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Human *ether-à-go-go* gene potassium channels are regulated by EGFR tyrosine kinase

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ABSTRACT

Human ether á-go-go gene potassium channels (hEAG1 or Kv10.1) are expressed in brain and various human cancers and play a role in neuronal excitement and tumor progression. However, the functional regulation of hEAG channels by signal transduction is not fully understood. The present study was therefore designed to investigate whether hEAG1 channels are regulated by protein tyrosine kinases (PTKs) in HEK 293 cells stably expressing hEAG1 gene using whole-cell patch voltage-clamp, immunoprecipitation, Western blot, and mutagenesis approaches. We found that the selective epidermal growth factor receptor (EGFR) kinase inhibitor AG556 (10 μM), but not the platelet growth factor receptor (PDGFR) kinase inhibitor AG1295 (10 μM) or the Src-family inhibitor PP2 (10 μM), can inhibit hEAG1 current, and the inhibitory effect can be reversed by the protein tyrosine phosphatase (PTP) inhibitor orthovanadate. Immunoprecipitation and Western blot analysis revealed that tyrosine phosphorylation level of hEAG1 channels was reduced by AG556, and the reduction was significantly countered by orthovanadate. The hEAG1 mutants Y90A, Y344A and Y485A, but not Y376A and Y479A, exhibited reduced response to AG556. Interestingly, the inhibition effect of AG556 was lost in triple mutant hEAG1 channels at Y90, Y344, and Y485 with alanine. These results demonstrate for the first time that hEAG1 channel activity is regulated by EGFR kinase at the tyrosine residues Tyr^{90}, Try^{344}, and Try^{485}. This effect is likely involved in regulating neuronal activity and/or tumor growth.

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1. Introduction

Human ether á-go-go gene potassium channels (hEAG1 or Kv10.1) are expressed in the nervous system [1,2] and various human cancers [3–6] and are believed to play a role in neuronal excitement [1,7] and tumor progression [3,4,6]. Knockdown of hEAG1 channels by hEAG1 siRNA suppressed cell proliferation in several human tumor cell lines [8], while activation of hEAGl channels by arachidonic acid enhanced cell proliferation in human melanoma cells [9]. Earlier studies demonstrated that hEAG1 was regulated by Ca^{2+}/calmodulin [10,11], calmodulin-dependent kinase II [12], and cyclic AMP [1], and membrane-associated guanylate kinase adaptor proteins [13].

Protein tyrosine kinases (PTKs), including both receptor PTKs (e.g. EGFR kinase, epidermal growth factor receptor kinase) and non-receptor PTKs (e.g. Src-family kinases), are important intracellular signals [14,15]. In addition to the mediation of cellular events such as cell growth, differentiation, embryonic development, metabolism, immune system function and oncogenesis [14,15], PTKs regulate ion channels activity [16,17], including K^+ channels [18–21], Na^+ channels [22], and Cl^- channels [23]. We recently found that human ether á-go-go related gene (hERG or Kv11.1) potassium channels were regulated by both EGFR kinase and Src-family kinases [19]; however, the potential regulation of the super-family hEAG1 channels by PTKs is unknown. The present study was therefore to determine whether and how hEAG1 channels stably expressed in HEK 293 cells are regulated by PTKs using whole-cell patch voltage-clamp, immunoprecipitation, Western blot, and site-directed mutagenesis approaches. We found that the EGFR kinase inhibitor AG556, but not the Src-family kinase inhibitor PP2, inhibited hEAG1 current by decreasing tyrosine phosphorylation of the channel.

2. Materials and methods

2.1. Cell culture, mutagenesis, and gene transfection

The plasmid hEAGl/pTracer CMV [8] was generously provided by Dr. L. Pardo and was transfected into HEK 293 cells (ATCC, Manassas, VA). The HEK 293 cell line stably expressing hEAG1 channels were established as previously described [24,25], and selected with 800 μg/ml zeocin (Invitrogen, Hong Kong, China). The cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Hong Kong) supplemented with 10% fetal bovine serum, 300 μg/ml zeocin.

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The predicted potential tyrosine phosphorylation sites of hEAG1 channels were examined using the software NetPhos 2.0 (www.cbs.dtu.dk/cgi-bin). The mutant hEAG1 channels were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions, and confirmed by full DNA sequencing analysis (Gene Centre, University of Hong Kong). The mutants Y90A, Y344A, Y376A, Y479A, Y485A, Y639A and Y639F were transiently expressed separately in HEK 293 cells using 10 μl of Lipofectamine 2000 with 4 μg of the plasmid in a 35 mm culture dish. The cells used for electrophysiology were seeded on a glass cover slip.

2.2. Solutions 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 current recordings, the pipette solution contained (mM) KCl 20, K-aspartate 110, $MgCl_2$ 1.0, HEPES 10, ethyleneglycoltetraacetic 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 (Bristol, UK). All other reagents were obtained from Sigma-Aldrich (St Louis, MO, USA). Stock solutions were made with dimethylsulfoxide (DMSO) for AG556 (100 mM), AG1295 (20 mM) and PP2 (10 mM). The stocks were divided into aliquots and stored at −20°C. Sodium orthovanadate stock solution (200 mM) was made with distilled water, and pH was adjusted to 9.0.

2.3. Electrophysiology

Cells on a coverslip were transferred to a cell chamber (0.5 ml) mounted on the stage of an inverted microscope (Diaphot, Nikon, Japan) and superfused at ~2 ml/min with Tyrode solution. Whole-cell currents were recorded as described previously [24,25]. 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 2–3 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 with the cell. After a gigaohm seal was obtained by negative pressure, the cell membrane was ruptured by applying a gentle suction to establish whole cell configuration. Series resistance (Rs, 3–6 MΩ) was compensated by 60–80% to minimize voltage errors. The liquid junction potential (13.5 mV) was not corrected throughout the experiment. Membrane currents were recorded 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. Current signals were low-pass filtered at 5 kHz and stored in the hard disk of an IBM compatible computer. All experiments were conducted at room temperature (22–23 °C).

2.4. Immunoprecipitation and Western blotting

The immunoprecipitation and Western blotting were performed following the procedure as described previously [19,20]. HEK 293 cells (~80% confluence) stably expressing hEAG1 channels were treated respectively with different compounds for 30 min at room temperature, and centrifuged (400×*g* for 10 min) at 4 °C. The cell pellet was then lysed with lysis buffer containing (mM) Tris 25, NaCl 150, NaF 1.0, EDTA 1.0, orthovanadate 1.0, phenylmethylsulfonyl fluoride 1.0, 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, USA), and diluted to equal concentrations. Proteins were immunoprecipitated overnight at 4 °C using 2 μg of anti-KCNH1 (i.e. anti-hEAG1) antibody (NBP1-42816, Novus Biologicals, Littleton, CO, USA) and 100 μl of protein A/G beads (DAM 1460243, Millipore, Billerica, MA, USA). 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 (1:1000, Cell Signaling Technology Inc., 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, Santa Cruz Biotechnology) for 2 h at room temperature. Blots were developed with enhanced chemiluminescence (ECL, Amersham Biosciences, Sweden) and exposed on an X-ray film (Fuji Photo Film GmbH). The blots were then stripped and reprobed with the anti-hEAG1 antibody to determine total hEAG1 channel proteins. The film was scanned, imaged by a Bio-Imaging System (Syngene, Cambridge, UK), and analyzed via GeneTools software (Syngene).

2.5. RNA interference

Short interference RNA (siRNA) molecules targeting human EGFR (sc-29301) were purchased from Santa Cruz Biotech. This siRNA is a mixture of three target-specific 20–25 nucleotide siRNAs designed to knock down human EGFR gene expression. HEAG1-HEK 293 cells at 40–50% confluence were transfected with siRNA molecules at a total of 40 nM using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's protocol. The silencer negative control #1 siRNA (Ambion, #AM4611; Austin, TX), which contains no known target in mammalian genomes, was used as negative control. After 72 h of transfection, the cells were used for immunoprecipitation and Western blotting analysis as described above.

2.6. Statistical analysis

Data are expressed as means ± S.E.M. Nonlinear curve-fitting was performed using Pulsefit (HEKA) and Sigmaplot (SPSS, Chicago, IL). Paired and/or unpaired Student's *t*-test was 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 PTKs inhibitors on hEAG1 current

The PTKs effects on hEAG1 current were investigated by applying the highly selective EGFR kinase inhibitor AG556 [26], the selective platelet growth factor receptor (PDGFR) kinase inhibitor AG1295 [27], and the Src-family kinases inhibitor PP2 [28]. Fig. 1A shows the time course of hEAG1 current recorded at +50 mV (Fig. 1A) in the absence and presence of 10 μM AG556. AG556 gradually reduced the current. The inhibitory effect reached a steady-state level within 5 min upon AG556 exposure, and recovered rapidly on washout. Voltage-dependent hEAG1 current (Fig. 1B) was also inhibited by 10 μM AG556 (8 min), and the effect recovered on washout. Fig. 1C illustrates the mean values of current–voltage (*I–V*) relationships of hEAG1 current in the absence and presence of 10 μM AG556. The current was significantly suppressed at potentials from +30 to +60 mV ($n=6$, $P<0.01$ vs. control).

Voltage-dependent variables (g) of hEAG1 current were calculated for each cell with I–V relationship curves as shown in Fig. 1C based on the formulation $g = I/(V_T - V_R)$, where I is the current at test potential (V_T), and V_R is the measured reversal potential (−70 mV). The normalized current (Fig. 2A) was fitted to a Boltzmann equation as

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