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Basal activity of GIRK5 isoforms

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Abstract

G protein-coupled inwardly rectifying K^+ channels (GIRK or Kir3) form functional heterotetramers gated by Gβγ subunits. GIRK channels are critical for functions as diverse as heart rate modulation and neuronal post-synaptic inhibition. GIRK5 (Kir3.5) is the oocyte homologue of the mammalian GIRK subunits that conform the K_{ACh} channel. It has been claimed that even when the oocytes express GIRK5 proteins they do not form functional channels. However, the GIRK5 gene shows three initiation sites that suggest the existence of three isoforms. In a previous work we demonstrated the functionality of homomultimers of the shortest isoform overexpressed in the own oocytes. Remarkably, the basal GIRK5-Δ25 inward currents were not coupled to the activation of a G-protein receptor in the oocytes. These results encouraged us to study this channel in another expression system. In this work we show that Sf21 insect cells can be successfully transfected with this channel. GIRK5-Δ25 homomultimers produce time-dependent inward currents only with GTPγS in the recording pipette. Therefore, alternative modes of stimulus input to heterotrimeric G-proteins should be present in the oocytes to account for these results.

Keywords: GIRK, Kir3; Inward rectifying potassium channel; *Xenopus* oocytes; Insect cells; G-protein; Isoforms

Introduction

Inwardly rectifying K^+ channels activated by G protein-coupled receptors decrease membrane excitability by hyperpolarizing the membrane potential, slowing membrane depolarization, and

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shortening the action potential waveform [1]. These tightly regulated K^+ channels are functionally expressed in the heart [2] and in the central nervous system [3]. The molecular mechanisms underlying G protein regulation of GIRK channels have been most extensively studied in the cardiac muscarinic K^+ channel, the acetylcholine (Ach)-activated GIRK channel (I_{KACh}) in atrial pacemaker cells and myocytes [4–6]. I_{KACh} is composed of GIRK1 and GIRK4 subunits. Abundant experimental evidence shows that the functional expression of GIRK channels requires heterotetramerization [1].

Five members of the GIRK group (GIRK1 through 5) have been identified so far. GIRK1 through GIRK4 have been cloned only from mammalian species [7–9] while GIRK5 has been cloned from *Xenopus laevis* oocytes [10,11]. GIRK5 shows 78% amino acid homology with GIRK4 [10]. Consistent with the idea that functional GIRK channels are heterotetramers, oocytes overexpressing GIRK5 in the absence of GIRK1 do not display Ach-stimulated currents [10].

The genomic sequence of GIRK5 shows three possible translation initiation sites (methionine 1, 5 and 25) according to the Kozak consensus sequences [12]. Previously, we cloned and showed the functionality of the shortest isoform in the oocytes [11].

In this work, specific primers were designed to isolate the cDNA coding for the other GIRK5 isoforms. We found that GIRK5 and GIRK5-Δ5 did not express functional channels in contrast to GIRK5-Δ25 in the oocytes. To further characterize the functional properties of the endogenous oocyte GIRK5-Δ25 isoform, we transfected this channel in the recombinant insect cell baculovirus expression system [13].

The results presented here confirm that GIRK5-Δ25 also produces stable, G-protein-activated homomultimers in insect cells. A possible role of the first 25 amino acids in the functional association of GIRK5 homotetramers is discussed.

Methods

The GIRK5 and GIRK5-Δ25 isoforms were obtained as described in [11]. The GIRK5-Δ5 was amplified by PCR with the primers 5′-AAG AGT CGA CAT GGC ACC AAG TCC TCA GTG-3′ (sense) and 5′-AGA GAC CAA AAA GAG ACG ATC GTC GCC TGT ATC AAA-3′ (antisense). The fragment amplified was processed like GIRK5 [11]. 25 ng of GIRK5, GIRK5-Δ5 and GIRK5-Δ25 cRNA transcribed in vitro (RNA polymerase SP6, transcription kit, Ambion) were injected into oocytes previously defolliculated for electrophysiogical assays. The full-length cDNA encoding GIRK5-Δ25 was subcloned into the pBlueBac4 vector at Xho I- Sal I sites (Invitrogen). Restriction mapping and sequence analysis confirmed the orientation of the cDNA. The recombinant baculovirus was produced following the manufacturer instructions (Invitrogen). The baculovirus was purified twice using the plaque assay and amplified. The titer of the recombinant virus was determined following the expression of β-galactosidase [13]. The final virus stock was maintained at 4 °C until use.

The insect Sf21 cell line (Invitrogen) from the armyworm caterpillar *Spodoptera frugiperda*, was kept in a monolayer culture at 27 °C in Grace's media (GIBCO). Cells were propagated every 3–4 days. The cells were infected with recombinant *Autographa californica* nuclear polyhedrosis (AcNPv) baculovirus containing the full-length cDNA for GIRK5-Δ25. The cells were infected in 35 mm plates at a MOI of 5 and kept at 27 °C after infection. Cells were used 2 days later for recordings. Experiments were carried out at room temperature (20–25 °C).

PCR assays

Sf21 cells were grown on 100 mm plastic tissue-culture dishes (Corning). Cells were infected either with GIRK5-Δ25 or bradykinin receptor (Bk2) recombinant baculoviruses. Total RNA was obtained from non-infected and infected cells with the trizol reagent (Life Technologies). Several RT-PCR experiments were performed (M-MLV reverse transcriptase, GIBCO). 5 μg of total RNA from oocytes, rat brain, kidney and insect cells were used to obtain their cDNAs. 10 ng cDNA of these samples and plasmids containing the channels GIRK5-Δ25, GIRK4 and GIRK1, respectively, were amplified with primers (GIBCO) designed for conserved GIRK subfamily regions. Pore H5 (160–172 amino acids in GIRK5): 5′G TCT GCA TTT CTT TTT TCT ATT GAA ACT GAG ACT ACA ATT G 3′ (sense) and at the C-terminal (283–294 amino acids in GIRK5): 5′AGA GAC CAA AAA GAG ACG ATC GTC GCC TGT ATC AAA G 3′ (antisense). Meanwhile the H5 amino acids among GIRKs are 92% (GIRK1) and 100% (the other GIRKs) homologous, the C-terminal primer displays 92% (GIRK4), 100% (GIRK3), 83% (GIRK2) and 75% (GIRK1) amino acid homology. We expect a 432 bp fragment. 5 μl of the PCR samples were run in a 1% agarose gel in TBE buffer. Specific primers for GIRK1 were also designed: 5′CAT CGT CGA CAT GTC TGC ACT CCG AAG GAA ATT TGG G 3′ (sense) and 5′GAT GGT GGC CTC GGT CTC GAT GAA GAA AAG GAA GGC 3′ (antisense).

Electrophysiology

Oocytes 3–4 days after cRNA injection were recorded using two-electrode voltage clamp technique (AxoClamp 2B, Axon Instruments). Whole-oocyte recordings were filtered at 4 kHz, and stored on the computer hard disk for off-line analysis with pCLAMP v. 6.4 (Axon Instruments) and Origin v. 5.0 (Micrococal). Electrodes showed resistances of 0.5–1 MΩ (3 M KCl). The bath solution contained (in mM): KCl 118, $CaCl_2$ 1.8, $MgCl_2$ 1 and HEPES 5 at pH7.4. Oocytes were voltage clamped at − 0 mV and voltage pulses from − 160 to 60 mV for 100 ms with increments of 20 mV were applied to elicit currents. Currents in Sf21 cells were measured by whole-cell patch clamp recording. Pipettes (glass 1.5/1.0 WPI) were pulled in two steps on a Narishige microelectrode puller and were not fire polished. Electrodes were filled with (in mM): L- Aspartic acid (monopotassium salt) 145, $MgCl_2$ 2, EGTA 2, MES 10 (pH 6.2), and they had resistances of 2–4 MΩ. 100 μM GTPγS (Sigma) was added to the pipette solution of the experiments indicated in the figure legends. The extracellular medium contained (in mM): L-aspartic acid 145, $MgCl_2$ 2, $CaCl_2$ 2, MES 10 (pH 6.2). The holding potential was − 10 mV. Voltage pulses were applied from − 160 to 30 mV in 10 mV steps for 300 ms. Currents were recorded using an Axopatch 200A amplifier, filtered at 5 KHz and digitized at 2.5 KHz. Currents were stored directly into the computer's hard disk through the Digidata 1200 interface (Axon Instruments). All data reported show the mean ± standard deviation from a number (n) of independent experimental observations.

Results

Inward K^+ currents were not detected in oocytes injected with cRNA of the GIRK5 or GIRK5-Δ5 isoforms (Fig. 1B, C and E). In contrast, oocytes injected with cRNA of the shortest isoform GIRK5-

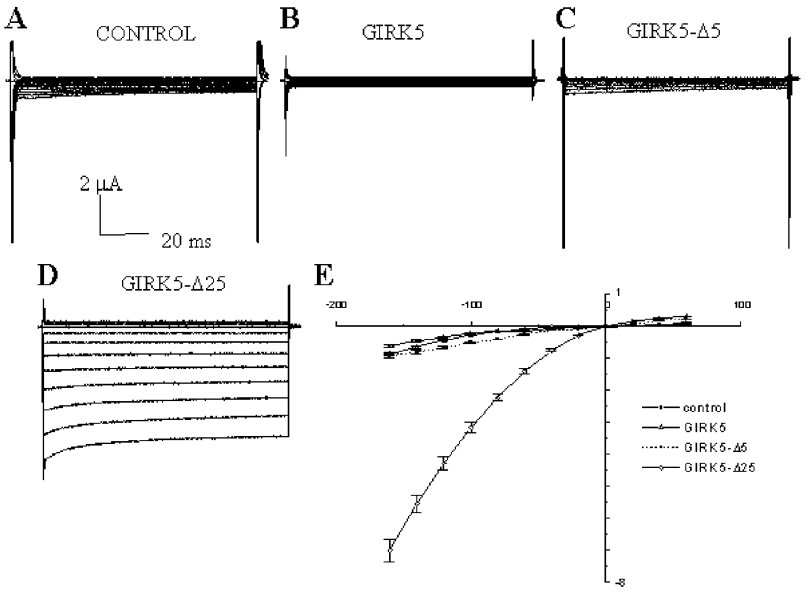


Fig. 1. Currents recordings of the GIRK5 isoforms in oocytes. Representative currents obtained from water-injected oocytes (control; A), oocytes injected with 25 ng of GIRK5 (B), GIRK5-Δ5 (C) and GIRK5-Δ25 (D) cRNAs. Voltage protocol stepped from − 160 mV to 60 mV in 20 mV increments applied during 100 ms from a holding potential of 0 mV. E: Current-voltage relationships from the currents shown above (n = 6). Whole-oocyte currents were measured in 118 mM extracellular KCl.

Δ25, expressed functional K^+ channels displaying a high basal activity (i.e. without receptor stimulation; Fig. 1D, Ref. [11]).

GIRK5-Δ25 high basal activity in the oocytes should come from channels activated by free endogenous Gβγ. We carried out an experiment with oocytes coinjected with GIRK5-Δ25 and the recombinant Gα cRNAs (Fig. 2) to clarify this fact. Accordingly with the above hypothesis, we observed an inhibition of GIRK5-Δ25 by Gα.

To further characterize the GIRK5-Δ25 isoform, this channel was infected in a baculovirus insect cell line. This expression system has been successfully used for the heterologous expression of ionic channels [15,16] and other membrane proteins [17,18].

Patch clamp experiments in the whole-cell configuration obtained from non-infected cells showed small currents (150 ± 75 pA at − 160 mV, n = 6, Fig. 3A). 100 μM GTPγS into the recording pipette resulted in a small increment in basal current (200 ± 50 pA, n = 6, Fig. 3A), which was not statistically significant ($p > 0.1$).

Sf21 cells infected with the recombinant baculovirus containing GIRK5-Δ25 (Fig. 3B, left), showed basal currents that were indistinguishable from the non-infected cells (180 ± 45 pA, n = 7); however, addition of 100 μM GTPγS in the recording pipette resulted in the appearance of large, inwardly rectifying currents (1600 ± 280 pA at − 160 mV, n = 7, Fig. 3B right).

To determine if Sf21 cells express endogenous GIRK channels, RT-PCR experiments were performed with several cDNA samples. We observed that primers designed at the conserved regions of GIRKs amplified the expected band of 432 bp for all the GIRKs assayed: GIRK5-Δ25, GIRK4 and GIRK1 (Fig. 3A, lanes 10, 11 and 12, respectively). Even when the antisense primer had the lowest homology with GIRK1, it also amplified this channel (lane 12). GIRKs are expressed in rat brain and according to this

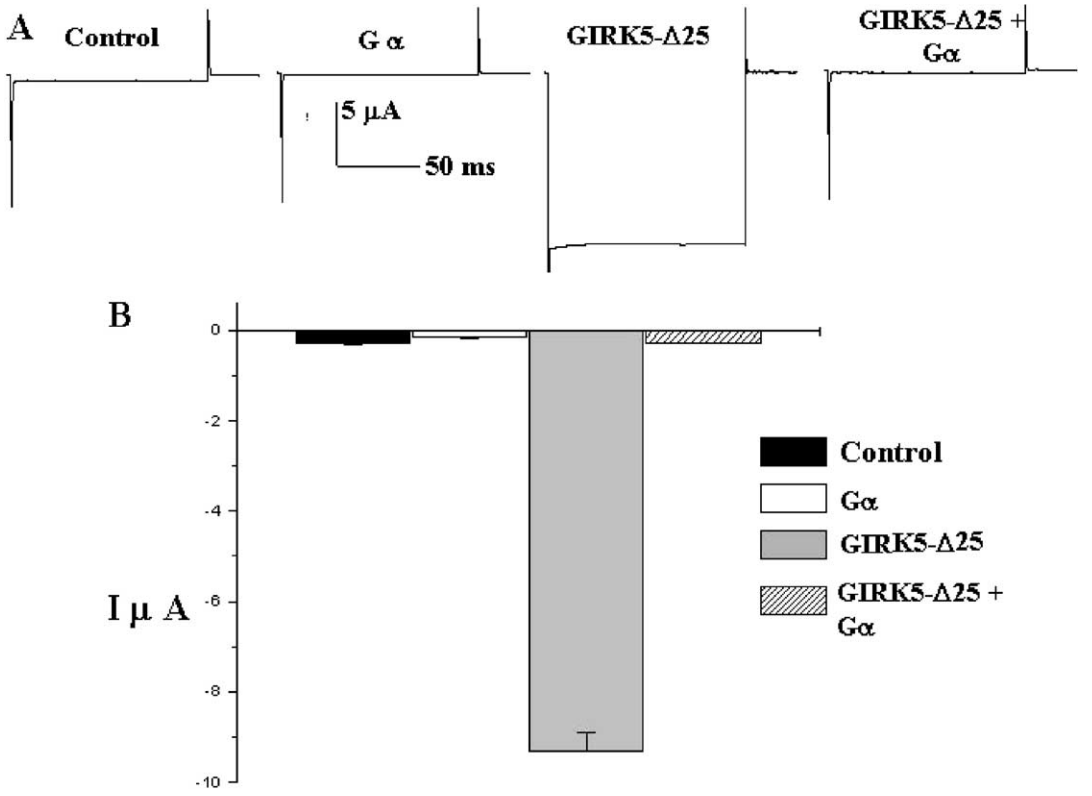


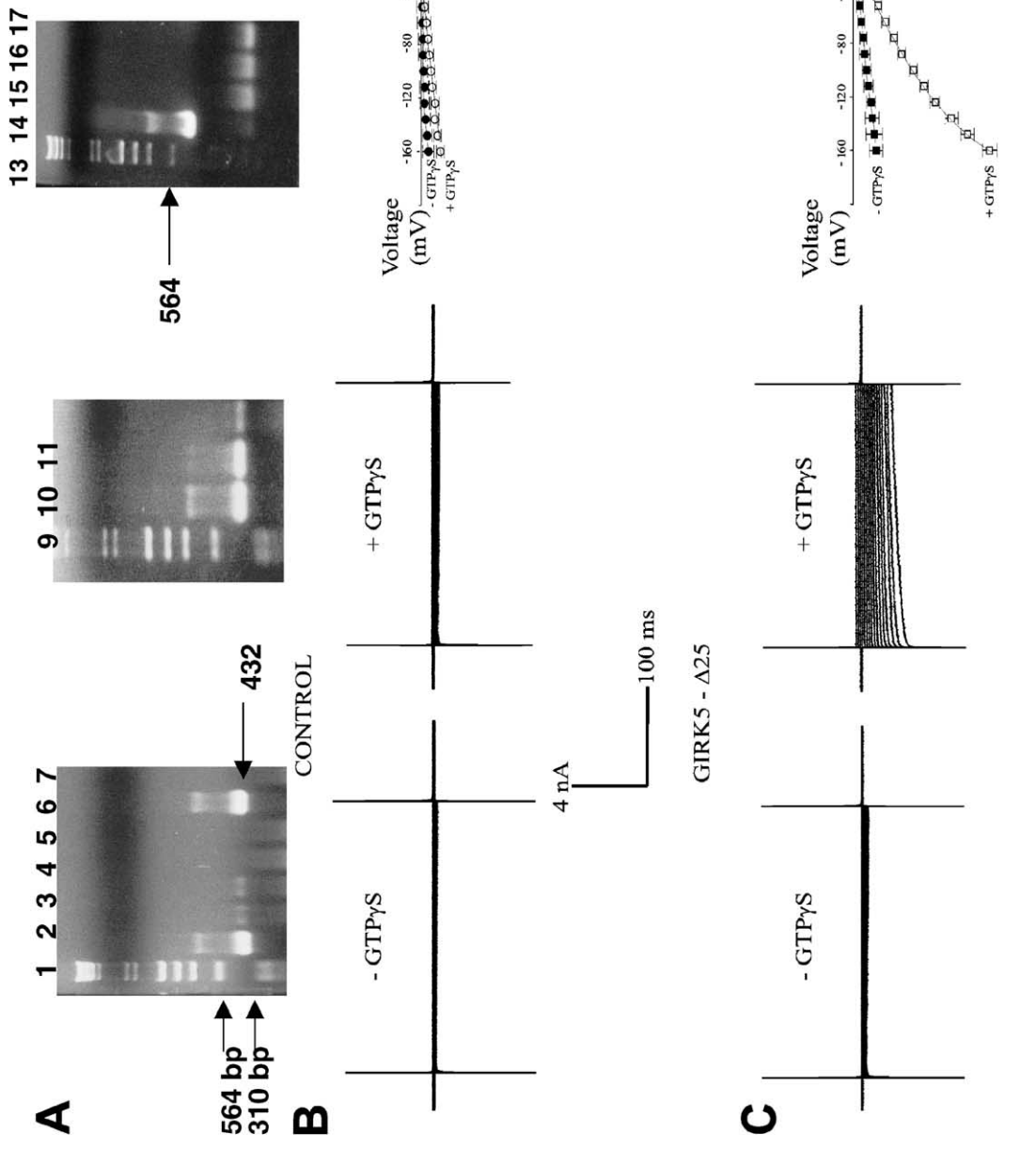
Fig. 2. Inhibition of the basal activity of GIRK5-Δ25 by Gα proteins. Currents were evoked at −40 mV with a holding potential of 0 mV in 118 mM K-gluconate. (A) Time-course traces of control oocytes and oocytes injected with: Gα, GIRK5-Δ25 and GIRK5-Δ25 + Gα cRNA, respectively. (B) Histogram of the above traces: control (n = 4), Gα (n = 5), GIRK5-Δ25 (n = 6) and GIRK5-Δ25 plus Gα protein (n = 5).

fact, our primers amplified a GIRK from rat brain cDNA (lane 4). Negative controls were in agreement with non cDNA amplification for a GIRK: kidney, non-infected insect cells and insect cells infected with the bradykinin receptor; lanes 5, 6, 8, respectively. GIRK1 specific primers (lanes 14–17) amplified only with the GIRK1-pBluescript SK- vector. Sf21 (lane 15), Sf21/GIRK5Δ25 (lane 16), Sf21/BK (lane 17) cDNAs did not give any GIRK cDNA band. In conclusion, the insect cells do not express an endogenous GIRK.

SF21 cells infected with baculovirus containing the GIRK5-Δ25 construct, did not display basal inward currents (Fig. 3B). Only after the addition of 100 μM GTPγS in the recording pipette, K^+ currents were observed (Fig. 3C), most probably because the dissociation of the endogenous Gi/Go-proteins.

In contrast to the results obtained with Sf21 cells, oocytes expressing GIRK5-Δ25 showed large basal K^+ currents (Fig. 1D), suggesting that in these cells there might be basal levels of GIRK channel modulators such as free Gβγ, Na^+, Mg-ATP and PIP_2 [19]. Coexpression of GIRK1/GIRK4 heteromultimers also displays significant basal activities in oocytes [10].

A representative time course of GIRK5-Δ25 activation with 100 μM GTPγS in Sf21 cells is illustrated in Fig. 4. As indicated in this figure, the maximum current amplitude is obtained between 7–10 minutes after disrupting the membrane and going into the whole-cell configuration with 100 μM


A
1 2 3 4 5 6 7
432
564 bp
310 bp
9 10 11
13 14 15 16 17
564
B
CONTROL
- GTPγS
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- GTPγS
+ GTPγS
4 nA
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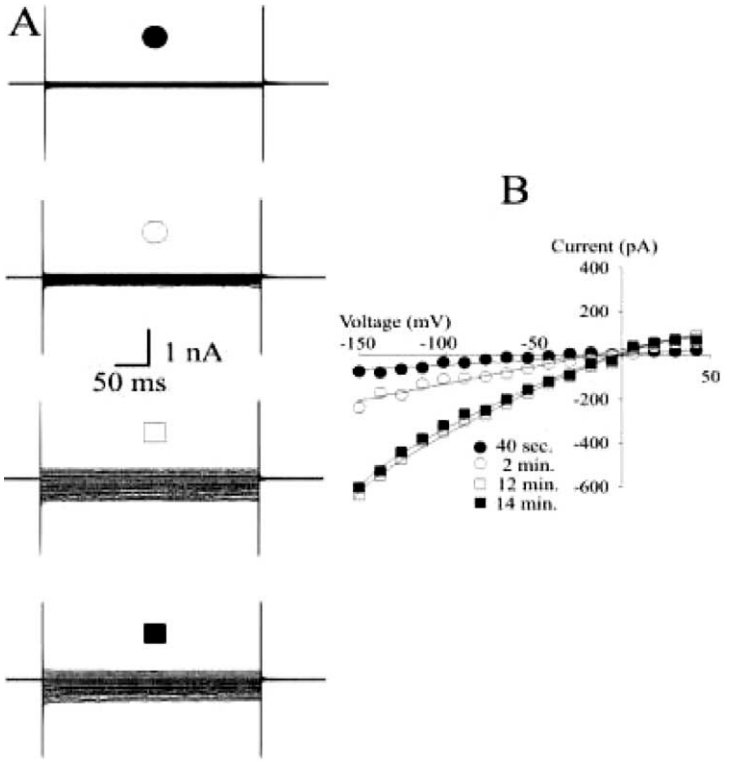

Fig. 4. Time course of current activation with GTPγS. A: Representative experiment illustrating the time course of the activation of inward current in Sf21 cells infected with the recombinant baculovirus containing the cDNA for GIRK5-Δ25. B: current-voltage relationships of the currents illustrated in *A* obtained 40 seconds (closed circle), 2 minutes (open circle), 12 minutes (open square) and 14 minutes (close square) after establishing the whole-cell configuration. No further current increments were observed after 10 minutes of going into whole-cell. Non-infected or infected Sf21 cells with the Bk2, did not show any significant current increment in the presence of GTPγS. The voltage protocol was similar to the one used in Fig. 2. The pipette and bath solutions contained 145 mM KCl.

GTPγS in the patch pipette (Fig. 4). Non-infected cells or cells infected with Bk2 produced no significant increment in current amplitude during the 30 minutes explored in these experiments (data not shown). These results indicate a time-dependent activation of the inwardly rectifying current in cells

Fig. 3. A: 1% agarose gel with the RT-PCR products. Lane 1, 9 and 13 correspond to the molecular weight markers λ/Hind III y φ X174 RF DNA/Hae III. Horizontal arrows indicate the size of two markers. GIRK5-Δ25 in pBF vector as positive control [2,10]; cDNA from: oocytes [3], rat brain [4], kidney rat [5], Sf21 cells non-infected [6], Sf21 cells infected with recombinant GIRK5-Δ25 [7] recombinant human bradykinin type 2 receptor [8]; GIRK4 [11] and GIRK1 [12]. RT-PCR with GIRK1 specific primers (lanes 14–17) GIRK1-pBluescript SK- vector [14], Sf21 [15], Sf21/GIRK5Δ25 [16], Sf21/BK [17] cDNAs. Functional expression of GIRK5-Δ25 in insect cells. B: Representative whole-cell patch-clamp recordings from non-infected insect cells in the absence (-GTPγS) and presence (+GTPγS) of 100 μM GTPγS. Current-voltage relationships illustrating the mean ± SD obtained from 7 independent observations for each condition. C: Representative whole-cell patch-clamp recordings from insect cells infected with the recombinant baculovirus containing the GIRK5-Δ25 cDNA. Current-voltage relationships illustrating the mean ± SD obtained from 7 independent observations for each condition. Cell recordings in the presence of GTPγS were obtained 10 minutes after establishing the whole-cell configuration. The voltage protocol is similar to the one described in Fig. 1 except that the voltage steps were every 10 mV. The pipette and bath solutions contained 145 mM KCl.

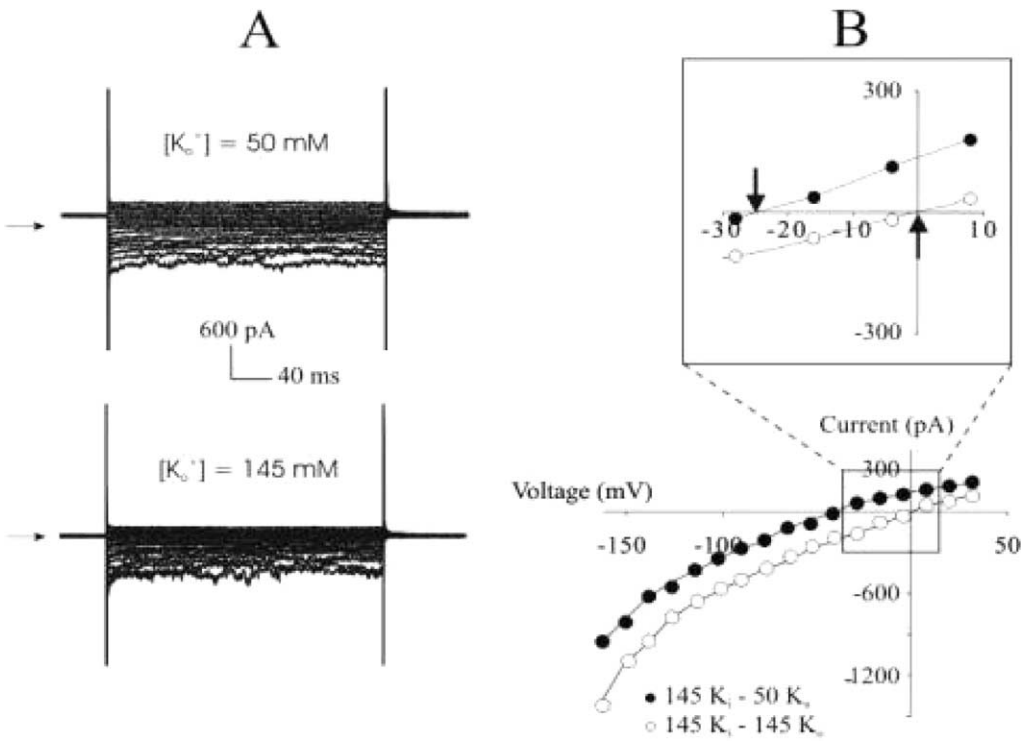

Fig. 5. GIRK5-Δ25 is a K^+ selective channel. A: Representative currents from Sf21 cells infected with the recombinant baculovirus containing the cDNA for GIRK5-Δ25 under two different extracellular K^+ concentrations. The cell was exposed to 145 mM KCl and the bath solution was replaced later with 50 mM KCl (+95 mM NaCl). Pipette solution contained 145 mM KCl and 100 μM GTPγS. Horizontal arrows indicate the zero current level. B: current-voltage relationships obtained from the cells shown in A. The inset is a magnification to illustrate the shift in the reversal potential indicated by the vertical arrows. The voltage protocol was similar to the one used in Fig. 3.

expressing GIRK5-Δ25. The 10-minute delay required to obtain full current activation may represent the dilution of GTPγS into the cell and the concomitant activation of the G-protein.

Selectivity experiments were performed to determine if the GTPγS-induced inwardly rectifying current from Sf21 cells was carried by K^+ ions. Fig. 5 shows a representative experiment in which the GTPγS-induced current was studied under two extracellular K^+ concentrations (50 and 145 mM). The inwardly rectifying current showed a reversal potential near 0 mV with symmetrical 145 mM K^+. Lowering extracellular K^+ concentration to 50 mM caused a shift of the reversal potential to -25 ± 3 mV (n = 7), a value near the predicted potential for a K^+ selective electrode according to the Nernst equation (-27 mV).

Discussion

Functional properties of GIRK isoforms or splice variants have been poorly studied [14]. The GIRK5 gene has three in-frame ATGs which could result in three GIRK5 proteins with variable N- terminal

lengths [10]. It has not been explored yet which of these isoforms are translated in vivo. However, oocytes overexpressing GIRK5 in the absence of GIRK1 do not show Ach-stimulated currents [10]. We have shown here that neither the full-length GIRK5 and the GIRK5Δ5 do not express functional channels whereas GIRK5-Δ25 produces constitutively active channels in the oocytes. Basal activities of homomultimers of a GIRK2 isoform (Kir3.2d) are very low in oocytes [20]. Interestingly, some authors may observe high basal activities of GIRK4 homomultimers in oocytes [21], while others only after its transfection in CHO cells [22].

Apparently, insect cells do not have free Gβγ since GIRK5-Δ25 does not display basal activity in this system. We added GTPγS in the patch-pipette to dissociate the Gβγ complex from Giα to produce inwardly rectifying K^+ currents. Therefore, GIRK5-Δ25 forms functional homotetramers in these cells, supporting the previously published observations using oocytes overexpressing this novel GIRK5 isoform [11].

The remarkably high basal activity of the GIRK5-Δ25 isoform suggest a possible role of the N-terminal domain (residues 1–25) in the channel modulation. Chimeras of the inwardly rectifying Kir2.1, Kir2.3 and Kir3.2 channels have shown that the N-terminal end (residues 1-49) of Kir2 is a crucial structural element for heteromultimeric subunit assembly between Kir2.1 and Kir2.3 channels [23]. The N-terminal domain (T1) controls heteromultimeric subunit assembly in Kv1.2 potassium channels [24].

Ongoing experiments may help to elucidate the role of the first 25 amino acids in the functional homotetramerization and the high basal activity of the GIRK5-Δ25 isoform. The insect cell baculovirus expression system may provide a good model to explore structural determinants of multimeric GIRK channel assembly.

Acknowledgements

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