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Regulation of human cardiac KCNQ1/KCNE1 channel by epidermal growth factor receptor kinase

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Running titles: PTKs on human cardiac $I_{Ks}$

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Abstract
The present study was to investigate whether/how the recombinant human cardiac $I_{Ks}$ could be regulated by epidermal growth factor receptor kinase in HEK 293 cells stably expressing hKCNQ1/hKCNE1 genes using the approaches of perforated patch clamp technique and immunoprecipitation and Western blot analysis. It was found that the broad spectrum isoflavone tyrosine kinase inhibitor genistein and the selective epidermal growth factor receptor kinase inhibitor tyrphostin AG556 suppressed the recombinant $I_{Ks}$, and their inhibition was countered by the protein tyrosine phosphatase inhibitor orthovanadate. The Src-family kinase inhibitor PP2 reduced the current, but the effect was not antagonized by orthovanadate. Immunoprecipitation and Western blot analysis revealed that tyrosine phosphorylation level of hKCNQ1 protein was decreased by genistein or AG556, but not by PP2. These results provide the novel information that epidermal growth factor receptor kinase, but not Src-family kinases, regulates the recombinant cardiac $I_{Ks}$ stably expressed in HEK 293 cells via phosphorylating KCNQ1 protein of the channel.

Keywords. Epidermal growth factor receptor kinase; protein tyrosine phosphorylation; recombinant cardiac hKCNQ1/hKCNE1 channel; electrophysiology
1. Introduction

It is well known that protein tyrosine kinases regulate cell growth and differentiation [1], and also play an important role in the modulation of ion channels [2]. By the use of tyrosine kinase inhibitors (e.g. isoflavone genistein, tyrphostin compounds) and/or protein tyrosine phosphatase inhibitor (e.g. orthovanadate), several ionic channels/currents were found to be regulated by protein tyrosine kinases, including L-type Ca\(^{2+}\) current (\(I_{\text{Ca,L}}\)) in cardiac myocytes [3], smooth muscle cells [4], GH3 cells [5], volume sensitive chloride current (\(I_{\text{Cl,vol}}\)) in dog [6] and in human [7] cardiac myocytes, Na\(^+\) current (\(I_{\text{Na}}\)) in cardiac myocytes [8,9] and epithelial cells [10], and K\(^+\) currents in mouse Schwann cells [11], rat cardiac myocytes [12]. In addition, cloned KCNQ K\(^+\) channels expressed in CHO cells [13] and hERG channel stably expressed in HEK 293 cells [14] were also modulated by PTKs.

However, regulation of the slow delayed rectifier potassium current \(I_{\text{Ks}}\) by PTKs is not fully understood. In an earlier report, cell swelling-induced increase of cardiac \(I_{\text{Ks}}\) in dog ventricular myocytes was prevented by the broad-spectrum PTK inhibitor genistein, but not by its PTK-inactive analogue daidzein, suggesting that PTKs mediate the augmentation of \(I_{\text{Ks}}\) [15]. Nevertheless, in guinea pig ventricular myocytes, genistein was found to inhibit \(I_{\text{Ks}}\) in a PTK-independent pathway [16]. Recent reports demonstrated that cardiac \(I_{\text{Ks}}\) in guinea pig cardiac myocytes and in HEK 293 cells stably expressing KCNQ1/KCNE1 genes is suppressed by PTK inhibitors (e.g. genistein, tyrphostin compounds) in a PTK-dependent way [17, 18]. However, it is unclear whether the regulation of cardiac \(I_{\text{Ks}}\) is related to direct interaction with the channel protein or which specific PTK is involved. The present study employed the broad spectrum PTK inhibitor genistein, the Src-family kinase inhibitor PP2, the selective EGFR (epidermal growth factor receptor) tyrosine kinase inhibitor tyrphostin AG556, and the protein tyrosine phosphatase inhibitor orthovanadate to determine which specific tyrosine kinase participates in the regulation of the recombinant cardiac \(I_{\text{Ks}}\) (hKCNQ1/hKCNE1) stably expressed in HEK 293 cells using perforated patch technique and immunoprecipitation and Western blot analysis. Our results demonstrated that EGFR tyrosine kinase, but not Src-family kinases, modulated the recombinant cardiac \(I_{\text{Ks}}\) via phosphorylating hKCNQ1 protein.

2. Materials and methods

2.1. Cell culture

The HEK 293 cell line stably expressing recombinant human cardiac KCNQ1/KCNE1 channel (\(I_{\text{Ks}}\)) [19] was cultured in Dulbecco’s modified eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 100 \(\mu\)g/ml hygromycin (Sigma-Aldrich, St Louis MO, USA). The cells were seeded on glass coverslips when used for electrophysiological recording.

2.2. Solutions and reagents

Tyrode solution contained (mM) NaCl 140, KCl 5.4, MgCl\(_2\) 1.0, CaCl\(_2\) 1.8, NaH\(_2\)PO\(_4\) 0.33, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10.0 and glucose 10 (pH was adjusted to 7.3 with NaOH). The pipette solution contained (mM) KCl 20, K-aspartate 110, MgCl\(_2\) 1.0, HEPES 10.0, pH was adjusted to 7.2 with KOH. The antibiotic amphotericin B (Sigma-Aldrich) was freshly added into the pipette solution (160-200 \(\mu\)g/ml) for perforated patch configuration [19].

3-(4-Chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP2) was purchased from Tocris (Bristol, UK). All other reagents were obtained from Sigma-Aldrich. Stock solutions were made with dimethylsulfoxide (DMSO) for genistein (100 mM),
daidzein (100 mM), tyrphostin AG556 (AG 556) (100 mM), PP2 (20 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.

2.3. Electrophysiology

Cells on a coverslip were transferred to an open cell chamber mounted on the stage of an inverted microscope and superfused with Tyrode solution at a rate of ~2 ml/min. Borosilicate glass electrodes (1.2-mm OD) were pulled with a Brown-Flaming puller (model P-97, Sutter Instrument, Novato, CA, USA) and had tip resistances of 1-2 MΩ when filled with the pipette solution. The currents were measured using an EPC-10 amplifier and Pulse software (Heka Elektronik, Lambrecht, Germany). A 3-M KCl-agar bridge was used as the reference electrode. The tip potential was zeroed before cell contact, the patch pipette solution contained 160-200 µg/ml amphotericin B to make perforated configuration. Access resistance gradually decreased, and became stable within 30 min. Only cells with constant series resistance (10–15 MΩ) were accepted for further study [19]. Electrical signals were low-pass filtered at 5 kHz and stored in the hard disk of an IBM compatible computer. All experiments were performed at room temperature (22-23 °C).

2.4. Immunoprecipitation and Western blot

The immunoprecipitation and Western blot analysis were performed as previously described [9, 14]. The HEK 293 cells stably expressing hKCNQ1/hKCNE1 genes were treated respectively with equivolume DMSO (control), 30 µM genistein, genistein plus 1 mM orthovanadate, 30 µM AG556, AG556 plus 1 mM orthovanadate, 1 mM orthovanadate, or cells with serum-free culture for 36 h, or the starved cells treated with 100 ng/ml EGF at room temperature. Following centrifugation at 4°C, the cell pellet was lysed with the lysis buffer containing (mM) 25 Tris, 150 NaCl, 100 NaF, 1.0 EDTA, 1.0 orthovanadate, 1.0 phenylmethylsulfonyl fluoride, and 1% Na deoxycholate, 0.1% SDS, 1% Triton X-100, 1 µg/ml leupeptin, and 1 µg/ml aprotinin. 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 2 µg of anti-KCNQ1 antibody (sc-20816, Santa Cruz Biotech, Santa Cruz, CA) and 100 µl of protein A beads (16-125, Millipore, Billerica, MA). Immunoprecipitated proteins bound to pelleted protein A 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-pTyr-100 antibody (1:1000, Cat. 9411, Millipore, Billerica, MA) 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 Biotech, CA) for 1 h at room temperature. Blots were developed with enhanced chemiluminescence (ECL, Amersham Biosciences) and exposed on X-ray film (Fuji Photo Film GmbH). The blots were then stripped and reprobed with the anti-KCNQ1 antibody to determine total KCNQ1 protein. The film was scanned, imaged by a Bio-Imaging System (Syngene, Cambridge, UK), and analyzed via GeneTools software (Syngene) [14].

2.5. Statistical analysis

Nonlinear curve-fitting was performed using Pulsefit (HEKA) and Sigmaplot (SPSS, Chicago, IL). Paired and/or unpaired Student’s t-test were used as appropriate to evaluate the statistical significance of differences between two group means, and analysis of variance was used for multiple groups. Group data are expressed as means ± SE. Values of P<0.05 were considered to be statistically significant.
3. Results

3.1. Effect of genistein on \(I_{Ks}\)

It was reported that the broad spectrum PTK inhibitor genistein inhibited cardiac \(I_{Ks}\) in guinea pig cardiac myocytes [17,16]. We confirmed whether genistein would reduce the recombinant cardiac \(I_{Ks}\) stably expressed in HEK 293 cells. Fig. 1A shows the time-course of the recombinant \(I_{Ks}\) recorded in a representative cell with a 3-s voltage step to +40 from −80 mV, then back to −40 mV as shown in the inset in the absence and presence of 30 μM genistein. Genistein gradually inhibited \(I_{Ks}\), and reached a steady-state effect within 8 min, and the effect recovered upon washout.

Fig. 1B displays the voltage-dependent recombinant \(I_{Ks}\) traces recorded with 3-s voltage steps to between −60 and +60 from −80 mV (20-mV increments, 0.05 Hz), then back to −40 mV as shown in the inset. Genistein reversibly inhibited the currents at all test potentials. Genistein at 30 μM reduced the \(I_{Ks}\) tail current (at test potential of +40 mV) to 8.3 ± 0.6 pA/pF (n=8, P<0.01 vs control) from 15.6 ± 1.3 pA/pF in control, and recovered to 14.6 ± 1.2 pA/pF upon washout. Kinetics of the recombinant \(I_{Ks}\) was not affected by genistein (data not shown). Genistein inhibited the current in a concentration-dependent manner (Fig. 1C), and the current was reduced by 11.1±1.1, 25.7±1.6, 52.3±3.7, and 72.6±4.5% with 3, 10, 30, and 100 μM genistein, respectively. These results indicate that genistein, as in native cardiac myocytes [17,16], inhibits the recombinant cardiac \(I_{Ks}\) expressed in HEK 293 cells.

In an earlier report, the observation of PTK-independent suppression of guinea pig cardiac \(I_{Ks}\) by genistein was based on that the PTK-inactive analogue daidzein produced a significant decrease of the current amplitude [16]. We also found that 100 μM daidzein induced a remarkable reduction of the recombinant \(I_{Ks}\); however, 30 μM genistein produced a more pronounced suppression of the current in the same cell (Fig. 2A). The current inhibition (26.4±4.5%, n=6, P<0.01 vs control) with 100 μM daidzein was much weaker (Fig. 2A) than that (Fig. 1C, 52.5±3.7%, n=8) with 30 μM genistein (P<0.01 vs daidzein 100 μM).

The protein tyrosine phosphatase inhibitor orthovanadate was then employed to observe whether it would antagonize the genistein effect to determine whether the genistein effect on recombinant \(I_{Ks}\) is PTK-dependent. Orthovanadate (1 mM) exhibited a slight increase of the recombinant \(I_{Ks}\) (n=8, 5.5 ±3.1%, P<0.05), consistent with the previous report in guinea pig cardiac myocytes [17]. Fig. 2B displays the time course of the recombinant \(I_{Ks}\) in a typical experiment in the absence and presence of 30 μM genistein, and genistein plus 1 mM orthovanadate. Genistein remarkably suppressed the current amplitude, and the effect was countered by co-application of genistein and orthovanadate. Fig. 2C illustrates the mean percent values of the recombinant \(I_{Ks}\) with genistein, and genistein plus orthovanadate. Genistein (30 μM) reduced \(I_{Ks}\) step to 49.2±3.1% of control, and the co-application of genistein with orthovanadate reversed the effect to 92.5±4.3% (n=6, P<0.01 vs genistein alone). These results suggest that the recombinant \(I_{Ks}\) inhibition by genistein is PTK-dependent.

To investigate which specific PTK is involved in the regulation of the recombinant \(I_{Ks}\), effects of the Src-related kinase inhibitor PP2 and the highly selective EGFR kinase inhibitor AG556 on the recombinant \(I_{Ks}\) and tyrosine phosphorylation level of hKCNQ1 protein are determined in the following experiments.

3.2. Effect of PP2 on \(I_{Ks}\)

To determine whether Src-related PTKs would regulate the recombinant \(I_{Ks}\), we tested the effect of the selective Src-family PTK inhibitor PP2 on the current amplitude. Fig. 3A shows the time course of the recombinant \(I_{Ks}\) recorded in a representative cell with the protocol shown in the inset in the presence of PP2. PP2 at 1 μM, a concentration 200 times higher [20] than the EC50 of inhibiting Src-related kinases, had no effect on the current.
However, PP2 at 20 μM substantially decreased the current, but the effect could not be countered by 1 mM orthovanadate (Fig. 3B). The $I_{Ks,\text{step}}$ was 610.5 ± 38.7 pA in control, 488.4 ± 37.5 pA (n = 6, P<0.01 vs control) with 20 μM PP2, and 505 ± 40.4 pA (P=NS vs PP2 alone) with PP2 plus 1 mM orthovanadate. These results suggest that Src kinases may not be involved in the regulation of the recombinant $I_{Ks}$ channel expressed in HEK 293 cells.

### 3.3. Effect of AG556 on $I_{Ks}$

Fig. 4A shows the time course of the recombinant $I_{Ks}$ recorded in a typical experiment with the voltage protocol shown in the inset in the absence and presence of 20 μM AG556 (a highly selective EGFR kinase inhibitor) [8,9,14], showed a remarkable suppression of the recombinant $I_{Ks}$. AG556 produced a significant inhibition of the recombinant $I_{Ks}$, and the effect recovered upon washout.

Fig. 4B displays the voltage-dependent $I_{Ks}$ traces recorded in a representative cell with the voltage protocol shown in the inset. The $I_{Ks}$ tail current at +40 mV was decreased to 10.4 ± 0.8 pA/pF (n = 8, P<0.01 vs control) with 20 μM AG556 from 17.1 ± 1.4 pA/pF of control, and recovered to 15.1 ± 1.2 pA/pF upon washout. The concentration response relationship for the inhibition of the recombinant $I_{Ks}$ by AG556 was evaluated at +40 mV (Fig. 4C). The $I_{Ks,\text{step}}$ was reduced by 9.1 ± 0.5, 27.4 ± 1.8, 53.3 ± 2.1 and 67.2 ± 4.1% (P<0.05 or P<0.01) with 3, 10, 30 and 100 μM AG556, respectively. These results indicate that the selective EGFR kinase inhibitor AG556, like genistein, decreased the recombinant $I_{Ks}$.

Fig. 5A shows that the tyrphostin PTK-inactive analogue tyrphostin 63 (A63, 100 μM) had no effect on the recombinant $I_{Ks}$, while AG556 (30 μM) produced a reversible inhibition of the current in a typical experiment. Similar results were obtained in seven other cells. Fig. 5B illustrates the time course of the recombinant $I_{Ks}$ recorded in a representative cell. AG556 at 30 μM induced a substantial decrease of the current, and the effect was fully countered by co-application of AG556 and 1 mM orthovanadate. Fig. 5C summarizes the mean percent values of $I_{Ks,\text{step}}$ (+40 mV) in the absence and presence of 30 μM AG556, and AG556 plus 1 mM orthovanadate. The current was reduced to 56.5 ± 5.8% of control with 30 μM AG556 (P<0.01 vs control), and reversed to 95.6 ± 4.2% of control with co-application of AG556 and 1 mM orthovanadate (P<0.01 vs AG556 alone). Orthovanadate significantly antagonized the inhibition of $I_{Ks}$ by the selective EGFR kinase inhibitor AG556, suggesting that EGFR kinase participates in regulating the recombinant $I_{Ks}$.

### 3.4. Effect of EGF on $I_{Ks}$

To further determine the potential regulation of $I_{Ks}$ by EGFR kinase, EGF (100 ng/ml) was directly applied in the bath solution. However, EGF was unable to enhance the recombinant $I_{Ks}$ (n = 6, data not shown). To demonstrate whether it is related to the saturation of basal tyrosine phosphorylation of the channel, cells were cultured in a serum-free medium for 36 h. Fig. 6A shows the time course of the recombinant $I_{Ks}$ (+40 mV) recorded in a representative cell with a 36-h starvation. The current was gradually increased by application of 100 ng/ml EGF. AG556 (20 μM) inhibited both EGF-induced current and basal current level. Fig. 6B illustrates the mean percent values of $I_{Ks,\text{step}}$ (+40 mV) in the absence and presence of 100 ng/ml EGF, and EGF plus 20 μM AG556. The current was increased to 114.1 ± 5.6% of control (n=6, P<0.05 vs control) with EGF (100 ng/ml), and reduced to 75.3 ± 6.7% of control (P<0.01 vs EGF alone or control) with co-application of EGF and 20 μM AG556. These results indicate that basal tyrosine phosphorylation level of the recombinant $I_{Ks}$ is saturated in cells with normal culture conditions (with 10% FBS) and EGF enhances the current only in starved cells.
3.5. Tyrosine phosphorylation level of KCNQ1

The tyrosine phosphorylation level of KCNQ1 protein of \( I_{Ks} \) was determined with immunoprecipitation and Western blot analysis. Fig. 7A shows the images of tyrosine phosphorylation level of KCNQ1 with different treatments (30 min). Genistein (30 \( \mu \)M) and AG556 (30 \( \mu \)M) reduced the phosphorylation level of KCNQ1 protein and the reduction was substantially reversed by co-application of 1 mM orthovanadate. PP2 (20 \( \mu \)M) did not decrease the tyrosine phosphorylation level. In addition, orthovanadate (1 mM) and EGF (100 ng/ml) had no effect on the tyrosine phosphorylation level of KCNQ1 in cells with normal culture conditions. The tyrosine phosphorylation level of KCNQ1 protein slightly reduced in cells with a serum-free culture for 36 h, and EGF countered the reduced phosphorylation level.

Fig. 7B summarizes the quantitative tyrosine phosphorylation levels of KCNQ1 protein. The tyrosine phosphorylation level of KCNQ1 protein was 72.8 ± 5.9% and 68.1%±6.7% of control with genistein and AG556, respectively (P<0.01 vs control). The phosphorylation level was 91.2 ± 7.7% and 93.6±7.9% of control respectively with genistein plus orthovanadate and AG556 plus orthovanadate (P<0.05 vs genistein alone or AG556 alone). PP2, orthovanadate, and EGF had no effect on the phosphorylation of KCNQ1 protein. The serum-free starvation decreased the phosphorylation level to 90.1±8.7% (P>0.05 vs control), and the reduction was reversed by application of EGF. These results indicate that the reduction of tyrosine phosphorylation in KCNQ1 protein caused by EGFR kinase inhibition can be countered by inhibiting protein tyrosine phosphatases. Basal KCNQ1 tyrosine phosphorylation is saturated; therefore, additional stimulation would not increase the phosphorylation level with EGF or orthovanadate, and a 36-h serum-free starvation induces a slight reduction of the phosphorylation in HEK 293 cells stably expressing hKCNQ1/hKCNE1 genes.

4. Discussion

The slowly delayed rectifier \( I_{Ks} \) is an important repolarizing current in mammalian hearts [21,22]. It has been demonstrated that serine/threonine protein kinases (e.g. PKA and PKC) regulate \( I_{Ks} \) [23,24]. Activation of PKA by cAMP increases \( I_{Ks} \) density and produces a rate-dependent shortening of action potential duration, which underlies the sympathetic nervous system regulation of cardiac function mediated by \( \beta \)-adrenergic receptor activation, and pharmacological therapeutics [25]. In addition, cardiac \( I_{Ks} \) or recombinant \( I_{Ks} \) was regulated by phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2) [26,27,28] and calmodulin [29]. Previous reports [15,17,30] and the present observation provide additional information that cardiac \( I_{Ks} \) is regulated by PTKs.

The earliest evidence of \( I_{Ks} \) regulation by PTKs was reported by Zhou and colleagues in dog ventricular myocytes, in which swelling-induced increase of \( I_{Ks} \) was counted by the PTK inhibitor genistein, but not by its PTK-inactive analogue daidzein [15]. However, the later study from Washizuka and co-workers showed a PTK-independent suppression of cardiac \( I_{Ks} \) by genistein based on the observation that the PTK-inactive analog daidzein also significantly decreased the current amplitude [16]. A recent study from Missan and colleagues confirmed that the genistein-induced reduction of \( I_{Ks} \) was PTK-dependent, because the current inhibition was remarkably antagonized by the protein tyrosine phosphatase inhibitor orthovanadate [17]. In addition, orthovanadate also countered the \( I_{Ks} \) suppression by the inhibitor tyrphostin A23 or A25 in guinea pig cardiac myocytes [17].

In the present study, we also employed pharmacological tools of PTK inhibitors, in which the selectivity is relative [2] and nonselective effect is reported previously [16], to determine the regulation of recombinant \( I_{Ks} \) by PTKs; however, the result with a wide range of
concentrations (3-100 μM) of the broad spectrum PTK inhibitor genistein supports the notion that the suppression of I_{Ks} by this inhibitor is PTK-dependent, since the current inhibition by the selected concentration (30 μM) of genistein was not only greater than that by 100 daidzein (a PTK-inactive analogue), but also antagonized by the protein tyrosine phosphatase inhibitor orthovanadate (Fig. 2). The further experiments demonstrated that the inhibition of I_{Ks} by the selective EGFR kinase inhibitor AG556 (30 μM), but not the Src-family kinase inhibitor PP2 (20 μM), was almost fully countered by orthovanadate (Fig. 5). Therefore, the reduction of I_{Ks} by 20 μM PP2 is likely mediated by PTK-independent inhibition, while the AG556 suppression of the current is mediated by EGFR kinase inhibition. Thus, the new finding of the present study is that EGFR kinase, but not Src-related kinases, participates in the regulation of recombinant I_{Ks}. The immunoprecipitation and Western blot analysis further revealed that PP2 had no effect on tyrosine phosphorylation level of KCNQ1 protein, whereas genistein and AG556 remarkably decreased the tyrosine phosphorylation level, and the reduced phosphorylation level was reversed by orthovanadate (Fig. 7). These results suggest that EGFR kinase, but not Src-related kinases, regulates I_{Ks} activity by phosphorylating α-subunit KCNQ1 protein of the channel.

It has been demonstrated that endogenous EGFRs are present in HEK 293 cells [31]. Our previous study demonstrated both EGFR kinase and Src-related kinases regulate the α-subunit hERG current of human cardiac I_{Kr} [14]; however, it is not the case for the recombinant cardiac I_{Ks}, because the inhibition of the recombinant I_{Ks} by PP2 could not be countered by the protein tyrosine phosphatase inhibitor orthovanadate (Fig. 3) and tyrosine phosphorylation level of KCNQ1 protein is not reduced by PP2 (Fig. 7). Our results are supported by the previous report in which KCNQ1 channel expressed in CHO cells is not modulated by Src-family kinases [32].

The present observation and previous studies [17, 30, 15] have demonstrated that there is no doubt that cardiac I_{Ks} is regulated by PTKs. However, the recent study from Missan and colleagues did not demonstrate the evidence that I_{Ks} channels are directly phosphorylated by PTKs based on the results that cells co-expressed KCNE1 with KCNQ1 mutants at Y51, Y111, Y171, Y184, Y395, Y461, Y662 with phenylalanine did not show a remarkable reduction of the channel inhibition by the PTK inhibitor A25 (compared to that of wild type KCNQ1) [18]. However, they did find that four mutants (at Y51, Y94, Y111 and Y382) of KCNQ1 showed a trend to decrease the current inhibition by the PTK inhibitor A25, though the reduction was not statistically significant [18], which is most likely that more than one tyrosine of the channel is involved in the phosphorylation.

In the present study, the immunoprecipitation and Western blot analysis provide a strong evidence of KCNQ1 tyrosine phosphorylation of the recombinant I_{Ks}. The phosphorylation level was decreased by genistein or AG556, and the reduction of the phosphorylation was countered by the protein tyrosine phosphatase inhibitor orthovanadate (Fig. 7), indicating a direct evidence that I_{Ks} is regulated by EGFR kinase.

Our patch clamp results showed that the recombinant I_{Ks} was enhanced by EGF only in the starved cells cultured with a serum-free medium for a 36-h (Fig. 6), but not in cells cultured with a normal medium (containing 10% serum). The biochemical experiments also showed that tyrosine phosphorylation level of the recombinant KCNQ1 was not enhanced by EGF or orthovanadate in cells cultured with a normal medium; however, the tyrosine phosphorylation level was reduced in the cells cultured with serum-free medium for a 36-h, even the reduction was not statistically significant. This suggests the basal tyrosine phosphorylation of KCNQ1 subunit in HEK 293 cells is saturated, and therefore is highly sensitive to the inhibition of EGFR kinase (Fig. 7).

It is well known that I_{Ks} plays an important role in cardiac repolarization [21,22]. In addition to the regulation by protein kinases PKA and PKC [23,24], cardiac I_{Ks} is modulated
by PIP2 [33,26,27,28] and calmodulin [29]. Dysfunction of $I_{KS}$, resulting from either the gene mutations or cardiac diseases such as chronic heart failure, can induce inherited or acquired long-QT syndromes which are often associated with life-threatening arrhythmias in emotional or physical stress [34,35,36,37], which is also related to abnormal phosphorylation of the $I_{KS}$ channel proteins [38]. Previous studies and the present observation provide the strong evidence that cardiac $I_{KS}$ could also be modulated by interaction with protein tyrosine phosphatases and EGFR tyrosine kinase. However, whether and how the alteration of $I_{KS}$ channel regulation by protein tyrosine phosphatases and/or EGFR tyrosine kinase participates in the pathophysiological and electrical remodeling in cardiac disorders remains to be studied.

Collectively, the present study has demonstrated the novel information that the recombinant human cardiac $I_{KS}$ stably expressed in HEK 293 cells is regulated by EGFR tyrosine kinase, but not by Src-family kinases.

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Fig. 1. Effect of genistein on the recombinant $I_{Ks}$. A. Time-course of the recombinant $I_{Ks}$ recorded in a typical experiment with 3-s voltage step to +40 from –80 mV, then back to –40 mV (to recorded $I_{Ks}$ tail current) as shown in the inset in the absence and presence of 30 μM genistein. Original $I_{Ks}$ traces at corresponding time points are shown in right of the panel. B. Voltage-dependent $I_{Ks}$ traces recorded in a representative cell with 3-s voltage steps to between –60 and +60 mV from –80 mV (20-mV increment, 0.05 Hz), then to –40 mV (inset) in the absence and presence of 30 μM genistein. Genistein reversibly inhibited the voltage-dependent current. C. Mean percent value of the recombinant $I_{Ks}$ (at +40 mV) inhibition by 3, 10, 30 and 100 μM genistein. The number in the parenthesis indicates the experimental number. *P<0.05; **P<0.01 vs control.
Fig. 2. Orthovanadate on genistein effect.  A. Time-course of the recombinant $I_{Ks}$ recorded in a typical experiment in the absence and presence of 100 μM daidzein or 30 μM genistein. Original current traces at corresponding time points are shown in right of the panel. B. Time-course of the recombinant $I_{Ks}$ recorded in a representative cell in the absence and presence of 30 μM genistein and genistein plus 1 mM orthovanadate (OV). Original current traces at corresponding time points are shown in right of the panel. C. Histogram shows the mean percent values of $I_{Ks, \text{step}}$ during control, in the presence of 30 μM genistein, and genistein plus 1 mM orthovanadate. n= 8, **P<0.01 vs control; #P<0.01 vs genistein alone.
Fig. 3. PP2 effect on the recombinant $I_{Ks}$. A. Time-course of the recombinant $I_{Ks}$ recorded in a representative cell with the protocol shown in the inset during control, in the presence of 1 μM PP2. Original current traces at corresponding time points are shown in right of the panel. B. Time-course of the recombinant $I_{Ks}$ recorded in a typical experiment in the presence of 20 μM PP2, and PP2 plus 1 mM orthovanadate (OV). Original current traces at corresponding time points are shown in right of the panel.
Fig. 4. Effect of AG556 on the recombinant $I_{Ks}$. A. Time-course of $I_{Ks}$ recorded in a representative cell in the absence and presence of 20 μM AG556 with the protocol shown in the inset. Original $I_{Ks}$ traces are shown in right of the panel. B. Voltage-dependent $I_{Ks}$ traces recorded in a representative cell with the voltage protocol as shown in the inset in the absence and presence of 20 μM AG556. C. Mean percent value of the recombinant $I_{Ks}$ (at +40 mV) inhibition by 3, 10, 30 and 100 μM AG556. The number in the parenthesis indicates the experimental number. *P<0.05; **P<0.01 vs control.
Fig. 5. Orthovanadate on AG556 effect. A. Time-course of $I_{Ks}$ recorded from a representative cell with the voltage protocol as shown in the inset in the absence and presence of 100 μM tyrphostin 63 (A63) or 30 μM AG556. Original current traces at corresponding time points are shown in right of the panel. B. Time-course of $I_{Ks}$ recorded in typical experiment in the absence and presence of 30 μM AG556 and AG556 plus 1 mM orthovanadate (OV). Original current traces at corresponding time points are shown in right of the panel. C. Histogram shows the mean percent values of the recombinant $I_{Ks}$ (at +40 mV) during control, in the presence of 30 μM AG556 and after co-application of AG556 and 1 mM orthovanadate. n=7, **P<0.01 vs control; #P<0.01 vs AG556 plus orthovanadate.
Fig. 6. EGF enhanced the recombinant $I_{Ks}$ in cells with serum-free culture. A. Time-course of $I_{Ks}$ recorded in typical experiment in the absence and presence of 100 ng/ml EGF and after co-application of EGF and 20 $\mu$M AG556. Original current traces at corresponding time points are shown in right of the panel. B. Histogram showing the mean percent values of the recombinant $I_{Ks}$ (at +40 mV) in cells with a 36-h serum-free culture during control, in the presence of 100 ng/ml EGF and after co-application of EGF and 20 $\mu$M AG556. n = 6, *P<0.05, **P<0.01 vs control; # P<0.01 vs EGF alone.
**Fig. 7. Tyrosine phosphorylation level of recombinant I$_{Ks}$.** A. Upper panel, protein lysates were immunoprecipitated with anti-KCNQ1 antibody, Western blots were then prepared and probed with the anti-phosphotyrosine (Tyr(P)) antibody. Lower panel, Western blots of cell lysates, probed with the anti-KCNQ1 antibody. B. Histogram summarizes the mean values of the relative levels of tyrosine-phosphorylated KCNQ1 protein analyzed by the densitometry. The density of the immunoprecipitation (as in A) was normalized to that of the Western blots. Relative phosphorylated protein level (n=4 experiments) is expressed as a percentage of the vehicle control. **$P<0.01$ vs vehicle control, #$P<0.05$ vs genistein or AG556 alone.