthovanadate. In HEK 293 cells stably expressing human *KCND3*, genistein, AG556 and PP2 significantly reduced the hKv4.3 current, and the reduction was antagonized by orthovanadate. Interestingly, orthovanadate also reversed the reduced tyrosine phosphorylation level of hKv4.3 channels by genistein, AG556 or PP2. Mutagenesis revealed that the hKv4.3 mutant Y136F lost the inhibitory response to AG556, while Y108F lost response to PP2. The double mutant Y108F-Y136F hKv4.3 channels showed no response to either AG556 or PP2.
Conclusion: Our results demonstrate that human atrial I_{to} and cloned hKv4.3 channels are modulated by EGFR kinase via phosphorylation of the Y136 residue and Src-family kinases via phosphorylation of the Y108 residue; tyrosine phosphorylation of the channel may be involved in regulating cardiac electrophysiology.
Key words. Human atrial myocytes; transient outward potassium current; hKv4.3; protein tyrosine kinases, protein tyrosine phosphatase, EGFR kinase, Src-family kinases

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1. Introduction

The transient outward potassium current I_{to} in human and canine hearts is mainly encoded by the Kv4.3 (*KCND3*) gene.[1, 2] Cardiac I_{to} is regulated by various endogenous molecules and signal pathways.[3] Alpha-adrenergic stimulation inhibits cardiac I_{to} in rabbit ventricular myocytes.[4] I_{to} in human cardiac myocytes and cloned hKv4.3 current expressed in mammalian cells are reduced by protein kinase C (PKC).[5, 6] Activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) slows Kv4.3 current inactivation and accelerates the recovery rate from inactivation of the current via directly phosphorylating S550 located in C-terminus.[7] Nitric oxide inhibited Kv4.3 channels expressed in CHO cells and human atrial I_{to} by activating adenylate cyclase and the subsequent activation of PKA and the serine/threonine phosphatase 2A.[8]

Our earlier study demonstrated that genistein, a broad-spectrum inhibitor of protein tyrosine kinases (PTKs), decreased I_{to} in rat ventricular myocytes by inhibiting PTKs; the inhibition effect was antagonized by orthovanadate, an inhibitor of protein tyrosine phosphatases (PTPs).[9] Consistent with our report, a recent study demonstrated that a functional interaction was present in between Kv4.3 channels expressed in HEK 293 cells and c-Src tyrosine kinase; furthermore, the tyrosine phosphorylation of hKv4,3 protein was mediated by SH2 and SH3 domains of c-Src kinases.[10] However, a more recent report showed that genistein inhibited Kv4.3 channels stably expressed in CHO cells via a tyrosine phosphorylation-independent mechanism.[11] This raises the question whether cardiac I_{to}/Kv4.3 channels are regulated by PTKs. In the present study, we utilized the multiple experimental approaches including electrophysiology, immunoprecipitation, Western blotting, and mutagenesis to investigate whether/how human atrial I_{to} and hKv4.3 channels stably expressed in HEK 293 cells are regulated by PTKs. We found that both Src-family kinases and EGFR kinase regulated human cardiac I_{to} and hKv4.3 channels via phosphorylating tyrosine residues in the N-terminus of the channel.

2. Materials and Methods

2.1 Human atrial myocyte preparation

Human atrial specimens of human right atrial appendage were obtained from patients undergoing coronary artery bypass grafting. The procedure for obtaining the tissues was approved by the Ethics Committee of the University of Hong Kong (UW-10-174), with the patients' consent. The investigation conforms with the principles outlined in the Declaration of Helsinki (see *Cardiovascular Research* 1997;35:2–4) for use of human tissue. Human atrial myocytes were enzymatically isolated from the procedure as described previously[12, 13] and in *Supplemental Methods*. The isolated myocytes were kept at room temperature in the KB medium[12, 13] at least 1 h before use.

2.2 Cell culture, mutagenesis and gene transfection

The pCDNA3.1/hKv4.3 plasmid was kindly provided by Dr. Klaus Steinmeyer (Sanofi-Aventis Deutschland GmbH), and transfected into HEK 293 cells (ATCC, Manassas, VA). The HEK 293 cell line stably expressing hKv4.3 channels was established as described previously[14, 15] and in Supplemental Methods.

2.3 Solutions and chemicals

Compositions of Tyrode solution and pipette solutions are described in *Supplemental Methods*. 3-(4-Chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d] pyrimidin-4-amine (PP2) was purchased from Tocris Bioscience (Bristol, UK). Other reagents were obtained from Sigma-Aldrich (St Louis, MO). Stock solutions were made in dimethyl sulfoxide (DMSO) for genistein (100 mM), AG556 (100 mM), PP2 (10 mM). The stocks were aliquoted and stored at -20°C. Sodium orthovanadate stock solution (100 mM) was made in distilled water and pH value was adjusted to 9.0 with HCl.

2.4 Electrophysiology

Current recording for I_{to} and Kv4.3 current and action potential recording were performed as described previously[12, 13] and in *Supplemental Methods*. Briefly, human atrial myocytes or HEK 293 cells on a cover slip were transferred to the cell chamber and superfused with Tyrode solution. After a gigaohm seal was obtained by negative pressure, the cell membrane was ruptured by applying a gentle

negative pressure to establish the whole-cell configuration. Series resistance (3-6 MΩ) was compensated by 80% to minimize voltage errors. Liquid junction potentials (~12 mV) between pipette and bath solutions were not corrected for the patch clamp recording. Membrane currents were measured using an EPC-10 amplifier and Pulse software (Heka Elektronik, Lambrecht, Germany). Command pulses were generated by a 12-bit digital-to-analog converter controlled by Pulse software. All current recording experiments were conducted at room temperature (22-23°C).

Action potentials in human atrial myocytes were recorded at 36°C using a perforated patch configuration by including 200 μg/ml amphotericin B (Sigma-Aldrich) in the K^+ pipette solution as described previously.[1] Electrical signals were low-pass filtered at 5 kHz and stored on the hard disk of an IBM compatible computer.

2.5 Immunoprecipitation and Western blotting

The immunoprecipitation and Western blotting were performed following the procedure described previously[15, 16] and in *Supplemental Methods*. Briefly, HEK 293 cells (~80% confluence) stably expressing hKv4.3 channels were treated respectively with different interventions for 30 minutes at room temperature, and centrifuged at 4°C. The cell pellet was then lysed with the lysis buffer.[15, 16] Protein quantification of lysates was made using a protein assay reader (Bio-Rad Laboratories, Hercules, CA), and diluted to equal concentrations. Proteins were immunoprecipitated overnight at 4°C using 0.8 µg of anti-hKv4.3 antibody (NeuroMab, USA) and 100 µl of protein A/G beads (Upstate). Immunoprecipitated proteins bound to pelleted protein A/G beads were washed thoroughly in PBS, denatured in Laemmli sample buffer, separated using SDS-PAGE, and electroblotted onto nitrocellulose membranes. The immunoblots were probed with anti-phosphotyrosine antibody (1:2000, Cell Signaling) overnight at 4°C in a blocking solution containing 5% BSA in TBS and Tween 20, and subsequently treated with goat anti-mouse IgG-HRP antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. Blots were developed with enhanced chemiluminescence (ECL, GE Healthcare, Hong Kong) and exposed of X-ray film (Fuji Photo Film GmbH). The blots were then stripped and reprobed with the anti-hKv4.3 channel antibody to determine total hKv4.3 channel proteins. The film was scanned, imaged by a Bio-Imaging System (Syngene, Cambridge, UK), and analyzed via Gene Tools software (Syngene).

2.6 Statistical analysis

The data are expressed as means±SEM. Paired and/or unpaired Student's t-test were used as appropriate to evaluate the statistical significance of differences between two group means, and ANOVA was used for multiple groups. Values of $P<0.05$ were considered to be statistically significant.

3. Results

3.1 Effects of PTK inhibitors on I_{to} in human atrial myocytes

Human atrial I_{to} was recorded in the presence of 2 μM DPO-1 to limit the contamination of I_{Kur} as described previously.[13] The effect of the broad spectrum PTK inhibitor genistein on I_{to} was first examined in human atrial myocytes. Figure 1A shows the voltage-dependent I_{to} recorded in a representative human atrial myocyte with voltage steps as shown in the inset before and after application of genistein (10 μM) in bath solution. The current was significantly inhibited by genistein (5 min), and the effect recovered on washout (8 min).

If genistein-induced reduction of human atrial I_{to} is related to PTK inhibition, the effect should be antagonized by the protein tyrosine phosphatase (PTP) inhibitor orthovanadate.[15, 16] We tested the effect of orthovanadate (1 mM) on I_{to}, but this compound had no effect on I_{to} (n=5, data not shown). However, orthovanadate partially reversed the I_{to} inhibition induced by 10 μM genistein in a representative myocyte (Fig. 1B). This suggests that PTK inhibition is partially involved in I_{to} reduction by genistein.

To determine which specific PTK is involved, the highly selective EGFR kinase inhibitor AG556 and the Src-family kinase inhibitor PP2 were tested. AG556 (10 μM) reversibly inhibited the voltage-dependent I_{to} in a representative human atrial myocyte (Fig. 1C). The time course of I_{to} recorded in a typical experiment with a voltage step to +50 mV exhibits a gradual reduction of the current during 10 μM AG556 application (8 min) and the current inhibition was reversed by co-application of AG556 with 1 mM orthovanadate (Fig. 1D). This suggests that EGFR kinase inhibition may suppress human atrial I_{to}.

Figure 1E shows the voltage-dependent I_{to} recorded in a representative myocyte in the absence and presence of 10 μM PP2. PP2 slightly reduced the current amplitude, and the inhibition was reversed on washout. Figure 1F displays the time course of I_{to} recorded in a typical experiment with the voltage step shown in the inset in the absence and presence of 10 μM PP2, and with co-application of 1 mM orthovanadate. PP2 gradually decreased I_{to} and the inhibition was partially reversed by orthovanadate.

Figure 1G illustrates the percentage values of human atrial I_{to} inhibition by genistein, AG556, or PP2 with and without co-application of orthovanadate. Genistein (10 μM) reduced I_{to} (at +50 mV) by 13.8% (n=7, P<0.05 vs. control), and the inhibition was reversed to 7.2% by 1 mM orthovanadate (P=NS vs. genistein alone). AG556 (10 μM) inhibited I_{to} (at +50 mV) by 35.4% (n=7, P<0.01 vs. control), and the inhibitory action was reversed by co-application of 1 mM orthovanadate to 9.3% (P<0.05 vs. AG556 alone). PP2 (10 μM) reduced the current (at +50 mV) by 12.9% (n=6, P<0.05 vs. control), and the inhibition was reversed by co-application of 1 mM orthovanadate to 7.7% (P=NS vs. PP2 alone). These results suggest that EGFR kinase and Src-family kinases likely participate in the regulation of human atrial I_{to}.

To determine the effect of these PTK inhibitors on inactivation kinetics of human cardiac I_{to}, the current (at +50 mV) was fitted to a mono-exponential equation in the absence and presence of genistein, AG556, or PP2 (Supplemental Fig. 1A). Genistein (10 μM) reduced the inactivation time constant to 34.8 ± 2.3 ms from 43.4 ± 2.4 ms of control (n=6, P<0.05), AG556 (10 μM) decreased the time constant to 14.5 ± 2.5 ms from 36.1 ± 4.3 ms of control (n=6, P<0.01), while PP2 (10 μM) reduced the time constant to 29.2 ± 1.8 ms from 38.8 ± 2.3 ms of control (n=6, P<0.05), and the effect was partially reversed by orthovanadate. These results suggest that the EGFR kinase inhibition increases the inactivation of human atrial I_{to}, and the effect of AG556 on I_{to} inactivation was stronger than that of genistein or PP2 (P<0.05).

3.2 Alteration of action potential profile by I_{to} reduction with PTK inhibitors

To investigate whether action potential profile would be affected with I_{to} inhibition by the EGFR inhibitor AG556 or the Src-family inhibitor PP2, action potentials were recorded in human atrial myocytes under conditions with 2 μM DPO-1 in current clamp mode in the absence and presence of AG556 or PP2. Figure 2B shows that AG556 (10 μM) significantly prolonged early repolarization, whereas PP2 (10 μM) induced a slight prolongation of the early repolarization (Fig. 2C) in human atrial myocytes. The effect was countered by orthovanadate. The mean values (Fig. 2B) of action potential duration at 20% and 50% repolarization (APD_{20} and APD_{50}) was increased by AG556 (n=6,

P<0.01 vs. control), and the effect was reversed by orthovanadate (P<0.05 or P<0.01 vs. GA556 alone). The slight alteration of APD with PP2 was not statistically significant (Fig. 2D, n=5, P=NS). These results suggest that EGFR kinase inhibitor AG556 delays early repolarization in human atrial myocytes and the effect may be related to its significant facilitation of I_{to} inactivation (Supplemental Figure 1).

3.3   Inhibition of hKv4.3 channels by PTKs inhibitors

It is well recognized that human cardiac Ito is encoded by Kv4.3 (*KCND3*) gene.[1-3] To investigate potential molecular basis of PTK regulation of cardiac I_{to}, we determined the current inhibition of genistein, AG556, and PP2, and their interaction with the PTP inhibitor orthovanadate in HEK 293 cells stably expressing hKv4.3 channels.

Figure 3A shows the time course of hKv4.3 current recorded in a representative cell in the absence and presence of 10 μM genistein. Similar to the observation in I_{to} of human atrial myocytes (Fig. 1A), genistein reversibly inhibited hKv4.3 current amplitude. Voltage-dependent hKv4.3 current was also inhibited by genistein, and the inhibitory effect was significantly antagonized by 1 mM orthovanadate (Fig. 3B). These results differed from the PTK-independent inhibition of genistein observed in CHO cells expressing hKv4.3 channels.[11] Our results support the notion that genistein may inhibit human atrial I_{to} and hKv4.3 channels via both PTK-dependent and PTK-independent mechanisms.

Figure 3C shows the time course of hKv4.3 current recorded in a representative cell. AG556 (10 μM) gradually inhibited hKv4.3 current and the inhibition was reversed on washout. The suppression of voltage-dependent hKv4.3 current by 10 μM AG556 was countered by 1 mM orthovanadate (Fig. 3D). Similar to the results observed in human atrial I_{to}, PP2 reversibly inhibited hKv4.3 current (Fig 3E), and the inhibitory effect was antagonized by orthovanadate (Fig. 3F).

Figure 3G illustrates the percentage values of hKv4.3 current (at +50 mV) in cells treated with genistein, AG556, PP2, or co-application of orthovanadate. Genistein (10 μM) inhibited hKv4.3 current at +50 mV by 14.5% (n=6, P<0.01 vs. control), and the inhibition effect was reversed by 1mM orthovanadate to 6.7% (P<0.05 vs. genistein alone). AG556 (10 μM) inhibited the current by 48.2 % (n=6, P<0.01 vs. control), and the inhibition effect was reversed by co-application of 1 mM orthovanadate to 8.9% (P<0.01 vs. AG556 alone). PP2 (10 μM) reduced the current by 12.5% (n=6, P<0.05 vs. control), and reduction was reversed by 1 mM orthovanadate to 2.9% (P<0.05 vs. PP2 alone). These results suggest that hKv4.3 channels expressed in HEK 293 cells, like I_{to} in human atrial myocytes, are regulated by both EGFR kinase and Src-family kinases. If this is the case, tyrosine phosphorylation level of hKv4.3 protein would be reduced by genistein, AG556, or PP2.

Similar results to those observed in human atrial I_{to}, these three PTK inhibitors facilitated the inactivation of hKv4.3 current (Supplemental Fig. 2). AG556 effect on the current inactivation was stronger than that of genistein or PP2, and the effect was partially reversed by orthovanadate. It is interesting to note that hKv4.3 current reduction and the effect on current kinetics induced by AG556 were independent of the β-subunit KChIP expression (Supplement Fig. 3).

3.4   Tyrosine phosphorylation level of hKv4.3 channels

Tyrosine phosphorylation level of hKv4.3 protein was investigated using immunoprecipitation and Western blotting analysis in hKv4.3-HEK 293 cells, but not in human atrial myocytes, because the number of isolated human atrial myocytes is not sufficient to perform this experiment. Figure 4A shows the tyrosine phosphorylation images of hKv4.3 channels in cells treated with 20 μM genistein, 20 μM AG556, or 20 μM PP2 alone, or with the addition of 1 mM orthovanadate.[16] Genistein, AG556, or PP2 significantly reduced the phosphorylation level of hKv4.3 channel protein, and the inhibitory effects were countered by pretreatment (30 min) with 1 mM orthovanadate. Orthovanadate alone did not increase phosphorylation level of hKv4.3 protein, suggesting that the phosphorylation level of hKv4.3 channel protein is saturated under basal control conditions.

Figure 4B summarizes the relative values of quantitative tyrosine phosphorylation level of hKv4.3 protein. Orthovanadate did not affect the saturated tyrosine phosphorylation of hKv4.3 protein. Genistein reduced the tyrosine phosphorylation level of kKv4.3 channel protein by 32.2% at 20 μM, n=6, P<0.01 vs. control) and by 23.3% at 10 μM (n=4, P<0.05 vs. vehicle control), and the reduction

was reversed by co-application of 1 mM orthovanadate to 11.1% and 4.5%, respectively (P<0.05 vs. genistein alone). AG556 inhibited the phosphorylation level by 64.3% at 20 µM (n=6, P<0.01 vs. control) and by 49.9% at 10 µM (n=4, P<0.01 vs. control), the inhibition was reversed by co-application of 1 mM orthovanadate to 9.2% and 1.7%, respectively (P<0.01 vs. AG556 alone). PP2 reduced the phosphorylation level by 36.7% at 20 µM (n=6, P<0.01 vs. control) and by 25.6% at 10 µM (n=4, P<0.05 vs. control), and the reduction was reversed by co-application of 1 mM orthovanadate to 11.6% and 1.5%, respectively (P<0.05 vs. PP2 alone). These results indicate that the tyrosine residues of hKv4.3 channels are phosphorylated by both EGFR and Src tyrosine kinases.

3.5 Tyrosine phosphorylation sites of hKv4.3 channels

To identify the potential PTK phosphorylation sites of hKv4.3 channels, tyrosines of hKv4.3 channels with high scores of tyrosine phosphorylation were replaced by phenylalanine. Four mutants (Y108F, Y136F, Y441F and Y528F) were generated using site-directed mutagenesis technique.[27, 28] Figure 4 displays the inhibitory sensitivity of these mutants to the EGFR kinase inhibitor AG556 or the Src-family kinase inhibitor PP2. Significant current inhibition by 10 µM AG556 was observed for WT, Y108F, Y441F, and Y528F, but not for the mutant Y136F (Fig. 5A). PP2 (10 µM) inhibited WT, Y136F, Y441F, and Y528F current, but not Y108F current (Fig. 5C).

To further determine if Y108 and Y136 are the key tyrosine sites for phosphorylation, we constructed the double mutant Y108F-Y136F. Interestingly, this mutant lost inhibitory response to both AG556 and PP2. The percentage changes of different hKv4.3 mutants in response to AG556 and PP2 are illustrated in Fig. 5B and Fig. 5D, respectively. These results indicate that Y108 (for Src-family kinases) and Y136 (for EGFR kinase) are involved in the tyrosine phosphorylation of hKv4.3 channels.

It is interesting to note that the percentage values of the peak current inhibition by AG556 (Fig. 5B, 46-48%) is stronger than those by PP2 (Fig. 5D, 10-15%). This effect is likely related to the different effects on current inactivation (AG556>PP2, Supplemental Fig 1 and Fig. 2) and/or different tyrosine phosphorylation inhibition (AG556>PP2, Fig. 4) at Y136 and Y108 of N-terminus of the channel.

If Y108 and Y136 are the dominant tyrosine residues responsible for tyrosine phosphorylation of hKv4.3 channels, replacement of tyrosine at these two sites would remarkably reduce tyrosine phosphorylation level. To test if it is the case, we performed additional experiment with immunoprecipitation and Western blot analysis. Figure 6A shows that the tyrosine phosphorylation level was dramatically reduced in HEK 293 cells expressing the double mutant Y108F-Y136F. Taken all together, these results indicate that the tyrosine phosphorylation sites of hKv4.3 channels are located at Y108 (for Src-family kinases) and Y136 (for EGFR kinase) of the N-terminus (Fig. 6B).

4. Discussion

In the present study, we have demonstrated that inhibition of EGFR kinase by AG556 and Src-family kinases by PP2 reduce I_{to}, and increases the current inactivation in human atrial myocytes and hKv4.3 channels stably expressed in HEK 293 cells. Tyrosine phosphorylation level of hKv4.3 channels is reduced by PTKs inhibitors genistein, AG556 and PP2. These effects are antagonized by the PTP inhibitor orthovanadate. The site-directed mutagenesis reveals that EGFR kinase is responsible for Y136 phosphorylation, whereas Src-family kinases are responsible for Y108 phosphorylation in hKv4.3 channels.

4.1 Regulation of ion channels by PTKs

PTKs, including receptor PTKs (e.g. EGFR kinase, epidermal growth factor receptor kinase) and nonreceptor PTKs (e.g. Src-family kinases), are important intracellular signals.[17] In addition to regulating cell growth, differentiation, embryonic development, metabolism, and oncogenesis.[18] PTKs modulate ion channels, including L-type Ca^{2+} channels,[19] voltage-gated Na^+ channels,[16, 20] and volume-sensitive Cl^- channels[21, 22] in mammalian heart myocytes as well as several types of K^+ channels in different types of cells,[9, 15, 23-25] and also TRPC channels.[26] Cardiac volume-sensitive Cl^- channels,[21, 22] voltage-gated Na^+ channels,[16, 20] and hERG channels,[15] are regulated by both EGFR kinase and Src-family kinases, whereas cardiac slowly-delayed rectifier K^+ channel current (I_{Ks}),[24]

and the inwardly-rectifier K^+ channels (Kir2.1 and Kir2.3)[27, 28] are modulated by EGFR kinase. A recent report demonstrated that hKv4.3 channels were regulated by Src-family kinases,[10] although the broad spectrum PTKs inhibitor genistein was found to inhibit this channel via a PTK-independent pathway.[11] The present study has provided the novel information that hKv4.3 channels and I_{to} in human atrial myocytes are regulated by both Src-family kinases and EGFR kinase.

4.2 Comparison with previous reports related to ion channels inhibition by genistein.

Genistein is a well-known broad spectrum PTKs inhibitor that may strongly inhibit the tyrosine activity of EGFR kinase and Src-family kinases and weakly inhibit PKA and PKC.[29, 30] Therefore, it has been widely used for studying ion channel regulation by PTKs.[9, 21, 23] However, PTK-independent effects of genistein were reported in native cardiac I_{Ks}[31] and I_{Kr},[32] neural I_{Na},[33] osteoclast I_{Kir},[34] Kir2.1 and Kir2.3 channels expressed in *Xenopus* oocytes or HEK 293 cells,[35] and Kv4.3 channels expressed in CHO cells.[11] The PTK-independent results of genistein were mainly found in experiments in which the PTK-inactive analog daidzein significantly inhibited the ionic currents and/or genistein showed a remarkable inhibition in the presence of other PTK inhibitors using only an electrophysiological technique without testing whether tyrosine phosphorylation levels of these ion channels are affected by genistein.[11, 32-34]

Our recent studies demonstrated the strong evidence that genistein inhibited recombinant cardiac I_{Ks},[24] hERG,[15] Kir2.1,[27] and Kir2.3[28] channels via a PTK-dependent mechanism. The inhibition of current amplitudes by genistein is not only accompanied with a reduction in tyrosine phosphorylation levels of these channels, but also antagonized by the PTP inhibitor orthovanadate.[15, 24, 27, 28] The present study provides additional evidence that genistein inhibits human cardiac I_{to} and hKv4.3 channels expressed in HEK 293 cells via PTK suppression. The results obtained by us and others support the notion that genistein exhibits both PTK-dependent and PTK-independent inhibition of ion channels. Thus, it is important to note that multiple techniques are required to conclude whether a pharmacological tool shows a non-specific effect.

4.3 Novel findings of the present study

Using multiple experimental techniques including electrophysiology, immunoprecipitation, Western blot analysis, and mutagenesis, we demonstrated that human atrial I_{to} and hKv4.3 channels were inhibited by the broad spectrum PTK inhibitor genistein, the selective EGFR kinase inhibitor AG556, and the Src-family kinase inhibitor PP2 via inhibiting PTKs, which is supported by the results in which the reduced current amplitude and tyrosine phosphorylation level are antagonized by the PTP inhibitor orthovanadate. Furthermore, the results showed that hKv4.3 channels, like hERG channels,[15] are regulated by both EGFR kinase and Src-family kinases, and the tyrosine phosphorylation is saturated under basal experimental conditions. It is unclear why this is different from a previous report in which increased hKv4.3 current amplitude and phosphorylation is observed by inhibiting PTP with bpV(phen) or application of c-Src kinase.[10] In addition, antagonistic regulation of $I_{Cl.vol}$ by EGFR kinase and Src-family kinases was observed in native cardiac myocytes, in which the inhibition of Src-family kinases with PP2 stimulates the current, while the inhibition of EGFR kinase with AG556 or AG1478 reduces the current.[21, 22] However, it is not the case for I_{to} and hKv4.3 channels. Although it is unclear how hKv4.3 channels are activated by EGFR kinase and Src-family kinases, our mutagenesis results revealed that the phosphorylation sites of both EGFR kinase and Src-family kinases were located respectively at Y136 and Y108 of the N-terminus of hKv4.3 channels. The tyrosine phosphorylation level of hKv4.3 channels was dramatically reduced when these two tyrosine residues were replaced by phenylalanine.

We found that the inhibition of tyrosine phosphorylation at Y136 by the EGFR inhibitor AG556 induced a stronger facilitation of the current inactivation than that at Y108 by the Src-family kinase inhibitor PP2. This effect subsequently resulted in remarkable decrease of I_{to} and prolongation of human atrial action potential at early repolarization (APD_{20} and APD_{50}) with AG556.

4.4 Significance and limitation of the present study

I_{to} is a voltage-gated current that is rapidly activated and inactivated in response to depolarization,[36] and plays an important role in cardiac action potential repolarization.[37] I_{to} is heterogeneously expressed in the transmural ventricular wall from endocardium, midmyocardium,

and epicardium in canine and human hearts, and therefore contributes significantly to the early repolarization (phase 1) of the action potentials in these regional myocytes to maintain normal cardiac heterogeneous electrophysiology.[38-40] It is believed that I_{to} contributes more to atrial repolarization than to ventricular repolarization in humans,[3] and reduced I_{to} can lead to electrical remodeling in diseased human cardiomyocytes.[41, 42]

Kv4.3 channel activity has been shown to be downregulated in pathological cardiac hypertrophy.[41-43] Interestingly, increasing Kv4.3 current density by *in vivo* overexpression of Kv4.3 gene has been found to abrogate cardiac hypertrophy.[44] In addition, cardiac Src-kinase activity increases in both physiological[43] and pathological heart hypertrophy.[45] Recent studies show that EGFR plays a critical role in physiological and pathophysiological processes of the heart.[46, 47] We therefore speculate that the level of cardiac EGFR kinase and Src-family kinase activity may be important in controlling the transition from reversible to irreversible heart hypertrophy.[48] In the present study, we demonstrate that hKv4.3 channel activity is decreased by both EGFR kinase and Src-family kinase inhibitors. This suggests that tyrosine phosphorylation of hKv4.3 channels may alter cardiac electrical activity, which is likely to play a compensatory role in which cardiac repolarization and excitability have been compromised.

In summary, the present study has provided evidence that hKv4.3 channel is positively regulated by EGFR kinase and Src-family kinases which phosphorylate Y136 residue and Y108 residue of the channel respectively, and the effect may be involved in the regulation of cellular electrical activity and thus may alter electrophysiological properties in human hearts.

Conflict of Interest: none declared.

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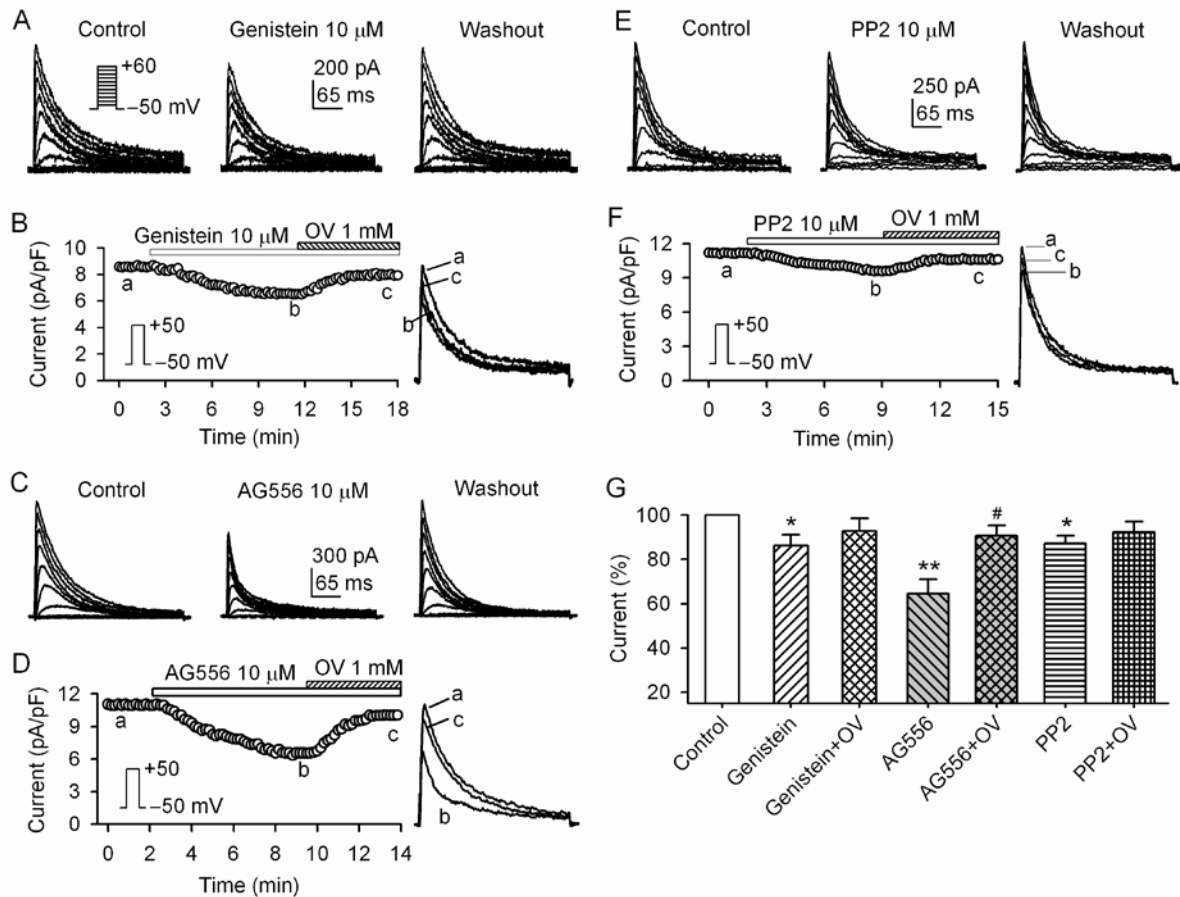
Figure 1. Inhibition of I_{to} by PTK inhibitors in human atrial myocytes. A. Voltage-dependent I_{to} traces recorded in a representative myocyte with the voltage protocol as shown in the inset in the absence and presence of 10 μM genistein. **B.** Time course of I_{to} recorded in a typical experiment with the voltage step (inset) in the absence and presence of 10 μM genistein, and genistein plus 1 mM orthovanadate (OV). Original current traces at corresponding time points are shown in right of the panel. **C.** Voltage-dependent I_{to} recorded in the absence and presence of 10 μM AG556, and upon washout. **D.** Time course of I_{to} recorded in a typical experiment during control, in the presence of 10 μM AG556, and AG556 plus 1 mM orthovanadate. Original currents at corresponding time points are shown in right of the panel. **E.** Voltage-dependent I_{to} current in a representative cell during control, in the presence of 10 μM PP2, and upon washout. **F.** Time course of I_{to} recorded with the voltage step shown in the *inset* in a representative myocyte during control, in the presence of 10 μM PP2, and PP2 plus 1 mM orthovanadate (OV). Original currents at corresponding time points are shown in right side of the panel. **G.** Mean percentage values of I_{to} at +50 mV in human atrial myocytes before (control) and after application of 10 μM genistein, 10 μM AG556, 10 μM PP2, and with co-application of 1 mM orthovanadate (n=6-7, *P<0.05, **P<0.01 vs. control, #P<0.05 vs. AG556 alone).
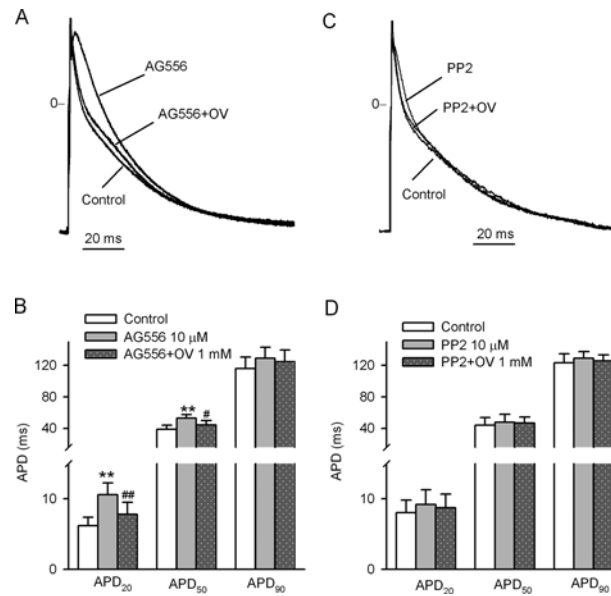
Figure 2. Effects of AG556 and PP2 on action potential profile in human atrial myocytes. A. Action potentials recorded in a representative myocyte during control, after addition of 10 μM AG556 (5 min) and AG556 plus 1 mM orthovanadate (OV). **B.** Mean values of APD20, APD50, and APD90 in control, AG556, and AG556 plus orthovanadate (n=6, **P<0.01 vs. control; #P<0.05, ##P<0.01 vs. AG556 alone). **C.** Action potentials recorded in a representative myocyte during control, after addition of 10 μM PP2 (5 min) and PP2 plus 1 mM orthovanadate (OV). **D.** Mean values of APD20, APD50, and APD90 in control, PP2, and PP2 plus orthovanadate (n=5, P=NS vs. control)
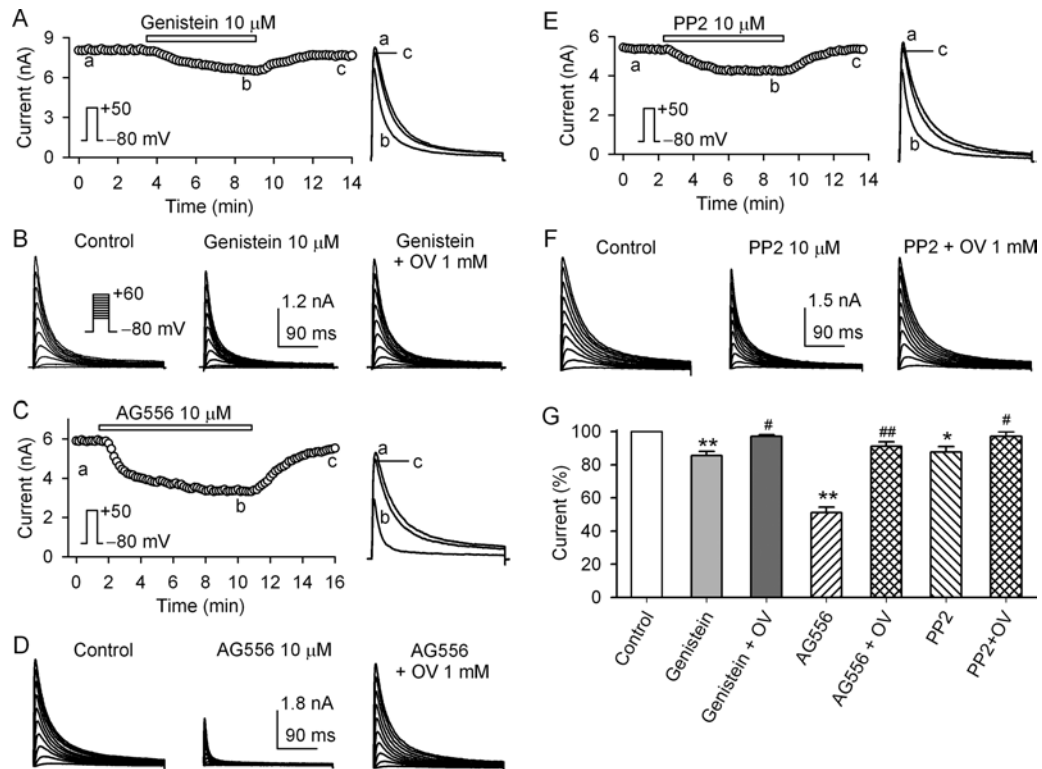
Figure 3. Effects of PTK inhibitors on hKv4.3 current stably expressed in HEK 293 cells. A. Time course of hKv4.3 current (left) and original current traces (right) in the absence and presence of genistein. **B.** Voltage-dependent hKv4.3 current was recorded in the presence of genistein, genistein plus orthovanadate (OV). **C.** Time course of hKv4.3 current (left) and original current traces (right) in the absence and presence of AG556. **D.** Voltage-dependent hKv4.3 current recorded during control, in the presence of AG556, and AG556 plus orthovanadate. **E.** Time course of hKv4.3 current and original currents recorded in the absence and presence of PP2. **F.** Voltage-dependent hKv4.3 current was recorded during control, in the presence of PP2, and PP2 plus orthovanadate. **G.** Percentage values of hKv4.3 current (at +50 mV) in the absence and presence of AG556 or AG556 plus orthovanadate, PP2, or PP2 plus orthovanadate (n=6, *P<0.05 or **P<0.01 vs. control, #P<0.05 vs. genistein or PP2 alone, ##P<0.01 vs. AG556 alone).
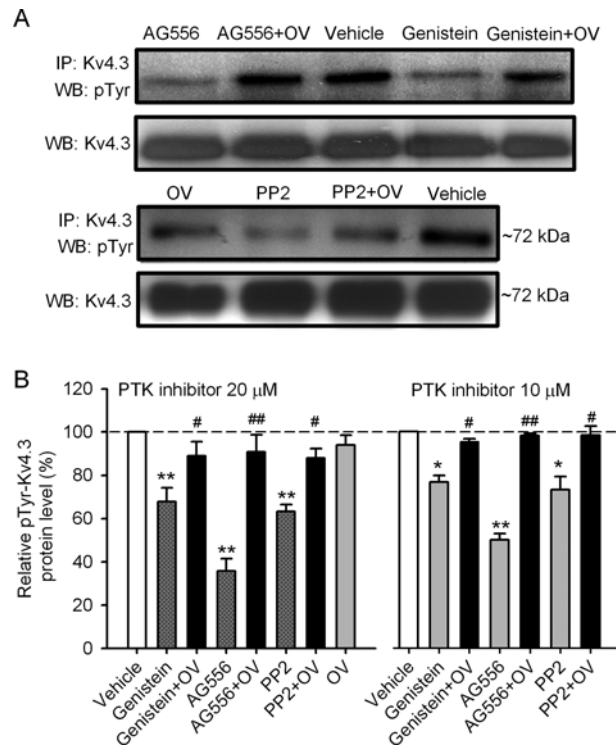
Figure 4. Tyrosine phosphorylation levels of hKv4.3 channels. A. Images of immunoprecipitation and Western blot in cells treated with vehicle (control), 20 μM AG556, AG556 plus 1 mM orthovanadate, 20 μM genistein, genistein plus orthovanadate, 1 mM orthovanadate alone, 20 μM PP2, and PP2 plus orthovanadate. **B.** Relative phosphorylated hKv4.3 levels were determined with pTyr-Kv4.3 image density divided by total hKv4.3 protein image density in cells treated with different compounds (20 μM PTK inhibitors) as described in A, and then normalized to vehicle (left, n=6, ** P<0.01 vs. vehicle control, #P<0.05 vs. genistein or PP2 alone, ##P<0.01 vs. AG556 alone), and in cells treated with 10 μM PTK inhibitors (right, n=4, ** P<0.01 vs. vehicle control, #P<0.05 vs. genistein or PP2 alone, ##P<0.01 vs. AG556 alone).
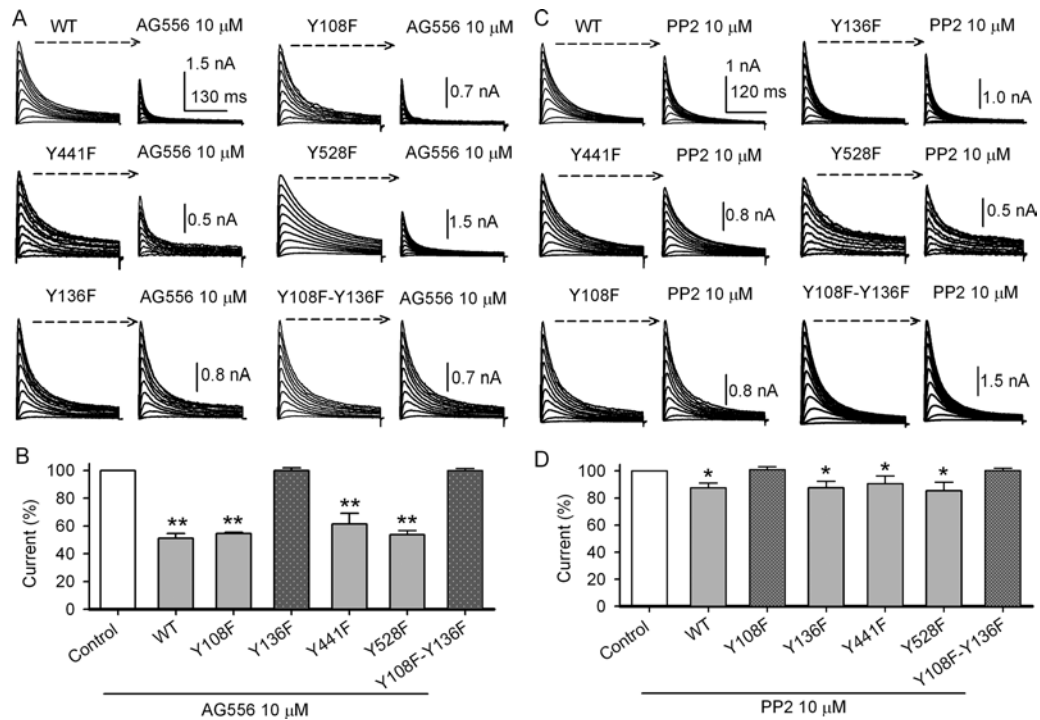
Figure 5. Mutant hKv4.3 channels and effects of AG556 and PP2. A. hKv4.3 currents recorded in HEK 293 cells expressing WT hKv4.3, Y108F, Y136F, Y441F, Y528F, and Y108F-Y136F mutants before and after application of 10 μM AG556. **B.** Percentage changes of hKv4.3 current (at +50 mV) in WT and different hKv4.3 mutants in response to 10 μM AG556 (n=5, **P<0.01 vs. control). **C.** hKv4.3 currents recorded in HEK 293 cells expressing WT hKv4.3, Y108F, Y136F, Y441F, Y528F, and Y108F-Y136F mutants before and after application of 10 μM PP2. **D.** Percentage changes of WT and different mutant hKv4.3 currents (at +50 mV) in response to 10 μM PP2 (n=5,*P<0.05 vs. control).
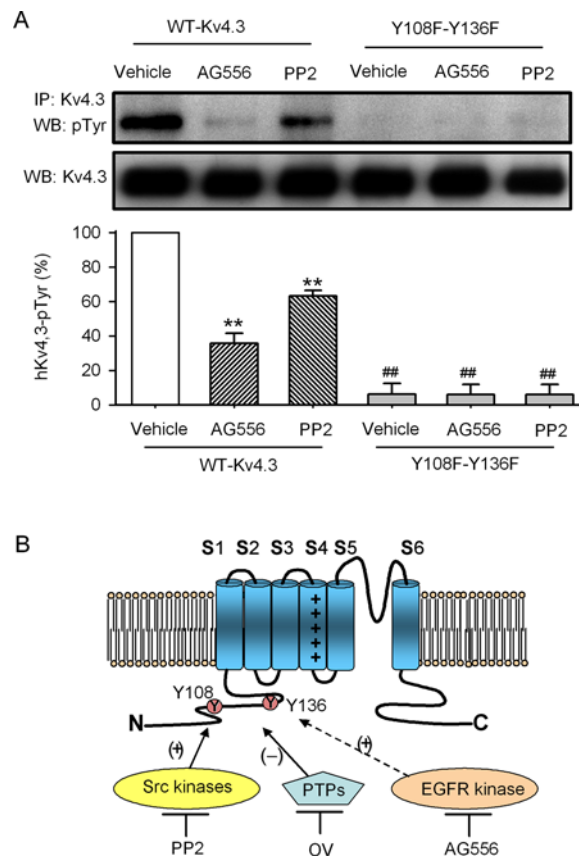
Figure 6. Tyrosine phosphorylation of the mutant Y108F-Y136F and schematic model for the regulation of hKv4.3 channel by EGFR and Src-family kinases. **A.** Upper panel: images of immunoprecipitation and Western blot in cells expressing WT or Y108F-Y136F treated with vehicle (control), 20 μM AG556, or 20 μM PP2. Lower panel: percentage values of phosphorylated hKv4.3 (pTyr-Kv4.3) level in cells as described in A were determined as in Fig. 4B (n=3, **P<0.01 vs. vehicle control, ##P<0.01 vs. WT vehicle control). B. Schematic model for regulation of hKv4.3 channels by EGFR and Src-family kinases. The tyrosine residues Y108 and Y136 of hKv4.3 channels are phosphorylated respectively by Src-family kinases and EGFR kinase. Phosphorylation of tyrosine by one or more steps favors hKv4.3 channel activation, whereas inhibition of phosphorylation may favor channel closure. AG556 inhibited EGFR tyrosine kinase and PP2 inhibited Src-family kinases.

Supplementary Materials

1. Supplementary Methods

1.1 Human atrial myocyte preparation

Atrial myocytes were enzymatically isolated from specimens of human right atrial appendage obtained from patients undergoing coronary artery bypass grafting. The experimental procedure for obtaining the human tissue was approved by the Ethics Committee of the University of Hong Kong (UW-10-174), based on the patients' consent. All patients were free from supraventricular tachyarrythmias, and the atria were grossly normal at the time of surgery. After excision, the samples were quickly immersed in oxygenated, normally Ca^{2+}-free cardioplegic solution for transport to the laboratory. Atrial myocytes were enzymatically dissociated as described previously.[1,2] The sliced cardiac tissue was gently agitated by continuous bubbling with 100% O_2 in a Ca^{2+}-free Tyrode solution (36 °C) for 15 min (5 min at a time in fresh solution). The chunks were then incubated for 50min in a similar solution containing 150-200 U/ml collagenase (CLS II, Worthington Biochemical, Freehold, NJ), 0.2 mg/ml proteinase (type XXIV, Sigma-Aldrich Chemical, St Louis, MO) and 1 mg/ml bovine serum albumin (Sigma-Aldrich). Subsequently, the supernatant was discarded. The chunks were re-incubated in a fresh enzyme solution with the same composition but no protease, microscope examination of the medium was performed every 10-15 min to determine the number and the quality of the isolated cells. When the yield appeared to be maximal, the chunks were suspended in a high K^+ medium containing (mM) 10 KCl, 120 K-glutamate, 10 KH_2PO_4, 1.8 $MgSO_4$, 10 taurine, 10 HEPES, 0.5 EGTA, 20 glucose, 10 mannitol, pH was adjusted to 7.3 with KOH. The isolated myocytes were kept at room temperature in the medium at least 1 h before use.

A small aliquot of the solution containing the isolated cells was placed in an open perfusion chamber (1-ml) mounted on the stage of an inverted microscope. Myocytes were allowed to adhere to the bottom of the chamber for 5-10 min and were superfused at 2-3 ml/min with Tyrode solution. Only quiescent rod-shaped cells with clear cross-striations were used. The study was conducted at room temperature (21-22 °C) except for specified.

1.2 Cell culture, mutagenesis and gene transfection

The pCDNA3.1/hKv4.3 plasmid was kindly provided by Dr. Klaus Steinmeyer (Sanofi-Aventis Deutschland GmbH), and transfected into HEK293 cells (ATCC, Manassas, VA). The HEK293 cell line stably expressing hKv4.3 channel was established as previously described,[3] and cultured in Dulbecco's modified eagle's medium (DMEM, Invitrogen, Hong Kong) supplemented with 10% fetal bovine serum. Clones for stably expressing hKv4.3 gene were selected using the culture medium containing 800 μg/ml G418, separated using cloning cylinders, and then maintained in culture

medium containing 400 µg/ml G418 (Invitrogen). Cells used for electrophysiology were seeded on a glass cover slip.

For the cells with co-expressing Kv4.3 and pCDNA3.1/KChIP (a gift from Dr. Jie Liu, Department of Pathophysiology, Medical School of Shenzhen University, China), 6 µg KChIP plasmids were transiently expressed in hKv4.3-HEK 293 cells with 1 µg green fluorescence plasmids in a 35 mm culture dish for 36 h. Whole-cell Kv4.3 current with KChIP was recorded with a fluorescence microscope.

The predicted potential tyrosine phosphorylation sites were examined using the software NetPhos 2.0 (www.cbs.dtu.dk/cgi-bin). The mutants of hKv4.3 channels were generated using a QuickChange site-directed mutagenesis kit following the manufacturer's instruction (Stratagene, USA), and then confirmed by DNA sequencing. The mutants Y108F, Y136F, Y441F and Y528F were transiently expressed in HEK293 cells with a 35 mm culture dish using 10 µl of Lipofectamine 2000 with 4 µg of pCDNA3.1/hKv4.3-mutant vector.

1.3 Solution and chemicals

Tyrode solution contained (mM) NaCl 140, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 1.8, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10.0 and glucose 10 (pH adjusted to 7.3 with NaOH). For whole-cell recordings, the pipette solution contained (mM) KCl 20, K-aspartate 110, $MgCl_2$ 1.0, HEPES 10, ethylene glycol tetraacetic acid (EGTA) 5, GTP 0.1, Na_2-phosphocreatine 5 and Mg-ATP 5 (pH adjusted to 7.2 with KOH).

3-(4-Chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d] pyrimidin-4-amine (PP2) was purchased from Tocris Bioscience (Bristol, UK). Other reagents were obtained from Sigma-Aldrich (St Louis, MO). Stock solutions were made with dimethyl sulfoxide (DMSO) for genistein (100 mM), AG556 (100 mM), PP2 (10 mM). The stocks were divided into aliquots and stored at −20°C. Sodium orthovanadate stock solution (100 mM) was made with distilled water, and pH value was adjusted to 9.0.

1.4. Electrophysiology

The transient outward potassium current Ito and hKV4.3 current were recorded with whole cell patch clamp technique as described previously.[1-3] Briefly, a small aliquot of the solution containing the isolated human atrial myocytes was placed in an open perfusion chamber (0.5-ml) mounted on the stage of an inverted microscope (Diaphot, Nikon, Japan). Myocytes were allowed to adhere to the bottom of the chamber for 5-10 min and were superfused at ~2 ml/min with Tyrode solution. Only quiescent rod-shaped cells with clear cross-striations were used for electrophysiological recording.

HEK 293 cells on a cover slip were transferred to the cell chamber and superfused with Tyrode solution. Whole-cell currents were recorded as described previously. Borosilicate glass electrodes

(1.2-mm OD) were pulled with a Brown-Flaming puller (model P-97, Sutter Instrument Co, Novato, CA) and had tip resistances of 1.5-3 MΩ when filled with the pipette solution. A 3 M KCl-Agar bridge was used as the reference electrode. The tip potential was zeroed before the patch pipette contacted the cell. After a gigaohm seal was obtained by negative pressure, the cell membrane was ruptured by applying a gentle negative pressure to establish the whole-cell configuration. Series resistance (Rs, 3-6 MΩ) was compensated by 80% to minimize voltage errors. Liquid junction potentials (~12 mV) between pipette and bath solutions were not corrected for the patch clamp recording. Membrane currents were measured using an EPC-10 amplifier and Pulse software (Heka Elektronik, Lambrecht, Germany). Command pulses were generated by a 12-bit digital-to-analog converter controlled by Pulse software. All experiments were conducted at room temperature (22-23°C).

Action potentials in human atrial myocytes were recorded at 36°C using a perforated patch configuration by including 200 μg/ml amphotericin B (Sigma-Aldrich) in the K^+ pipette solution as described previously.[1] Electrical signals were low-pass filtered at 5 kHz and stored on the hard disk of an IBM compatible computer.

1.5 Immunoprecipitation and Western blotting

The immunoprecipitation and Western blotting were performed following the procedure described previously.[4-5] HEK 293 cells (~80% confluence) stably expressing hKv4.3 channels were treated respectively with 20 µM genistein, 1 mM orthovanadate plus 20 µM genistein, 20 µM AG556, orthovanadate plus 20 µM AG556, 20 µM PP2, and orthovanadate plus PP2 for 30 minutes, at room temperature, and centrifuged at 4°C. The cell pellet was then lysed with the lysis buffer containing (mM) 20 Tris (pH=7.5), 150 NaCl, 1.0 EDTA, 1.0 EGTA, 1% Triton X-100, 2.5 Sodium pyrophosphate, 1.0 β-glycerophosphate, 1.0 orthovanadate, 1 µg/ml leupeptin. Protein quantification of lysates was made using a protein assay reader (Bio-Rad Laboratories, Hercules, CA), and diluted to equal concentrations. Proteins were immunoprecipitated overnight at 4°C using 0.8 µg of anti-hKv4.3 antibody (NeuroMab, USA) and 100 µl of protein A/G beads (Upstate). Immunoprecipitated proteins bound to pelleted protein A/G beads were washed thoroughly in PBS, denatured in Laemmli sample buffer, separated using SDS-PAGE, and electroblotted onto nitrocellulose membranes. The immunoblots were probed with anti-phosphotyrosine antibody (1:2000, Cell Signaling) overnight at 4°C in a blocking solution containing 5% BSA in TBS and Tween 20, and subsequently treated with goat anti-mouse IgG-HRP antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. Blots were developed with enhanced chemiluminescence (ECL, GE Healthcare, Hong Kong) and exposed of X-ray film (Fuji Photo Film GmbH). The blots were then stripped and reprobed with the anti-hKv4.3 channel antibody to determine total hKv4.3 channel proteins. The film was scanned, imaged by a Bio-Imaging System (Syngene, Cambridge, UK), and analyzed via Gene

Tools software (Syngene).

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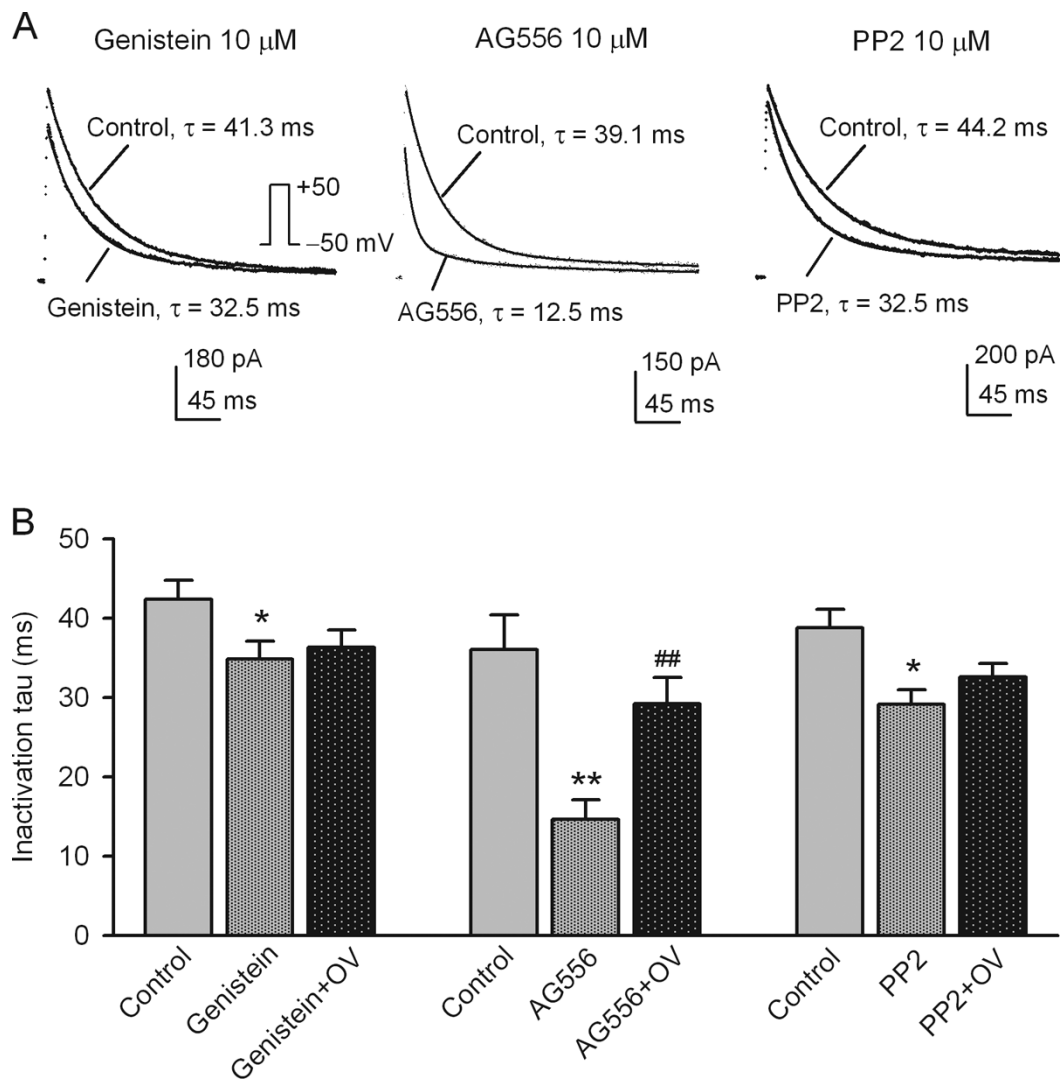
2. Supplementary Figures

Figure S1. Effects of PTK inhibitors on inactivation kinetics of I_{to} in human atrial myocytes. **A.** Inactivation of I_{to} was fitted to mono-exponential function in different individual human atrial myocytes with the inactivation time constant (τ) shown. Genistein, AG556, and PP2 (10 µM each) reduced inactivation time constant. **B.** Mean values of inactivation time constant of I_{to} in the absence (control) and presence of genistein (n=6), AG556 (n=7), PP2 (n=6) or plus orthovanadate (OV). *$P<0.05$, **$P<0.01$ vs. control; ##$P<0.01$ vs. Ag556 alone. AG556 decreased the time constant more than genistein or PP2 ($P<0.05$ vs. genistein or PP2).
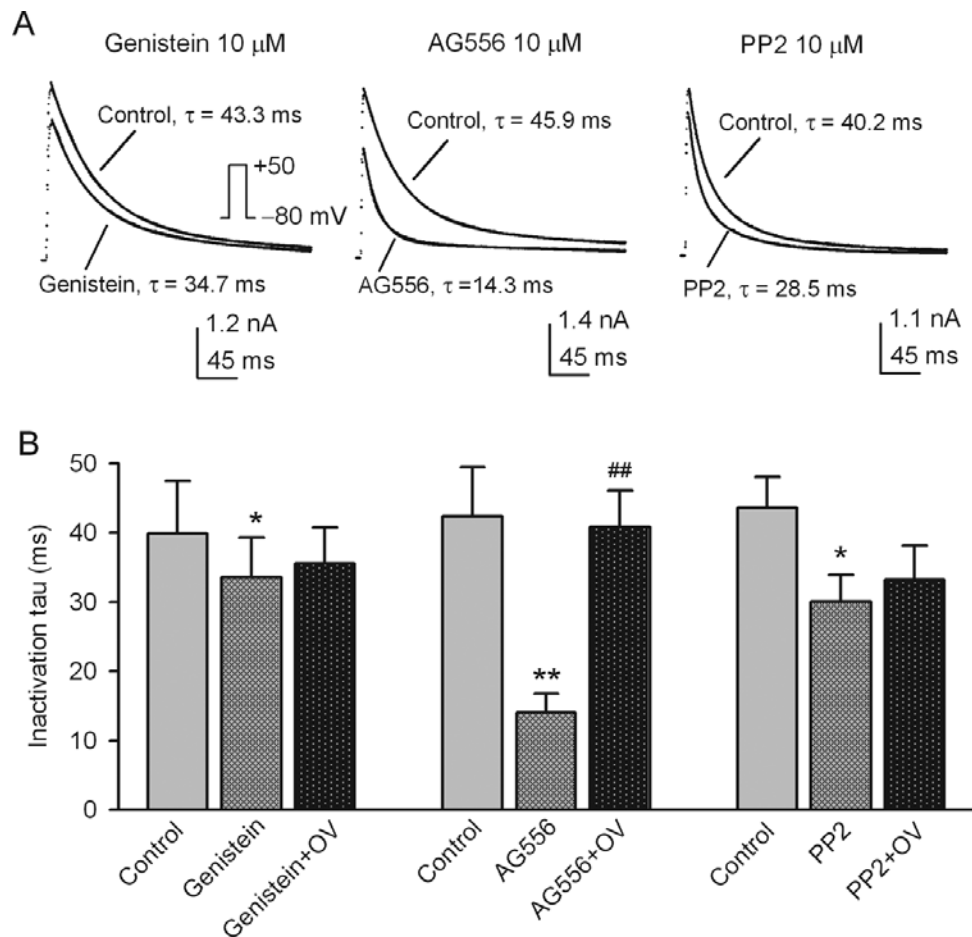
Figure S2. Effects of PTK inhibitors on inactivation kinetics of hKv4.3 in HEK 203 cell line.
A. Inactivation of I_{to} was fitted to mono-exponential function in different individual cells with the inactivation time constant (τ) shown. Genistein (10 μM) AG556 (10 μM), and PP2 (10 μM) reduced inactivation time constant. **B.** Mean values of inactivation time constant of hKv4.3 current in the absence (control) and presence of genistein (n=6), AG556 (n=7), PP2 (n=6) or plus orthovanadate (OV, 1 mM). *P<0.05, **P<0.01 vs. control; ##P<0.01 vs. AG556 alone. AG556 decreased the time constant more than genistein or PP2 (P<0.05 vs. genistein or PP2).
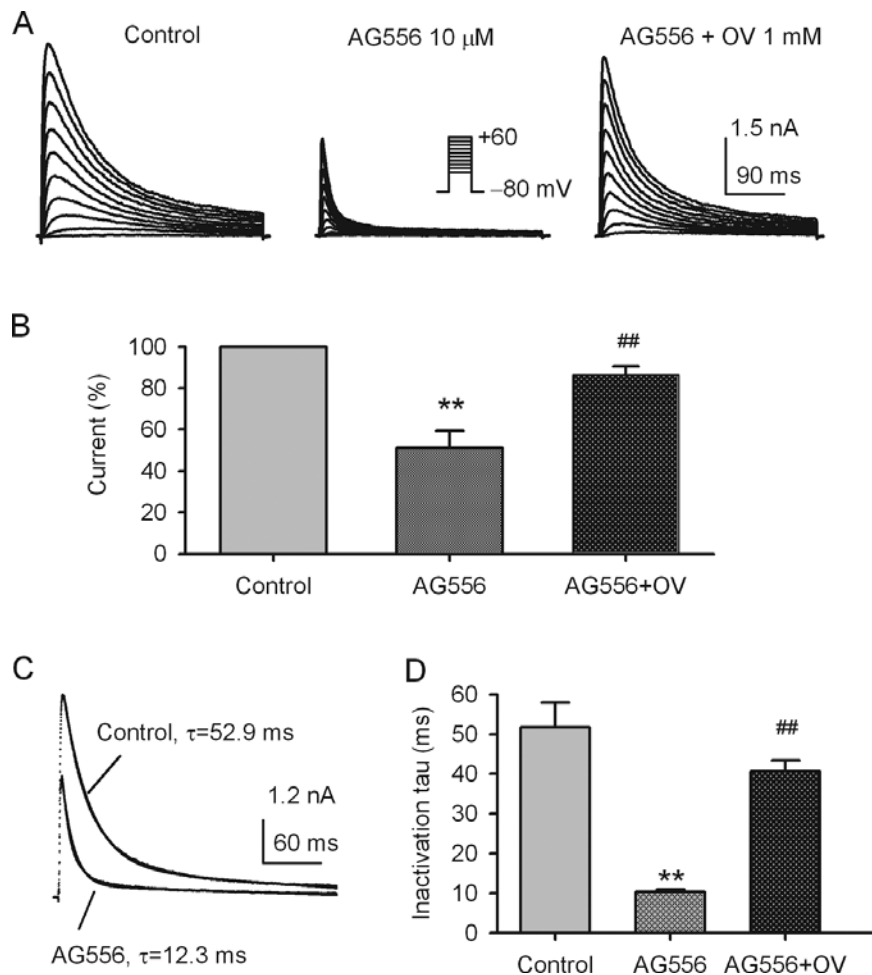
Figure S3. Human Kv4.3 channels with co-expression of KChIP and response to the EGFR kinase inhibitor AG556. **A.** Representative current traces of Kv4.3 channels with KChIP was recorded with the voltage protocol as shown in the inset in a typical experiment in the absence (control) and presence of 10 μM AG556 or AG556 plus 1 mM orthovanadate (OV). **B.** Percentage values of current (+50 mV) in cells expressing both hKv4.3 and KChIP gene. N=5, **$P<0.01$ vs. control; ##$P<0.01$ vs. Ag556 alone. **C.** The current of Kv4.3 plus KChIP was fitted to mono-exponential equation in the absence and presence of 10 μM AG556. **D.** Mean values of inactivation time constant of hKv4.3 plus KChIP current in the absence (control) and presence of AG556 (n=5 or plus orthovanadate (OV, 1 mM). **$P<0.01$ vs. control; ##$P<0.01$ vs. AG556 alone. No difference was observed for the effects of AG556 on the Kv4.3 current in cells expressing Kv4.3 alone and Kv4.3 with KChIP.