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RESEARCH PAPER

Epidermal growth factor receptor tyrosine kinase regulates the human inward rectifier potassium $K_{IR}2.3$ channel, stably expressed in HEK 293 cells

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BACKGROUND AND PURPOSE

The detailed molecular modulation of inward rectifier potassium channels (including the $K_{IR}2.3$ channel) is not fully understood. The present study was designed to determine whether human $K_{IR}2.3$ ($K_{IR}2.3$) channels were regulated by protein tyrosine kinases (PTKs).

EXPERIMENTAL APPROACH

Whole-cell patch voltage-clamp, immunoprecipitation, Western blot analysis and site-directed mutagenesis were employed to determine the potential PTK phosphorylation of Kir2.3 current in HEK 293 cells stably expressing *Kir2.3* gene.

KEY RESULTS

The broad-spectrum PTK inhibitor genistein (10 μM) and the selective epidermal growth factor (EGF) kinase inhibitor AG556 (10 μM) reversibly decreased $K_{IR}2.3$ current and the effect was reversed by the protein tyrosine phosphatase inhibitor, orthovanadate (1 mM). Although EGF (100 ng·mL^{-1}) and orthovanadate enhanced $K_{IR}2.3$ current, this effect was antagonized by AG556. However, the Src-family tyrosine kinase inhibitor PP2 (10 μM) did not inhibit $K_{IR}2.3$ current. Tyrosine phosphorylation of $K_{IR}2.3$ channels was decreased by genistein or AG556, and was increased by EGF or orthovanadate. The decrease of tyrosine phosphorylation of $K_{IR}2.3$ channels by genistein or AG556 was reversed by orthovanadate or EGF. Interestingly, the response of $K_{IR}2.3$ channels to EGF or AG556 was lost in the $K_{IR}2.3$ Y234A mutant channel.

CONCLUSION AND IMPLICATIONS

These results demonstrate that the EGF receptor tyrosine kinase up-regulates the $K_{IR}2.3$ channel via phosphorylation of the Y234 residue of the WT protein. This effect may be involved in the endogenous regulation of cellular electrical activity.

Abbreviations

EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; PTKs, protein tyrosine kinases; PTPs, protein tyrosine phosphatases

Introduction

Inwardly rectifying potassium (K_{IR}) channels (nomenclature follows Alexander *et al.*, 2009) are characterised by passing current more readily in the inward direction and are expressed in many types of cells and play a critical role in regulating membrane potential and/or cell excitability (Murata *et al.*, 2001; Bichet *et al.*, 2003). K_{IR} channels exhibit

a subunit topology of two transmembrane domains surrounding a pore region (Murata *et al.*, 2001). There are seven K_{IR} subfamilies (K_{IR}1.x to K_{IR}7.x) identified by cloning (Bichet *et al.*, 2003). The K_{IR}2.x subfamily includes K_{IR}2.1, K_{IR}2.2, K_{IR}2.3 and K_{IR}2.4 channels (Makhina *et al.*, 1994; Morishige *et al.*, 1994; Perier *et al.*, 1994; Raab-Graham *et al.*, 1994; Ashen *et al.*, 1995; Inagaki *et al.*, 1995). It is particularly interesting that K_{IR}2.3 channels are highly expressed in both heart and brain (Perier *et al.*, 1994; Bichet *et al.*, 2003). Unlike other members of the Kir2.x family, K_{IR}2.3 channels are directly coupled to G proteins, which allows these channels to contribute to neurotransmission and cell–cell communications (Cohen *et al.*, 1996). In addition, K_{IR}2.3 channels are modulated by several intra- and extra-cellular signal molecules including ATP (Collins *et al.*, 1996), protons (Coulter *et al.*, 1995) and protein kinase C (Zhu *et al.*, 1999a).

Protein tyrosine kinases (PTKs), including receptor PTKs, such as the epidermal growth factor receptor kinase (EGFR kinase) and non-receptor PTKs, such as the Src-family kinases, provide important intracellular signalling mechanisms (Hunter, 2000). In addition to the mediation of cellular events such as cell growth, differentiation, embryonic development, metabolism, immune system function and oncogenesis (Hunter, 2000), protein phosphorylation at tyrosine residues modulates ion channels (Levitan, 1994), including Ca^{2+} channels (Ogura *et al.*, 1999), the voltage-gated Na^+ channel (Ahern *et al.*, 2005; Liu *et al.*, 2007), volume-sensitive Cl^- channels (Du *et al.*, 2004b) as well as voltage-gated K^+ channels (Davis *et al.*, 2001; Tiran *et al.*, 2003; Zhang *et al.*, 2008; Dong *et al.*, 2010). Protein kinases and phosphatases are believed to be the yin and yang of protein phosphorylation and signalling, that is, the level of protein phosphorylation is dependent on the balance between the kinases and phosphatases (Levitan, 1994; Hunter, 2000). It is not known whether K_{IR}2.3 channels are regulated by the PTKs and protein tryrosine phosphatases (PTPs). The present study was therefore designed to determine whether and how K_{IR}2.3 channels are regulated by PTKs and PTPs in HEK 293 cells stably expressing the *Kir2.3* gene. We found that K_{IR}2.3 channels were regulated by the interplay of EGFR kinase and PTPs at the Y234 residue of the channel.

Methods

Cell culture, mutagenesis and gene transfection

The pCDNA3.1/hKir2.3 plasmid was kindly provided by Dr Carol A Vandenberg (University of California at Santa Barbara, CA, USA.) (Perier *et al.*, 1994), and transfected into HEK293 cells (ATCC, Manassas, VA, USA). The HEK 293 cell line stably expressed hK_{IR}2.3 channels was established as previously described (Tang *et al.*, 2007; Zhang *et al.*, 2009), and cultured in Dulbecco's modified Eagle's medium (Invitrogen, Hong Kong, China) supplemented with 10% foetal bovine serum, 400 $\mu g \cdot mL^{-1}$ G418 (Invitrogen). The predicted potential tyrosine phosphorylation sites were examined using the software NetPhos 2.0 (http://www.cbs.dtu.dk/cgi-bin). The mutants of K_{IR}2.3 channels were generated using a QuickChange site-directed mutagenesis kit following the manufacturer's instruction (Stratagene, La Jolla, CA, USA), and then confirmed with DNA sequencing, and the mutants Y234A, Y329A and Y333A were transiently expressed in HEK 293 cells using 10 μL of Lipofectamine 2000 with 4 μg of pCDNA3.1/hK_{IR}2.3-mutant vector. Cells used for electrophysiology were seeded on a glass cover slip.

Solutions

Tyrode solution contained (mM) NaCl 140, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 1.8, 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, MgCl2 1.0, HEPES 10, EGTA 5, GTP 0.1, Na_2-phosphocreatine 5 and Mg-ATP 5 (pH adjusted to 7.2 with KOH).

Electrophysiology

Cells on a coverslip were transferred to a cell chamber (0.5 mL) mounted on the stage of an inverted microscope (Diaphot, Nikon, Tokyo, Japan), and were superfused at ~2 $mL \cdot min^{-1}$ with Tyrode solution. Whole-cell currents were recorded at 22–23°C, as described previously (Tang *et al.*, 2007; Zhang *et al.*, 2009).

Immunoprecipitation and Western blot

The immunoprecipitation and Western blotting were performed as described previously (Zhang *et al.*, 2008). Briefly, cells were treated, respectively, with 1 mM orthovanadate, 100 $ng \cdot mL^{-1}$ EGF, 10 μM genistein, 1 mM orthovanadate plus 10 μM genistein, 100 $ng \cdot mL^{-1}$ EGF plus 10 μM genistein, 10 μM AG556, 1 mM orthovanadate plus 10 μM AG556, and EGF plus 10 μM AG556 for 30 min at room temperature, and centrifuged at 4°C. The cell pellet was then lysed with the lysis buffer described previously (Zhang *et al.*, 2008). Protein quantification of lysates was made using a protein assay reader (Bio-Rad Laboratories, Hercules, CA, USA), and diluted to equal concentrations. Proteins were immunoprecipitated overnight at 4°C using 2 μg of anti-K_{IR}2.3 antibody (#sc-23632, Santa Cruz Biotech., Santa Cruz, CA, USA) and 100 μL of protein A agarose bead (#16–125, Millipore, Billerica, MA, USA). Immunoprecipitated proteins bound to pelleted protein A-beads were washed thoroughly in PBS, denatured in Laemmli sample buffer, separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The immunoblots were probed with an anti-phosphotyrosine antibody (1:1000, P-Tyr-100 #9411, Cell Signalling Tech, Danvers, MA, USA) 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, #sc-2005, Santa Cruz Biotech.) for 1 h at room temperature. Blots were developed with enhanced chemiluminescence (GE Healthcare, Hong Kong, China) and exposed on X-ray film (Fuji Photo Film GmbH, Düsseldorf, Germany). The blots were then stripped and reprobed with the anti-K_{IR}2.3 antibody to determine total K_{IR}2.3 channel proteins. The film was scanned, imaged by a Bio-Imaging System (Syngene, Cambridge, UK) and analysed via GeneTools software (Syngene).

Statistical analysis

The data are expressed as means ± SEM. Paired and/or unpaired Student's *t*-tests were used as appropriate to evalu-

ate 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.

Materials

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, USA). Stock solutions were made with dimethyl sulphoxide (DMSO) for genistein (100 mM), daidzein (100 mM), AG556 (100 mM), AG 1295 (20 mM), PP2 (10 mM). EGF was reconstituted using 10 mM acetic acid containing 0.1% BSA to 20 $\mu g \cdot mL^{-1}$ stock solution. The stocks were divided into aliquots and stored at –20°C. Sodium orthovanadate stock solution (200 mM) was made with distilled water and the pH adjusted to 9.0.

Results

Inhibition of $K_{IR}2.3$ current by PTK inhibitors

Figure 1 shows the effects of the broad-spectrum PTK inhibitor genistein on $K_{IR}2.3$ channels stably expressed in HEK 293 cells. Genistein (10 μM) inhibited voltage-dependent $K_{IR}2.3$ current elicited by the voltage steps, as shown in the inset in a representative cell, and the inhibitory effect was fully reversed by washout (Figure 1A). Figure 1B displays the time course of $K_{IR}2.3$ current recorded in a typical experiment with a 200-ms voltage step from –40 to –120 mV in the absence and presence of genistein, and with co-application of genistein and orthovanadate. The current was substantially suppressed by 10 μM genistein, and the inhibition was fully reversed by 1 mM orthovanadate. Similar results were obtained in assays of voltage-dependent $K_{IR}2.3$ current (Figure 1C, $n = 6$). Figure 1D illustrates the current-voltage (*I-V*) relationships of $K_{IR}2.3$ current activated by a 3 s ramp (0 to –120 mV, from a holding potential of –40 mV) during control, in the presence of genistein, genistein plus orthovanadate or Ba^{2+}. The current was inhibited by 10 μM genistein, and the inhibitory action was antagonized by co-application of 1 mM orthovanadate. The cardiac inward rectifier K^+ channel (I_{K1}) blocker Ba^{2+} (Li and Dong, 2010) almost fully suppressed $K_{IR}2.3$ current at 0.5 mM ($n = 5$). In a total of eight cells, genistein (10 μM) decreased $K_{IR}2.3$ current (at –120 mV) by 27.5% ($P < 0.01$ vs. control), and the inhibition was reversed by 1 mM orthovanadate to 2.2% ($P < 0.01$ vs. genistein alone) (Figure 1E). These results suggest that the inhibitory effect of genistein on $K_{IR}2.3$ current is related to PTK inhibition.

Figure 2 illustrates the effects of the selective EGFR tyrosine kinase inhibitor AG556 on $K_{IR}2.3$ current. AG556 (10 μM) reversibly inhibited the voltage-dependent $K_{IR}2.3$ current activated by the voltage protocol shown in the inset (Figure 2A, $n = 5$). Figure 2B displays the time course of $K_{IR}2.3$ current in a typical experiment in the absence and presence of 10 μM AG556 and AG556 plus orthovanadate. The current was reduced by 10 μM AG556, and this inhibition was reversed by 1 mM orthovanadate. The reduction of voltage-dependent $K_{IR}2.3$ current by AG556 was also antagonized by orthovanadate (Figure 2C, $n = 6$). Figure 2D illustrates the *I-V*

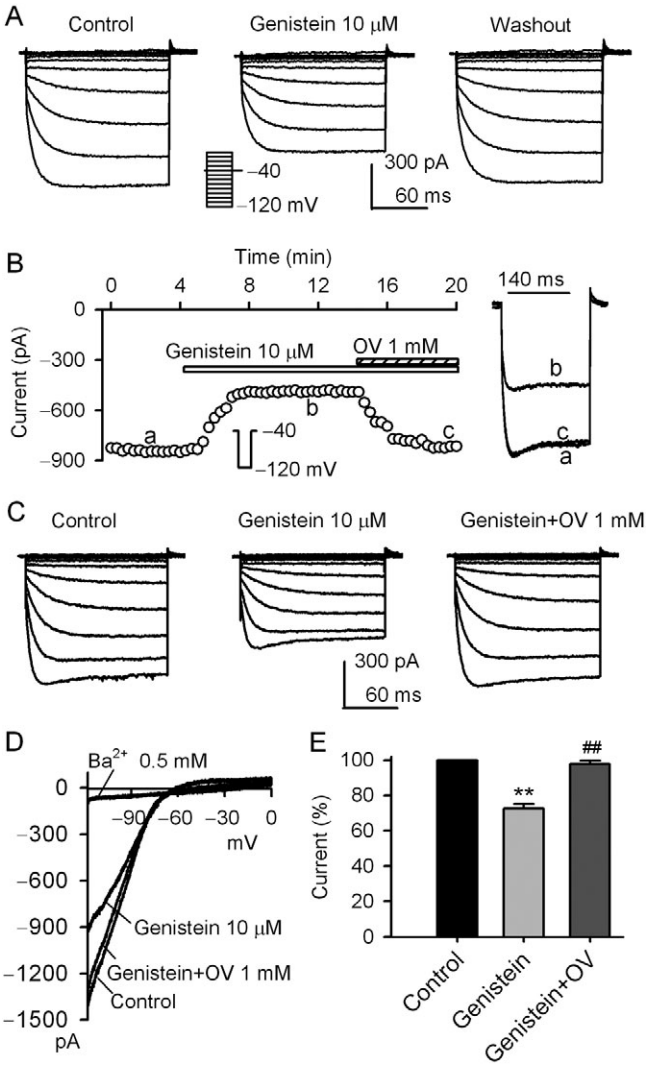

Figure 1

Inhibition of $K_{IR}2.3$ current by genistein. (A) Voltage-dependent $K_{IR}2.3$ current recorded in a representative cell with 200-ms voltage steps to between –120 to 0 mV from a holding potential of –40 mV (0.1 Hz, inset) in the absence and presence of 10 μM genistein. (B). Time-course of $K_{IR}2.3$ current recorded in a representative cell with 200-ms voltage step to –120 mV from a holding potential of –40 mV in the absence and presence of genistein (10 μM), genistein plus 1 mM orthovanadate (OV). The current was measured from the zero level to steady-state current at end of voltage step. Original current traces at corresponding time points are shown in right of the panel. (C) Voltage dependent $K_{IR}2.3$ current recorded in a typical experiment with the voltage protocol as shown in the inset of panel A during control, in the presence of 10 μM genistein and genistein plus 1 mM orthovanadate. (D) *I–V* relationships of $K_{IR}2.3$ current recorded by a 3 s ramp (0 to –120 mV from a holding potential of –40 mV) in control, 10 μM genistein application, genistein plus 1 mM orthovanadate and 0.5 mM Ba^{2+}. (E) Summary values of $K_{IR}2.3$ current (–120 mV) as percent control, in cells before (control) or after 10 μM genistein and genistein plus 1 mM orthovanadate ($n = 8$, $^{**}P < 0.01$ vs. control, $^{\#\#}P < 0.01$ vs. genistein alone).

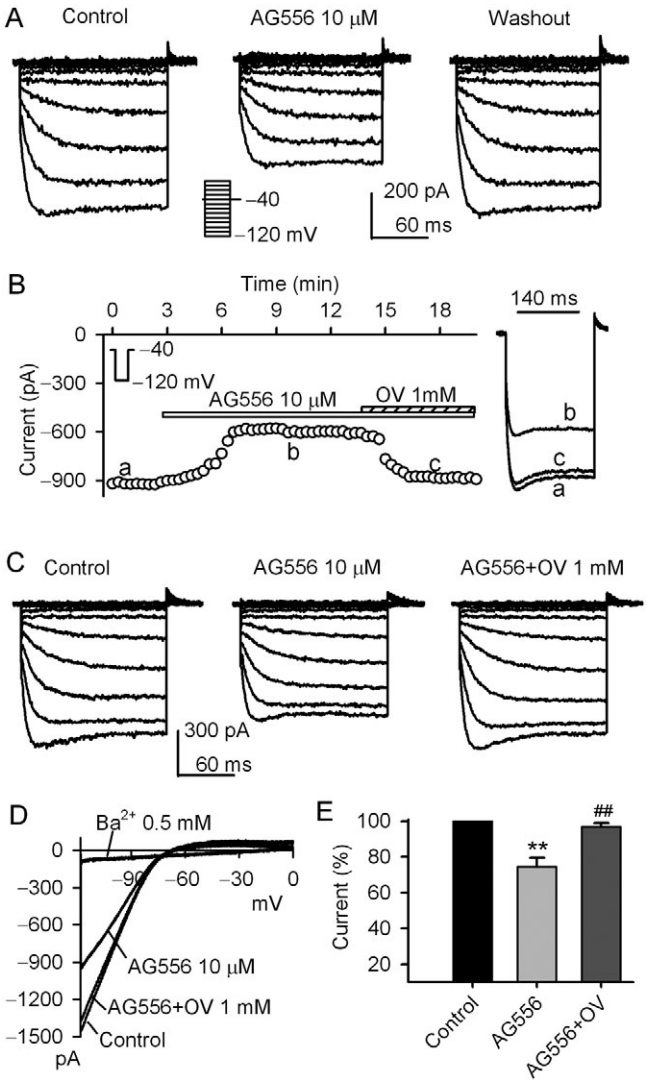

Figure 2

Effect of AG556 on $K_{IR}2.3$ current. (A) Voltage-dependent $K_{IR}2.3$ current recorded in a representative cell with the protocol shown in the inset in the absence and presence of 10 μM AG556. (B) Time-course of $K_{IR}2.3$ current recorded in another cell in the absence and presence of 10 μM AG556, AG556 plus 1 mM orthovanadate (OV). Original current traces at corresponding time points are shown in right of the panel. (C) Voltage-dependent $K_{IR}2.3$ current recorded in a representative cell during control, in the presence of 10 μM AG556 and AG556 plus 1 mM orthovanadate. D. *I-V* relationships of $K_{IR}2.3$ current recorded by a ramp protocol in control, 10 μM AG556, AG556 plus 1 mM orthovanadate and 0.5 mM Ba^{2+}. (E) Summary values of $K_{IR}2.3$ current (−120 mV) as percent control, in cells before (control) or after, 10 μM AG556 and AG556 plus 1 mM orthovanadate (n = 8, $^{**}P < 0.01$ vs. control, $^{\#\#}P < 0.01$ vs. genistein alone).

relationships of $K_{IR}2.3$ current activated by a 3 s ramp (0 to −120 mV from a holding potential of −40 mV) during control, in the presence of AG556, AG556 plus orthovanadate, or Ba^{2+}. The current was inhibited by 10 μM AG556, and the inhibition was antagonized by 1 mM orthovanadate. Ba^{2+} (0.5 mM) almost fully suppressed $K_{IR}2.3$ current (n = 5). In a total of nine cells, AG556 (10 μM) decreased $K_{IR}2.3$ current (at −120 mV) by 25% ($P < 0.01$ vs. control), and the inhibition was reversed by 1 mM orthovanadate to 3.1% ($P < 0.01$ vs. genistein alone) (Figure 2E). These results suggest that the inhibition of $K_{IR}2.3$ current by genistein or AG556 is related to the inhibition of EGFR kinase.

Effects of EGF on $K_{IR}2.3$ channels

To demonstrate whether EGF can up-regulate $K_{IR}2.3$ current, 100 ng·mL^{-1} EGF was applied in bath solution. Figure 3A displays the time-course of $K_{IR}2.3$ current recorded in a typical experiment using a voltage step from −40 to −120 mV (inset). The current was gradually increased after application of 100 ng·mL^{-1} EGF in the bath solution and the effect partially recovered on washout (n = 5). Figure 3B shows the voltage-dependent $K_{IR}2.3$ current recorded in another cell with a voltage protocol shown in the inset in the absence and the presence of EGF. EGF (100 ng·mL^{-1}) enhanced $K_{IR}2.3$ current, and the effect partially recovered on washout (n = 7). This result suggests that the increase of Kir2.3 current by EGF is likely to be related to an enhanced tyrosine phosphorylation of the channel by EGFR kinase.

Effect of orthovanadate on $K_{IR}2.3$ channels

If the enhancement of $K_{IR}2.3$ current by EGF is mediated by the increased tyrosine phosphorylation of $K_{IR}2.3$ channels, inhibition of PTPs might also increase the tyrosine phosphorylation and up-regulate the channel activity by reducing the dephosphorylation of the channel. Figure 3C shows the time-course of $K_{IR}2.3$ current in another cell in the absence and presence of the PTP inhibitor orthovanadate (1 mM). Orthovanadate exhibited a reversible enhancement of $K_{IR}2.3$ currents. The same effect was observed in voltage-dependent $K_{IR}2.3$ current (Figure 3D, n = 6).

Figure 3E illustrates the time-course of $K_{IR}2.3$ current recorded in a typical experiment, in which EGF (100 ng·mL^{-1}) increased $K_{IR}2.3$ current, and co-application of EGF with 1 mM orthovanadate produced an additional increase of the current. The same results were obtained in voltage-dependent $K_{IR}2.3$ current (Figure 3F). Figure 3G shows the *I–V* relationships of $K_{IR}2.3$ current activated by a ramp protocol during control, in the presence of 100 ng·mL^{-1} EGF, EGF plus 1 mM orthovanadate or 0.5 mM Ba^{2+}. EGF (100 ng·mL^{-1}) enhanced $K_{IR}2.3$ current and this effect was further potentiated by co-application of EGF and 1 mM orthovanadate. Ba^{2+} almost fully inhibited $K_{IR}2.3$ current Figure 3H summarizes the increases of $K_{IR}2.3$ current at −120 mV with 100 ng·mL^{-1} EGF and EGF plus 1 mM orthovanadate. These results indicate that $K_{IR}2.3$ current is up-regulated by tyrosine phosphorylation of the channel via activating EGFR kinase or inhibiting PTPs.

Interaction of EGF and orthovanadate with AG556

The increase of voltage-dependent $K_{IR}2.3$ current by 100 EGF (Figure 4A) or 1 mM orthovanadate (Figure 4B) was fully antagonized by 10 μM AG556. Summary data from these experiments are shown in Figure 4C. These results suggest that $K_{IR}2.3$ channels may be up-regulated by the increase of

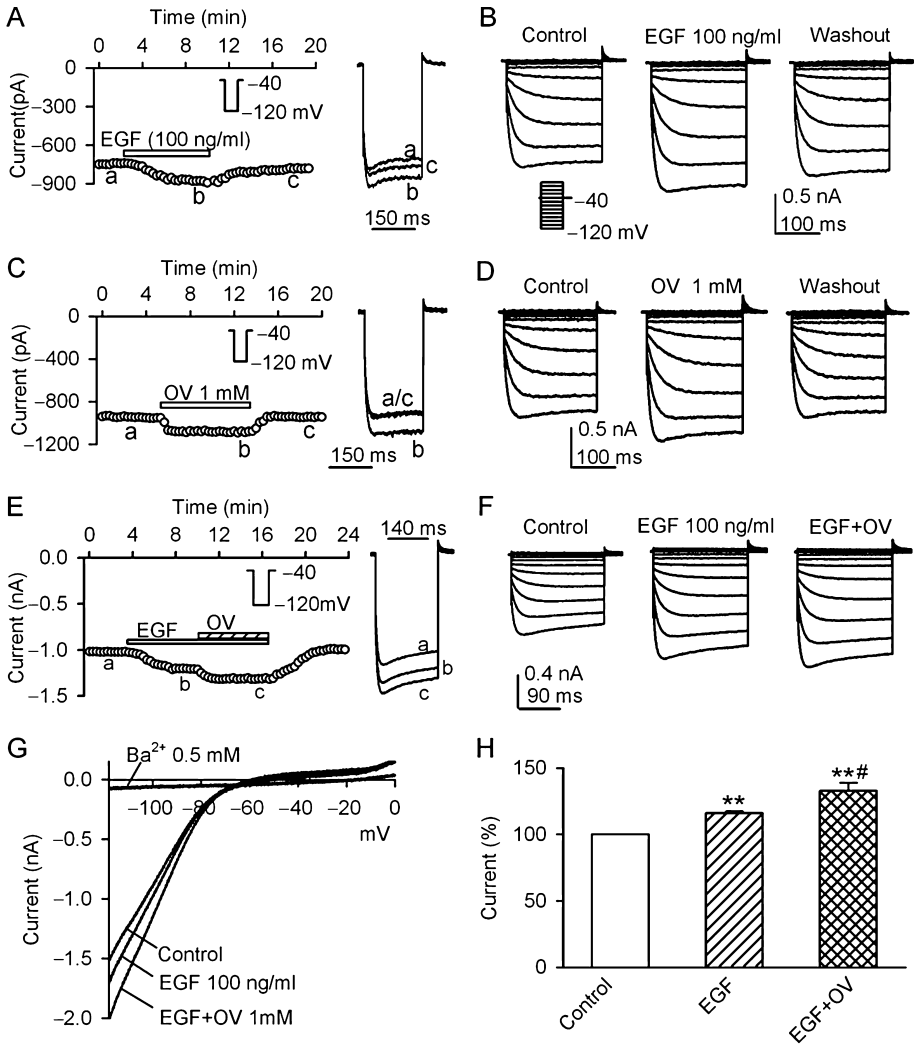

Figure 3

Effects of EGF and orthovanadate on $K_{IR}2.3$ current. (A) Time-course of $K_{IR}2.3$ current recorded in a representative cell with the voltage step as shown in the inset, in the absence and presence of 100 ng·mL^{-1} EGF. Original current traces at corresponding time points are shown in right of the panel. (B) Voltage-dependent $K_{IR}2.3$ current traces recorded in a typical experiment with 200-ms voltage steps from –40 mV to between –120 and 0 mV under control conditions, in the presence of EGF (100 ng·mL^{-1}) and washout. (C) Time-course of $K_{IR}2.3$ current recorded in another cell with the voltage protocol shown in the inset, in the absence and presence of 1 mM orthovanadate (OV). Original current traces at corresponding time points are shown in right of the panel. (D) Voltage-dependent $K_{IR}2.3$ current traces recorded with the voltage protocol as shown in the inset of panel B during control, in the presence of 1 mM orthovanadate and washout. (E) Time-course of $K_{IR}2.3$ current recorded in a typical experiment in the absence and presence of EGF (100 ng·mL^{-1}), EGF plus orthovanadate. Original current traces at corresponding time points are shown in right of the panel. (F) Voltage-dependent $K_{IR}2.3$ current recorded in a representative cell during control, in the presence of 100 ng·mL^{-1} EGF and EGF plus 1 mM orthovanadate. (G). *I-V* relationships of $K_{IR}2.3$ current recorded by a ramp protocol in control, EGF (100 ng·mL^{-1}), EGF plus 1 mM orthovanadate and 0.5 Ba^{2+}. (H) Summary values of $K_{IR}2.3$ current, as percent control, in cells before (control) or after 100 ng·mL^{-1} EGF and EGF plus 1 mM orthovanadate. **$P < 0.01$ vs. control; #$P < 0.01$ vs. EGF alone ($n = 6$).

tyrosine phosphorylation via either activating EGFR kinase, as with EGF, by or reducing dephosphorylation by inhibiting PTPs, as with orthovanadate.

Other PTK inhibitors on $K_{IR}2.3$ current

Tyrphostin AG1295 (10 μM), a selective inhibitor of the platelet-derived growth factor receptor (PDGFR) kinase, and PP2 (10 μM), a membrane-permeable inhibitor of the Src-family PTKs, had no inhibitory effect on $K_{IR}2.3$ current (Figure 5). These results suggest that the PDGFR kinase and Src-family kinases are not involved in the regulation of the $K_{IR}2.3$ channels.

Tyrosine phosphorylation level of $K_{IR}2.3$ channels

To demonstrate the tyrosine phosphorylation level of $K_{IR}2.3$ channels, immunoprecipitation and Western blot analysis were employed in cells pre-treated with the agents already

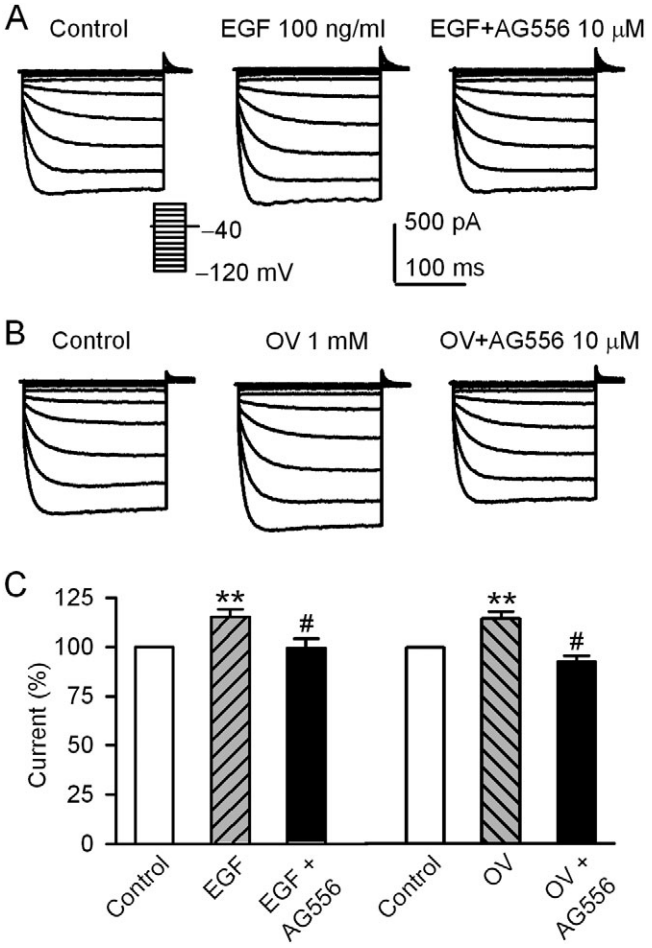

Figure 4

AG556 and EGF or orthovanadate effect on $hK_{IR}2.3$ current. (A) Voltage-dependent $K_{IR}2.3$ current recorded with the protocol as shown in the inset in a typical cell during control, in the presence of 100 ng·mL^{-1} EGF, EGF plus 10 μM AG556. (B) Voltage-dependent $K_{IR}2.3$ current recorded in a representative cell during control, in the presence of 1 mM orthovanadate (OV), orthovanadate plus 10 μM AG556. (C) Summary values of $K_{IR}2.3$ current, as percent control, in cells before (control) or after 100 ng·mL^{-1} EGF, EGF plus 10 μM AG556, 1 mM orthovanadate and orthovanadate plus 10 μM AG556. **$P < 0.01$ vs. control; #$P < 0.01$ vs. EGF alone or orthovanadate alone ($n = 6$).

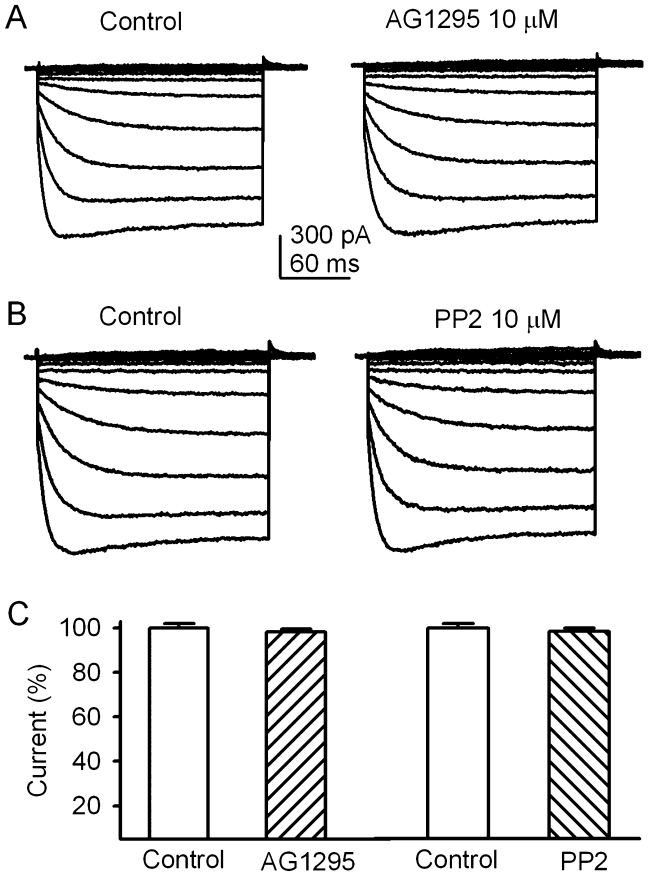

Figure 5

AG1295 and PP2 had no effect on $K_{IR}2.3$ channels. (A) $K_{IR}2.3$ current traces recorded with the voltage protocol as shown in inset of Figure 4A in a representative cell before (control) and after addition of 10 μM AG1295. (B) $K_{IR}2.3$ current traces recorded in a typical experiment before (control) and after addition of 10 μM PP2. (C) Summary values of $K_{IR}2.3$ current (−120 mV) as percent control, in cells before (control) or after 10 μM AG1295 ($n = 5$) or 10 μM PP2 ($n = 5$).

studied. EGF (100 ng·mL^{-1}) or orthovanadate (1 mM) increased tyrosine phosphorylation of $K_{IR}2.3$ channels, whereas genistein (10 μM) and AG556 (10 μM) reduced the level of phosphorylation. This reduction was substantially reversed by pre-treatment with 100 ng·mL^{-1} EGF or 1 mM orthovanadate (Figure 6A). The quantitative tyrosine phosphorylation of $K_{IR}2.3$ channels after different treatments are summarised in Figure 6B. Phosphorylation of $K_{IR}2.3$ channels was increased by 1 mM orthovanadate or 100 ng·mL^{-1} EGF ($n = 4$, $P < 0.01$ vs. control), and decreased by 10 μM genistein or 10 μM AG556 ($P < 0.01$). Orthovanadate and EGF reversed the decrease in phosphorylation by genistein ($P < 0.01$ vs. genistein alone) or by AG556 ($P < 0.01$ vs. AG556 alone). These results indicated that the $K_{IR}2.3$ channel protein is phosphorylated by interaction of EGFR tyrosine kinase and PTPs.

PTK phosphorylation sites of $K_{IR}2.3$ channels

To detect the potential tyrosine phosphorylation site(s) of $K_{IR}2.3$ channels, three mutants (Y234A, Y329A and Y333A) were generated using a site-directed mutagenesis technique and their responses to EGF or the EGFR kinase inhibitor AG556 were examined. The currents recorded with a 200-ms voltage step to −110 from −40 mV in HEK 293 cells transfected with corresponding genes are illustrated in Figure 7. The mutant Y234A channel was not affected by 100 ng·mL^{-1} EGF, unlike the WT $K_{IR}2.3$ channels or the other mutant channels Y329A and Y333A (Figure 7A; summary data in Figure 7B). The selective EGFR kinase inhibitor AG556 reduced the current amplitude of WT $K_{IR}2.3$, Y329A and Y333A mutant channels, but not that carried by the Y234A mutant channel (Figure 7C, 7D).

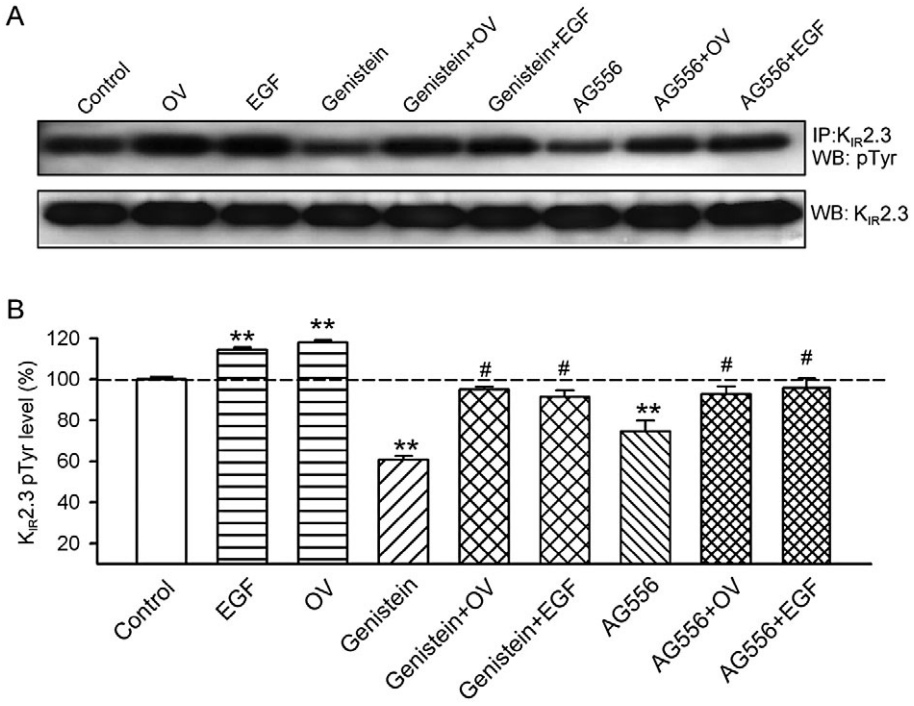

Figure 6

Tyrosine phosphorylation levels of $K_{IR}2.3$ channels. (A) Upper panel, protein lysates were immunoprecipitated with anti-$K_{IR}2.3$ antibody, and then Western blots were prepared and probed with the anti-phosphotyrosine (Tyr(P)) antibody. Lower panel, Western blots of cell lysates, probed with the anti-$K_{IR}2.3$ channel antibody. (B) Histogram summarizes the mean values of the relative levels of tyrosine-phosphorylated $K_{IR}2.3$ channel protein analysed by densitometry. The amount of protein from the immunoprecipitation (as in A) was normalized to those from the Western blots. Phosphorylated protein levels ($n = 5$ experiments) are expressed as a percentage of the vehicle control. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. vehicle control, $\#P < 0.05$ vs. genistein or AG556 alone.

Tyrosine phosphorylation level of the mutant Y234A channel protein

To further confirm whether the protein tyrosine phosphorylation of $K_{IR}2.3$ channels was at the site of Y234, we determined the tyrosine phosphorylation of the mutant Y234A protein in HEK 293 cells, stably expressing the mutant gene (Figure 8A and B). Phosphorylation of the Y234A channel, in response to EGF and/or orthovanadate, was almost totally lost, whereas the WT $K_{IR}2.3$ channels and the mutants Y329A and Y333A showed the expected increased phosphorylation with EGF or orthovanadate. These results indicate that the function of the $K_{IR}2.3$ channel protein is regulated by the interplay of EGFR kinase and PTPs via tyrosine phosphorylation at the Y234 residue, as illustrated by Figure 8C.

Discussion

It is well recognized that the activation of ion channels is regulated by tonic activity of several protein kinases and phosphatases to catalyse reversible phosphorylation and dephosphorylation events (Levitan, 1994). We and others have previously demonstrated that PTKs regulate several types of ion channels (Davis *et al.*, 2001; Du *et al.*, 2004b; Gao *et al.*, 2004; Zhang *et al.*, 2008), including L-type Ca^{2+} channel (Ogura *et al.*, 1999), voltage-gated Na^{+} channel (Ashen *et al.*, 1995; Liu *et al.*, 2007), volume-sensitive Cl^{-} channel (Du *et al.*, 2004b) and cardiac K^{+} channels (Gao *et al.*, 2004; Missan *et al.*, 2006; Zhang *et al.*, 2008; Dong *et al.*, 2010) in different types of cells. The present study has demonstrated that the $K_{IR}2.3$ channels are regulated by the EGFR tyrosine kinase. Although a recent study reported that genistein showed a non-specific inhibitory effect on rat $K_{IR}2.3$ channels expressed in *Xenopus* oocytes and HEK 293 cells with an IC_{50} of 16.9–19.4 μM (Zhao *et al.*, 2008), which is close to that in the present study (IC_{50}: 14.3 μM, $n = 5$, data not shown), the results from the present study strongly support the notion that genistein at a low concentration of 10 μM reduced tyrosine phosphorylation of $K_{IR}2.3$ channels, because the reduction of $K_{IR}2.3$ current by 10 μM genistein, like that after the selective EGFR kinase inhibitor, AG556 (10 μM), was almost fully reversed by the PTP inhibitor, orthovanadate (Figure 1). The IC_{50} of AG556 for maximum inhibition of $K_{IR}2.3$ currents was 8.9 μM in the present observation ($n = 5$, data not shown).

On the other hand, an earlier study reported that rat $K_{IR}2.1$ channels were inhibited by the PTP inhibitor perorthovanadate or the PTK activator EGF or insulin (Wischmeyer *et al.*, 1998). In addition, $K_{IR}2.1$ channels expressed in *Xenopus* oocytes were reduced by tyrosine (v-Src) decaging, which is related to the interaction of clathrin adaptor protein and tyrosine-based sorting motif of $K_{IR}2.1$ and also membrane endotocytosis (Tong *et al.*, 2001). Moreover, Du and col-

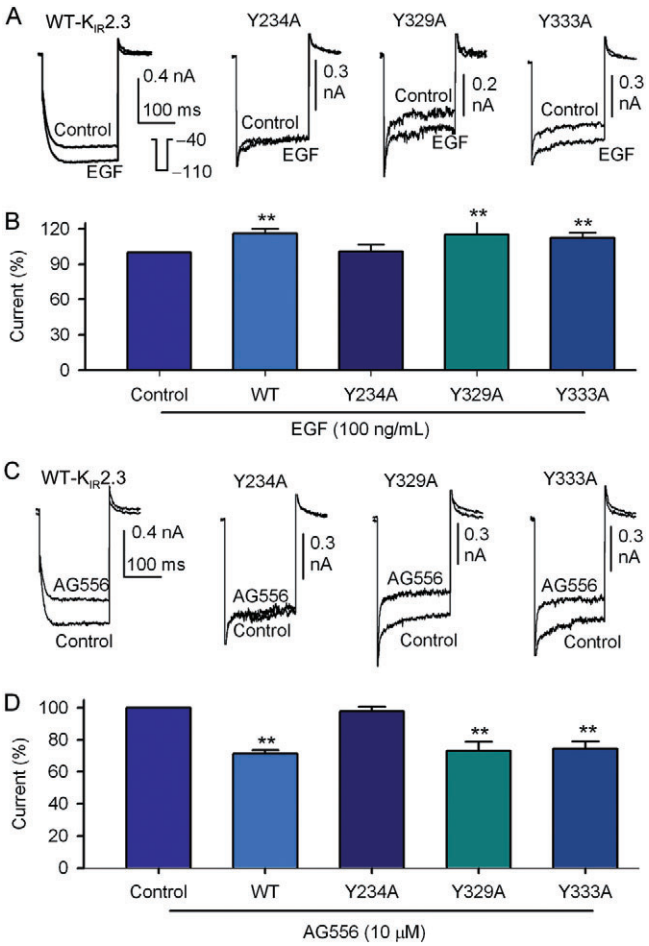

Figure 7

Single point mutations of $K_{IR}2.3$ channels. (A) $K_{IR}2.3$ current recorded in HEK 293 cells expressing WT Kir2.3, Y234A, Y329A and Y333A mutants with a 200-ms voltage step to –110 from –40 mV (inset) before (control) and after application of 100 ng·mL^{-1} EGF (5 min). (B) Summary values of $K_{IR}2.3$ current, as percent control, in cells expressing WT (n = 13), Y234A (n = 5), Y329A (n = 5) and Y339A (n = 13) mutant channels in response to 100 ng·mL^{-1} EGF. (C) $K_{IR}2.3$ current recorded in HEK 293 cells expressing WT Kir2.3, Y234A, Y329A and Y333A mutants with voltage protocol shown in the inset of panel A before (control) and after application of 10 μM AG556 (5 min). (D) Summary values of $K_{IR}2.3$ current, as percent control, in cells expressing WT (n = 6), Y234A (n = 5), Y329A (n = 5) and Y339A (n = 5) mutants in response to 10 μM AG556, $^{**}P < 0.01$ vs. control or Y234A mutant.

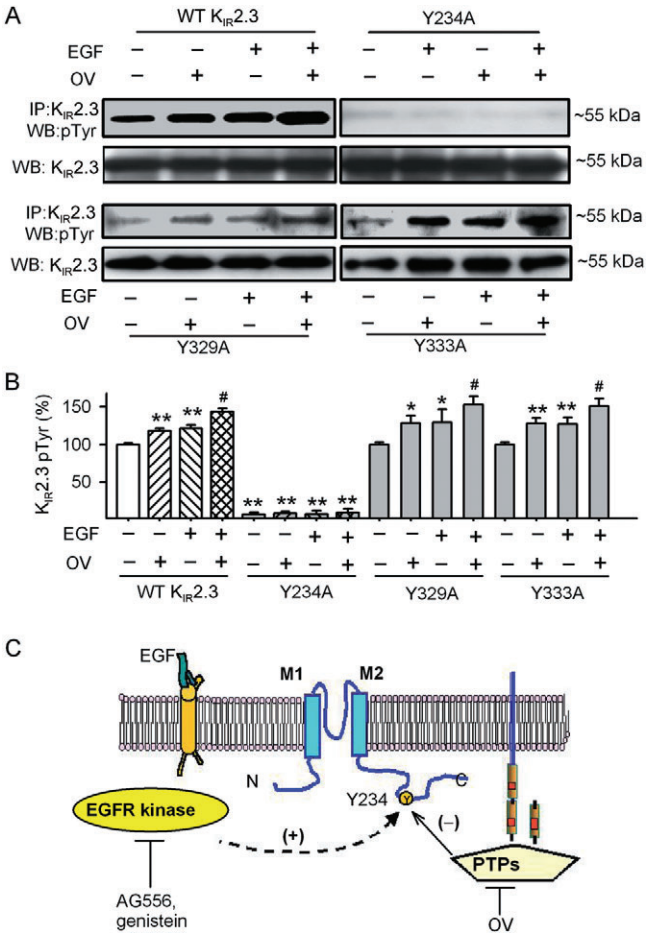

Figure 8

Tyrosine phosphorylation of the mutant Y234A channels and a model scheme for the regulation of $K_{IR}2.3$ channels by EGFR tyrosine kinase and PTPs. (A) Tyrosine phosphorylation of $K_{IR}2.3$ channels was detected with immunoprecipitation and Western blot in WT and mutant $K_{IR}2.3$ channels. (B) Histogram summarizes the mean values of the relative levels of tyrosine phosphorylated $K_{IR}2.3$ channel protein analysed by densitometry. The amount of protein from the immunoprecipitation (as in A) was normalized to those from the Western blots. Relative phosphorylated protein level (n = 4 experiments) is expressed as a percentage of the vehicle control. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle control (without EGF or orthovanadate) in WT $K_{IR}2.3$; $^{\#}P < 0.05$ vs. EGF or OV. (C) Tyrosine phosphorylation site on $K_{IR}2.3$ channels and signalling molecules are postulated. The tyrosine of the $K_{IR}2.3$ protein is phosphorylated by EGFR kinase and dephosphorylated by PTPs. Tyrosine phosphorylation at Y234 by one or more steps (dashed line) favours $K_{IR}2.3$ channel opening, whereas dephosphorylation favors closure. AG556 or genistein inhibited EGFR tyrosine kinase and reduced phosphorylation, while orthovanadate (OV) inhibited PTPs and decreased dephosphorylation of the channel.

leagues reported that the recombinant K_{IR} channels including $K_{IR}2.1$ and $K_{IR}2.3$ channels co-expressed with muscarinic M_1 and EGF receptors in *Xenopus* oocytes were up-regulated by phosphatidylinositol 4,5-bisphosphate and inhibited by acetylcholine or EGF (Du *et al.*, 2004a). However, this contrasts with our present observations, in which EGFR kinase activation up-regulated, while EGFR kinase inhibition decreased $K_{IR}2.3$ currents expressed in HEK293 cells, via direct tyrosine phosphorylation of the channel. It is unclear whether these differences result from the different expression system or subtle variation of channel types and/or different experimental conditions.

The important finding of the present observation was that $K_{IR}2.3$ current was reversibly augmented by either activating EGFR kinase with EGF or inhibiting PTPs with orthovanadate, and the EGF-induced activation of $K_{IR}2.3$ channels was potentiated by orthovanadate (Figure 3). The increase of $K_{IR}2.3$ current by EGF or orthovanadate was fully reversed by the

EGFR kinase inhibitor AG556 (Figure 4). These results strongly support the notion that $K_{IR}2.3$ channels are regulated by the interplay of EGFR kinase and PTPs. However, we did not find inhibition (Figure 5) of $K_{IR}2.3$ current by the PDGFR kinase inhibitor AG1295 (10 μM, 20 times higher than IC_{50} for inhibiting PDGFR tyrosine kinase) (Naraghi *et al.*, 2000; Zhang *et al.*, 2008) or the inhibitor of Src-family kinases PP2 (10 μM, 50 times higher than IC_{50} for inhibiting Src-kinases) (Zhu *et al.*, 1999b), indicating that these tyrosine kinases are not involved in the regulation of $K_{IR}2.3$ channels.

The simplest explanation for the actions of genistein, AG556, EGF, and orthovanadate and their interaction is that phosphorylation and/or dephosphorylation of a critical tyrosine residue (Y234) on $K_{IR}2.3$ channels or a signalling molecule, directly or indirectly, increases or reduces the channel activity. In this model (Figure 8C), activation of EGFR kinase by EGF increases $K_{IR}2.3$ channel activity by enhancing tyrosine phosphorylation of the channel, whereas inhibition of EGFR kinase by AG556 or genistein decreases the channel activity by reducing tyrosine phosphorylation. In addition, inhibition of PTPs with orthovanadate increases $K_{IR}2.3$ channel activity by allowing its unopposed phosphorylation. This may also explain why orthovanadate antagonized the effect of AG556 or genistein. Moreover, when the Y234 residue of the $K_{IR}2.3$ channel was replaced by alanine, the mutant channel was no longer sensitive to stimulation by EGF or inhibition by the EGFR kinase inhibitor AG556 (Figure 7).

The regulation of $K_{IR}2.3$ channels by the interplay of EGFR kinase and PTPs is further supported by the results from immunoprecipitation and Western blot analysis. Tyrosine phosphorylation of $K_{IR}2.3$ channel protein was enhanced by EGF or orthovanadate, while being reduced by genistein or AG556. The reduced phosphorylation of $K_{IR}2.3$ channels by genistein or AG556 was reversed by EGF or orthovanadate (Figure 6). The strongest evidence was the dramatic reduction of tyrosine phosphorylation and the lack of response to EGF and/or orthovanadate in the mutant Y234A channel, but not in the other mutant channels, Y329A and Y333A (Figure 8A and B).

Alterations in the electrical properties of the $K_{IR}2.3$ mutant channels were observed, leading to a rapid onset of the currents with voltage changes and a fast inactivation component (Figure 7A and C). These alterations in the mutants is more likely to be related to channel general function, rather than a specific action on tyrosine phosphorylation, because the mutant Y329A and Y333A channels exhibited the same electrical responses to the EGFR kinase inhibitor AG556 and the activator EGF (Figure 7), and also similar phosphorylation responses to those of the WT $K_{IR}2.3$ channels (Figure 8).

A number of endogenous signalling molecules, including ATP (Collins *et al.*, 1996), protein kinase C (Zhu *et al.*, 1999a), G-protein βγ subunits (Cohen *et al.*, 1996) and protons (Coulter *et al.*, 1995; Munoz *et al.*, 2007) have been reported to inhibit $K_{IR}2.3$ channels. The present study provides the novel evidence that EGFR kinase activated the $K_{IR}2.3$ channels. Because K_{IR} channels play a critical role in setting the resting membrane potential and firing pattern in a variety of cell types (Murata *et al.*, 2001; Bichet *et al.*, 2003), activation of $K_{IR}2.3$ channels may be important for decreasing cellular excitability, and this is most likely to be of particular significance in tissues of the brain and heart where $K_{IR}2.3$ channels are abundantly expressed (Perier *et al.*, 1994).

Collectively, the present study has provided the novel evidence that $K_{IR}2.3$ channels are regulated by the interaction of EGFR kinase (but not PDGFR kinase and Src-family kinases) and PTPs. PTPs negatively, while EGFR kinase positively, modulate these channel stably expressed in HEK 293 cells via phosphorylation of the Y234 residue of the channel. Such effects may be involved in the endogenous regulation of cellular electrical activity.

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Conflict of interest

None is declared.

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