Phosphorylation of a tyrosine at the N-terminus regulates the surface expression of GIRK5 homomultimers

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Abstract The G protein-coupled inwardly rectifying GIRK5 and Δ5GIRK5 splicing variants do not express functional potassium channels. In contrast, Δ25GIRK5 forms functional homomultimers in Xenopus laevis oocytes. A tyrosine is present at the N-term of the non-functional isoforms. We studied the effect of endogenous tyrosine phosphorylation on the GIRK5 surface and functional expression. Unlike wild type channels, GIRK5Y16A and Δ5GIRK5Y16A mutants displayed inwardly rectifying currents and inhibitors of Src tyrosine kinase promoted the trafficking of GIRK5 to the cell surface. This is the first evidence that endogenous phosphorylation of a tyrosine residue in a GIRK channel inhibits its surface expression.

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1. Introduction

Inwardly rectifying $K^+$ channels activated by Gi/Go protein-coupled receptors (GIRK or Kir 3.x) decrease membrane excitability by hyperpolarizing the membrane potential, slowing membrane depolarization, and shortening the action potential waveform [1]. These tightly regulated $K^+$ channels are functionally expressed in the heart [2], the central nervous system [3] and the smooth muscle [4]. The molecular mechanisms underlying G protein regulation of GIRK channels have been extensively studied in the acetylcholine activated GIRK1/GIRK4 heteromultimer (I_{K(Ca)}) from atrial pacemaker cells and myocytes [5–7]. Experimental evidence shows that functional expression of GIRK channels requires homo or heterotrimerization [1,7–12]. Four GIRK members have been identified so far in mammalian species: GIRK1 through GIRK4 [12–15].

In contrast to the widely characterized GIRKs from mammals, few functional studies exist of these channels in non-excitable cells where G-protein coupled receptors play a crucial role in cell development and differentiation.

Oocytes have been an important model system for studying cell cycle regulation and the functional characterization of heterologously expressed membrane proteins [16]. GIRK5 (Kir3.5) was identified in Xenopus laevis oocytes and shows a longer NH2 end compared with their mammals homologues [17]. The splicing variants of GIRK5 comprise three isoforms: GIRK5, Δ5GIRK5 and Δ25GIRK5 (Fig. 1A). Δ25GIRK5 gives rise to functional channels with an outstanding basal activity (non-receptor coupled) in oocytes [18] and insect cells [19]. Intriguingly, the isoforms GIRK5 and Δ5GIRK5 are not functional. These isoforms present a tyrosine (Y16) within a consensus phosphorylation site at their N-terminus (Fig. 1A) [20].

Ion channel phosphorylation plays an important role in the modulation of membrane excitability by altering the gating and ion permeation of ligand-gated receptors and voltage-gated calcium, sodium and potassium channels [21]. The activity of some potassium channels such as the inward rectifier Kir 2.1 [22,23], the voltage-gated Kv1.2 [24], Kv1.3 [25–27], Kv1.5 [28], Kv7.3, Kv7.4, Kv7.5 [29,30], and the hyperpolarization-activated HCN channels [31] is suppressed by tyrosine phosphorylation.

The aim of this work was to study the effect of endogenous phosphorylation of tyrosine 16 on the functional expression of GIRK5 channels in the oocytes.

Our study provides the first evidence that endogenous phosphorylation of a tyrosine at the N-terminus of a GIRK channel regulates its trafficking to the surface and, then, its functional expression.

2. Materials and methods

2.1. DNA clones, mutagenesis and fusion proteins

The open reading frames (ORF) of GIRK5 (U42207), Δ5GIRK5 and Δ25GIRK5 cDNAs were obtained by PCR as in previous studies [18,19]. Site-specific mutagenesis of tyrosine 16 to an alanine residue (GIRK5Y16A and Δ5GIRK5Y16A) was introduced by the polymerase chain reaction using the mutant primers: (a) 5′ AAA GAT TGG CTG AGT CAC C 3′ (sense) and (b) 5′ GGT GAC TCA GCC AAT CTT T 3′ (antisense). The SP6 (sense) and 5′ AGA GAC CAA AAAAGAG ACG ATC GTCGCC TGT ATC AAA 3′ (antisense) were used as flanking primers. Constructs encoding EGFP-GIRK5, EGFP-GIRK5Y16A and EGFP-Δ25GIRK5 fusion proteins were made by adding the ORF of the enhanced green fluorescent protein (pEGFP-C1; Clontech) at the N-terminal. All GIRK5 cDNAs were subcloned in the pRSPS6013A3-UWE vector (pBF) and sequenced [18,19].

2.2. cRNA synthesis and injection of oocytes

Gα2, m2ACh receptor and all GIRK5 cRNAs were synthesized from linearized plasmid vectors using the mMessage mMachine kit (Ambion Corporation, Austin, TX). Xenopus laevis oocytes stage V and VI were obtained as described in [20]. Control oocytes were injected with water. 5–20 ng of all GIRK5 cRNAs was injected in the oocytes. The cells were maintained in a ND96 solution as described in [18].
2.3. Electrophysiological procedures

Oocytes were registered with the two-electrode voltage clamp (TEVC) technique 2–4 days after injected as described [26]. Voltage pulse protocols were performed using consecutive 100 ms step changes from $-160$ to $+60$ mV with increments of 20 mV. Oocytes were clamped at a holding potential of 0 mV. $10\, \mu$M carbachol (carbamylcholine chloride, Sigma) was used to activate the m2 muscarinic receptor (m2ACh). TEVC was performed using the amplifier Geneclamp 500, a Digidata 1322A interface with pClamp8 software (Axon Instruments). Analysis was performed with the pClamp8 software and Origin v. 5.0 (Microcal). Data were sampled at 5–10 kHz and filtered at 1–5 kHz. The mean standard deviation from a number ($n$) of independent experimental observations is shown. ANOVA and $t$-student tests were used to compare significant differences ($p < 0.05$).

2.4. Confocal microscopy

Oocytes injected with the EGFP-GIRK5, EGFP-GIRK5Y16A and EGFP-$\Delta25$GIRK5 cRNAs were examined four days after their injection. EGFP fluorescence was excited at 488 nm with an argon laser beam and viewed with a Nikon TMD 300 inverted microscope equipped with a 10× dry lens. Fluorescent emissions of 520 nm were collected using a long pass filter. XY scans were obtained at approximately the midsection of each egg. Six sections of an oocyte membrane were recorded and the signal averaged for each cell.

2.5. Protein tyrosine kinase inhibitors assays

Genistein and geldanamycin (Alomone labs, Israel) were dissolved in dimethyl sulfoxide (Me$_2$SO; Baker) and stored at $-20^\circ$C. The final concentration of Me$_2$SO applied to the oocytes was less than 0.02%. Control oocytes and those injected with GIRK5, GIRK5Y16A, $\Delta25$GIRK5 and EGFP-GIRK5 cRNAs, were incubated with the inhibitors by 1 h. TEVC recordings of oocytes were then performed.

2.6. Western blotting

The membrane fraction of oocytes injected with the EGFP-GIRK5 cRNA was obtained according to [32]. The pellet was rinsed in 20 $\mu$L of sample buffer and loaded onto 10% SDS-polyacrylamide gel as described in [33], and electrophoretically transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Life Science). Pre-stained molecular mass markers were purchased from Amersham Life Science. A monoclonal antibody against EGFP was used for immunodetection (Clontech, Palo Alto, CA). The secondary antibody was a horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham Life Science). Antigen-antibody complexes were detected by autoradiography-enhanced chemiluminescence (ECL Advance, Amersham Life Science).

3. Results

Expression of recombinant $\Delta25$GIRK5 displayed a basal activity of $7.02 \pm 0.2\, \mu$A while GIRK5 and $\Delta5$GIRK5 did not show significant differences with control oocytes: $1.4 \pm 0.2$, $1.7 \pm 0.2$ and $1.6 \pm 0.3\, \mu$A at $-160$ mV, respectively (Fig. 1B and C). There is a tyrosine within a putative consensus site for phosphorylation at the NH2-end of GIRK5 and $\Delta5$GIRK5 (Fig. 1A; 28). In order to investigate if this tyrosine has a role in controlling the activity of these GIRK5 isoforms, mutants GIRK5Y16A and $\Delta5$GIRK5Y16A were tested. Both mutants produced inwardly rectifying currents: GIRK5Y16A, $8.5 \pm 0.2\, \mu$A and $\Delta5$GIRK5Y16A, $8 \pm 0.4\, \mu$A at $-160$ mV (Fig. 1B and C). Therefore, these results reveal that Y16, either if it is or not phosphorylated controls the activity of GIRK5 and $\Delta5$GIRK5.
Opening of GIRK channels can happen by direct binding of Gβγ subunits released from the Gαi/oβγ heterotrimers [1,2,9]. In order to test whether or not the basal activities of GIRK5Y16A and Δ25GIRK5 result from their activation by endogenous membrane-bound Gβγs, coexpression of Gαi2 was performed. As predicted, both channels were inhibited (75%) as a result of overexpressing Gαi2 (Fig. 2A and B).

To estimate if the overall expressed channels were activated by endogenous Gβγs, we studied their activation by recombinant m2ACh. Carbachol caused an increment of more than two times with respect to the basal activity of both GIRK5Y16A and Δ25GIRK5. In contrast, activation of m2ACh had no effect on GIRK5 functional expression (Fig. 2C).

To further correlate channel inhibition with loss of surface expression, we proceed to determine the presence of GIRK5 channels at the plasma membrane. Then, we tagged the channels with the EGFP to study their surface expression by laser-scanning confocal microscopy. Control oocytes and those injected with EGFP-GIRK5 cRNA did not show any fluorescence, whereas EGFP-GIRK5Y16A and EGFP-Δ25GIRK5 both appeared at the cell surface (Fig. 3B). Furthermore, these constructs also displayed basal activity: EGFP-GIRK5Y16A, 6.38 ± 1.85 μA (n = 11) and EGFP-Δ25GIRK5, 5.22 ± 1.05 μA (n = 11) at −160 mV (Fig. 3C). Therefore, Y16 controls the surface trafficking of GIRK5 channels to the plasma membrane.

Since endogenous Src-like tyrosine kinase activity has been determined in *Xenopus laevis* oocytes [34], we studied the effect of two tyrosine kinase (PTK) inhibitors. Oocytes injected with GIRK5, GIRK5Y16A and Δ25GIRK5 cRNAs were
incubated with 20 μM genistein or 75 nM geldanamycin by one hour. Both inhibitors did not produce significant changes in the basal activities of GIRK5Y16A and Δ25GIRK5 (Fig. 4).

Importantly, basal activity of GIRK5 was recorded for the first time: 2.6 ± 0.3 μA with genistein and 4.8 ± 0.6 μA with geldanamycin (Fig. 4). These results strongly suggest that PTKs inhibitors allow the dephosphorylation of GIRK5 at Y16 by endogenous phosphatases in the oocytes [22].

Finally, to further confirm that endogenous phosphorylation of tyrosine 16 inhibits the surface expression of GIRK5 two approaches were followed. First, oocytes injected with EGFP-GIRK5 cRNA were incubated without and with geldanamycin by one hour. Only the oocytes incubated with the PTK inhibitor showed fluorescence (Fig. 5B) and also displayed inward currents of 2.8 ± 0.4 μA at −160 mV (Fig. 5C). Second, a Western blot analysis was performed with the above oocytes. EGFP-GIRK5 appeared in the membrane fraction exclusively in the oocytes incubated with geldanamycin. The protein level of EGFP-GIRK5 was similar to the isoform EGFP-Δ25GIRK5 and the mutant EGFP-GIRK5Y16A (Fig. 5D). These results strongly suggest that basal phosphorylation of Y16 in GIRK5 inhibits its trafficking to the plasma membrane.

4. Discussion

To explain the lack of functionality of the wild type GIRK5, we studied the role of endogenous tyrosine phosphorylation in oocytes overexpressing this channel. Our findings show that phosphorylation of tyrosine 16 at the N-terminus of GIRK5 avoids its trafficking to the surface and, as a consequence, its functional expression. Several studies have demonstrated that tyrosine phosphorylation by Src can modulate channel activity. For example, the NMDA channel currents are increased [36] whereas Kv1.3 and Kv1.5 currents are suppressed [25,28,37]. An endogenous Sre is also involved in regulating the number of apical small conductance K⁺ channels in the cortical collecting ducts in kidneys from rats on a K-deficient diet [22]. In ROMK1 (Kir2.1) channels, Src mediated phosphorylation of tyrosine 337 at the carboxy terminus induces their internalization in oocytes [35].

Activation of a tyrosin kinase receptor by a neurotrophic factor accelerates GIRK1 and GIRK4 deactivation [38]. However, inhibition of these channels happens by the presence of an intrinsic GTPase-activating protein domain that it is unmasked by phosphorylation of two tyrosine residues [39].

In conclusion, phosphorylation of GIRK5 by endogenous PTKs regulates its surface trafficking. GIRK5 may have a contribution in establishing the resting membrane potential in oocytes. Further studies should be performed to identify and determine the molecular interactions of the endogenous PTKs that modulates GIRK5 surface expression in these cells. Understanding the basis for the modulation of GIRKs by tyrosine phosphorylation will provide additional insight into the processes regulating the activity of these physiologically significant channels.

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