Functional properties of a truncated recombinant GIRK5 potassium channel

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Abstract

Xenopus laevis oocytes codify a G-protein-activated inward rectifier potassium channel (GIRK5 or Kir3.5). Coinjection of other GIRKs, the muscarinic m2 receptor, or Gβγ protein cRNAs is required to observe functional GIRKx-GIRK5 heteromultimers in oocytes. Studies with GIRK2 isoforms have shown that the size of the amino or carboxyl terminus plays a crucial role on giving functional K+ channels. In this work we studied the properties of a GIRK5 with 25 amino acids deleted toward its amino-terminal domain. Injection of GIRK5-v25 cRNA alone displayed large basal and transient inward rectifying currents in oocytes. The instantaneous currents reached a stationary level after a long duration voltage pulse (10 s). For this relaxation, fast (τ1) and slow (τ2) time constants were estimated at different voltages. Recovery from inactivation followed a monoexponential function (τ = 0.95 ± 0.07 s). By contrast with other inward rectifier channels, blockade of GIRK5-v25 by extracellular Ba2+ was voltage-independent (KD = 102 ± 2 μM), suggesting the presence of a Ba2+ site at the external channel vestibule. To confirm this hypothesis, the Ba2+ sensitivity of two charged mutants GIRK5-v25(N129E) and GIRK5-v25(K157E) at each of the external loops was determined. GIRK5-v25(N129E) and GIRK5-v25(K157E) showed a 100-fold and 2-fold higher affinity to Ba2+, respectively, supporting the existence of this Ba2+ binding site. © 2001 Published by Elsevier Science B.V.

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1. Introduction

The G-protein-activated inwardly rectifying K+ channels (GIRK or Kir3 subfamily) are critical mediators of cell excitability and effectors of many G-protein-coupled receptors [1]. For example, acetylcholine binds to cardiac muscarinic receptors (m2) and then activates IKACH, slowing heart rate. This atrial channel exists as a heteromultimer composed of two GIRK1 and two GIRK4 subunits [2]. So far the GIRK conducting K+ channels have been observed as an aggregation of two different GIRK subunits. GIRK2 and GIRK3 are found in the brain where they possibly form heteromeric channels with GIRK1. Coexpression of GIRK1 and GIRK2 produces functional channels in Xenopus oocytes like the
native G-protein-coupled inward rectifiers found in brain [3]. In contrast, some GIRK2 isoforms and GIRK4 alone also form functional Gβγ-gated homomultimers in various heterologous expression systems [2–5]. The mutants GIRK1(F137S) and GIRK4(S143T) form homomeric functional channels with activity comparable to the wild-type heteromeric G-protein-gated channels [6].

Alternative splicing at the 3′-end of the open reading frame of GIRK3.2 cRNA generates the four isoforms GIRK3.2a, GIRK3.2b, GIRK3.2c and GIRK3.2d [7–9]. GIRK3.2d is 18 amino acids shorter at the N-terminal end than of Kir3.2c and can form functional channels. In contrast, Kir3.2c can only form functional GIRK channels with GIRK3.2a or GIRK3.1 [9]. It has been stated that both the N- and C-terminal ends of Kir3.2 isoforms play complex roles in the regulation of channel activity in GIRK channels composed of homomeric assemblies of GIRK3.2 isoforms [9].

The GIRK5 (Kir3.5) has been cloned from Xenopus laevis oocytes, the most widely used expression system [10]. Oocytes overexpressing GIRK5 in the absence of GIRK1 show very small Ach-stimulated currents [10]. The GIRK5 gene has three in-frame ATGs which could give rise to three GIRK5 proteins with different N-terminal sizes [10]. Whether all or some of these GIRK5 isoforms are synthesized in vivo is still an open question.

A small endogenous inward rectifying K⁺ current (Xir) can be recorded in some Xenopus oocytes [11,12]. Therefore, we suspected that Xir could comprise an assembly of GIRK5 isoforms. To establish the functionality of likely GIRK5 isoforms derived from oocytes, we isolated by RT-PCR a GIRK5 homologue that was 25 amino acids shorter at its N-terminal end (GIRK5-v25). Oocytes injected with cRNA for GIRK5-v25 expressed large basal currents in the absence of coexpressed muscarinic receptor (i.e. m2) or G-protein subunits.

GIRK5-Δ25 channels displayed an instantaneous component with a prolonged inactivation profile (10 s) at high K⁺ without Na⁺. GIRK5-Δ25 channels were also blocked by extracellular Ba²⁺ in a voltage-independent manner. In order to study the GIRK5-Δ25 Ba²⁺ binding site, we focused on the possible electrostatic interaction between Ba²⁺ and two amino acid residues at the external loops of GIRK5-Δ25. Therefore, we mutated to the negatively charged residue glutamate (E) the amino acids N129 and K157. We obtained a decrease by two orders of magnitude in the $K_d$ for Ba²⁺ block with the GIRK5-Δ25(N129E) mutant. By contrast, GIRK5-Δ25(K157E) had only a slight change in its $K_d$. These results suggest that the amino acid residue position in the vestibule of a Kir channel is crucial in the electrostatic interaction with Ba²⁺.

Preliminary results of this study were presented in abstract format [12].

2. Materials and methods

2.1. Molecular biology

Total RNA was obtained from 0.8 g of defolliculated Xenopus oocytes following the cesium chloride method [13]. RT-PCR experiments were performed to synthesize cDNA from 5 μg total RNA (M-MLV reverse transcriptase, Gibco). Two primers were designed based on the amino acid sequence homology with GIRK4 and the GIRK5 clone previously isolated from X. laevis oocytes [10]. The nucleotide sequences for these primers (Gibco) were: 5′-CAT CGT CGA CAT GGC AAG GGA TTT AAG GGT CTC TAT G-3′ (sense) and 5′-GAA ATG TAT CAA TGT TTT TCT GCA GTC AGT CTG GCT GTG-3′ (antisense). A 1214 bp fragment was amplified by PCR either with the Taq polymerase (Gibco) or the High Expand enzyme (Boehringer Mannheim). The same result was obtained with the RNA from other frogs (n = 3). The fragment was purified from an agarose gel (1%) by the Gene Clean method and subcloned in the pRSSP6013A3-UWE vector, pBF. DH5α cells were transformed. The full GIRK5 codifying region was amplified by PCR with the primers 5′-AAA GGT CGA CAT GAT TCC TGT ATC AAA G-3′ (antisense) and 5′-AGA GAC CAA AAA GAG ACG ATC GTC GCC TGT ATC AAA G-3′ (antisense). The fragment (300 bp) and the PBF-GIRK5-Δ25 construct were incubated with SalI and XhoI. Ligation was performed with T4 DNA Ligase (Gibco). Purified DNAs (Maxi-prep, Qiagen) were sequenced automatically (ABI Prism 310, Perkin Elmer) and manually (thermo-sequenase, Amersham Life Science).
5–20 ng of GIRK5 wild-type and GIRK5-Δ25 cRNA transcribed in vitro (RNA polymerase SP6, Gibco) were injected into oocytes for electrophysiological assays. X. laevis females were purchased from Carolina Biological Supply Company, USA. Defolliculated oocytes were obtained according to [14].
2.2. Electrophysiological assays

Recordings were made 1–3 days after cRNA injection, with a two-microelectrode voltage-clamp technique (Geneclamp 500, Axon Instruments). Borosilicate microelectrodes were filled with 3 M KCl and had resistances of 0.5–5 MΩ. Membrane potential and current signals were digitized at a rate of 10 kHz with a 12-bit AD/DA converter (TL-1 Labmaster, Axon Instruments) and filtered through a four-pole low-pass Bessel filter at a frequency of 2–5 kHz. Membrane potential and current signals were stored and analyzed off-line in a Pentium PC with pCLAMP v. 6.0, Microsoft Excel v. 2000 and SigmaPlot, v. 2.0. Pooled data were expressed as mean ± S.E. Unless otherwise indicated, current

Fig. 2. I-V relationship of GIRK5-Δ25. (A) The current-voltage relationships are shown for the instantaneous current (left) and for the current at the end of the pulse (270 ms, right) at different external K⁺ concentrations (118 (○), 75 (▲), 50 (■) and 20 (●) mM). The instantaneous component of the current was the value measured when the slope of capacitive current changed. This figure illustrates the substantial decay of GIRK5-Δ25 currents with time. Long pulses (>11 s), were required in order to reach the steady-state current level. (B) The protocol that was applied to subtract the leak current is shown at the top. The inward currents were measured with a long pulse (13 s) at −140 mV to inactivate completely the GIRK5-Δ25 channels. Then, several current traces were registered from −150 to −110 mV with 10 mV steps to develop only leak currents. The linear part of these traces allowed an estimate of the membrane resistance; the leak current was then subtracted off-line from the experimental data.
traces are shown after subtraction of the linear components. The leak component was subtracted off-line with a protocol applied after a long pulse to obtain only leak current (Fig. 2A, see legend for details). All experiments were carried out at room temperature (22–24°C). Oocytes were placed in a 1 ml chamber and perfused with solution. Electrode impalement and measurement of oocyte passive properties were

Fig. 3. Selectivity and permeation properties of GIRK5-A25. (Top) $E_k$ as a function of $[K^+]_o$. Continuous line represents the linear regression fit to the experimental data; the fitted slope was $50.0 \pm 2.9$ mV. Dotted line indicates the Nernst equation prediction with a slope of 58 mV for a $K^+$ selective electrode. (Bottom) The slope conductance as a function of $[K^+]_o$. The linear regression fit (continuous line) had a slope of 0.586.
performed in normal Ringer solution (mM): NaCl 117, KCl 2.5, CaCl₂ 1.8, HEPES 5, pH 7.4. For selectivity experiments, Na⁺ was replaced with variable K⁺ concentrations (2.5, 20, 50, 75, and 118 mM). Functional expression of GIRK5 and GIRK5-Δ25 was studied in high K⁺ (118 mM).

3. Results

3.1. Isolation of GIRK5-Δ25 from X. laevis oocytes

Since the GIRK5 gene had been already cloned [10], the easiest step to obtain a truncated variant was by RT-PCR. To overcome the intrinsic problems of this technique, we worked with three independent total RNA samples derived from different frogs. In our study primers were designed to amplify the coding sequence from the third start codon of the GIRK5 cDNA previously reported [10]. We obtained the GIRK5 cDNA and GIRK5 with 25 amino acids deleted at the N-terminal domain, GIRK5-Δ25. We sequenced the three clones isolated from different frogs. Based on the same oligonucleotide sequence of these samples, using either the Taq polymerase or the more reliable High Expand enzyme, we concluded that no artifactual variants were amplified with this procedure.

In comparison with the GIRK5 channel previously cloned [10], the GIRK5-Δ25 isolated in this work showed an arginine at position 429 instead of a leucine and a lack of a glycine at position 430 at the end of the intracellular carboxyl domain. One clone out of three had a threonine at position 190 instead of an isoleucine in the M2 domain. These results suggest that the GIRK5 gene displays polymorphism.

3.2. Functional expression in oocytes

Variable amounts of GIRK5-Δ25 cRNA (5-20 ng) were injected into oocytes, yielding an approximately linear relationship between the amount of cRNA injected and the current observed (not shown). Inward inactivating currents were recorded in high K⁺ (118 mM; Fig. 1) at the second day with an injection of 10 ng of GIRK5-Δ25 cRNA. Oocytes expressing inward potassium currents higher than 5 nA were used for recording. GIRK5-Δ25 homomultimers showed an instantaneous current that decayed slowly to a steady state and increased in amplitude with the hyperpolarizing pulse.

As more GIRK5-Δ25 cRNA was injected more current was measured. This also happened with the recording time (3-4 days after injection). We have also injected the GIRK5 wild-type cRNA (i.e. the full gene) into the oocytes, and inward currents were not observed under the same conditions (not shown).

3.3. K⁺-dependent activation and permeation properties of GIRK5-Δ25 homomultimers

I-V relationships for the instantaneous currents and for the currents at the end of the 10 s pulse were estimated for GIRK5-Δ25 channels in variable [K⁺]₀ (Fig. 2). Whole-cell currents were recorded in 2.5, 20, 75 and 118 mM [K⁺]₀. Currents were elicited

Table 1

Comparison of the time constants for the fast (τ₁) and the slow (τ₂) components of GIRK5-Δ25 and mutants

<table>
<thead>
<tr>
<th>E_m (mV)</th>
<th>GIRK5-Δ25</th>
<th>GIRK5-Δ25(N129E)</th>
<th>GIRK5-Δ25(K157E)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>τ₁ (ms)</td>
<td>τ₂ (ms)</td>
<td>τ₁ (ms)</td>
</tr>
<tr>
<td>−160</td>
<td>55.07 ± 0.36</td>
<td>465.91 ± 19.6</td>
<td>50.06 ± 1.15</td>
</tr>
<tr>
<td>−145</td>
<td>60.04 ± 0.76</td>
<td>476.04 ± 16.9</td>
<td>70.88 ± 0.23</td>
</tr>
<tr>
<td>−130</td>
<td>69.33 ± 0.84</td>
<td>502.54 ± 21.1</td>
<td>78.44 ± 0.45</td>
</tr>
<tr>
<td>−115</td>
<td>74.31 ± 1.49</td>
<td>509.19 ± 33.6</td>
<td>77.71 ± 1.12</td>
</tr>
<tr>
<td>−100</td>
<td>82.40 ± 3.45</td>
<td>529.58 ± 39.8</td>
<td>95.67 ± 7.56</td>
</tr>
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</table>

The relaxation was measured in 118 mM [K⁺]₀ (n = 3; mean ± S.E.).
from a holding potential (HP) of 0 mV, with the pulse protocol shown at the top of Fig. 1. Off-line subtraction of leak currents was performed with the protocol shown in Fig. 2B (for details see figure legend). Like other inward rectifier potassium channels, the current amplitude of GIRK5-\Delta25 channels was dependent on the [K$^+$]_o at all voltages and the I-V curves were shifted with respect to [K$^+$]_o.

The zero current potential was calculated for each I-V curve at all [K$^+$]_o (n = 3) and it was compared with the value predicted by the Nernst equation (Fig. 3, top). The experimental points were very close to the ones estimated except for concentrations lower than 20 mM. Endogenous Na$^+$ and Cl$^-$ currents may become apparent in the oocytes at low [K$^+$]_o.

Fig. 4. Steady-state inactivation of the inward rectifying K$^+$ current. (A) Traces show unsubtracted membrane currents elicited with the pulse protocol shown at the top, in 118 mM [K$^+$]_o. (B) The steady-state inactivation induced by a 10 s prepulse was evaluated measuring the instantaneous current at the test pulse (−150 mV) and plotted as a function of the prepulse potential (see text for more details).
The logarithmic scale had a slope value of 50.0 ± 2.9 mV, close to the 58 mV/decade change in $[K^+]_o$ for a $K^+$ selective electrode (dotted line), indicating that GIRK5-Δ25 channels were mainly selective for potassium ions.

Fig. 3 (bottom) shows the conductance dependence of GIRK5-Δ25 channels on $[K^+]_o$. The slope conductance was determined by fitting the linear part (between −150 and −70 mV) of the instantaneous I-V curve at the different $[K^+]_o$ and plotted in a double logarithmic scale. This relationship was well described by the power function $g_{\text{slope}} = A \left([K^+]_o\right)^{0.386}$, where
A = 0.36 ± 0.13 nS, \([K^+]_o\) is in mM and 0.586 is the slope of the linear regression line. This value was close to 0.5, indicating that the conductance was approximately proportional to the square root of \([K^+]_o\), which is typical of an inward rectifier potassium channel [15].

3.4. Voltage-dependent inactivation

In general, the small decay of Kir currents before they reach the steady state has been attributed to a combination of extracellular K⁺ depletion, external Na⁺ blockade, and a voltage-dependent inactivation [15–17].
GIRK5-Δ25, GIRK5-Δ25(K157E) and GIRK5-Δ25(N129E) currents showed a prominent decay at test potentials negative to −50 mV even at high [K⁺]o with nominally zero [Na⁺]o (Table 1). We studied this process with a standard inactivation protocol of two pulses shown in Fig. 4A. Prepulses to selected membrane potentials between −150 and 0 mV from a HP of 0 mV were applied during 10 s

Fig. 7. Sensitivity and voltage independence of GIRK5-Δ25 and the charge mutants GIRK5-Δ25(K157E) and GIRK5-Δ25(N129E) to Ba²⁺ block. Effect of Ba²⁺ on the instantaneous (A) and the late current at 750 ms (B) of GIRK5-Δ25, GIRK5-Δ25(N129E) and GIRK5-Δ25(K157E) (empty, gray and filled symbols, respectively) at two voltages, −160 mV (squares) and −80 mV (circles). Recordings were performed in 118 mM of [K⁺]o at several Ba²⁺ concentrations. The pulse protocol was the same as in Fig. 1. Mean ± S.E. values of unblocked current fraction (I/Icontrol) were plotted at −160 and −80 mV (n=5) as a function of Ba²⁺ concentration.
to allow the current to reach the steady state (I_{ss}). The peak current elicited at the test pulse of −150 mV and after subtracting I_{ss} was normalized to the maximal current observed without prepulse (I/I_{max}), to determine the steady-state inactivation. A sigmoidal relationship was observed, revealing the fraction of channels that was not inactivated at the different prepulse potentials (Fig. 4B). Complete inactivation was observed when prepulses were more negative than −3150 mV during 10 s test pulses. These recordings were performed at high [K+]o without Na+.

The recovery time course at 0 mV from the inactivation of GIRK5-Δ25 at −160 mV (Fig. 5A) was determined using a standard two-pulse protocol (upper part). The inactivating prepulse to −160 mV was followed by increasing recovery periods at 0 mV preceding the −160 mV test pulse. The instantaneous current at the onset of the test pulse was compared and normalized with the current obtained at the pre-pulse. These data were plotted as a function of time (Fig. 5B). The time course of the recovery at 0 mV could be fitted by a monoexponential function with a time constant of 0.95 ± 0.07 s (n = 4). In other Kir channels this recovery also follows a monoexponential time course [18].

3.5. Sensitivity to Ba^{2+} blockade

The high sensitivity to barium blockade is one pharmacological property of Kir channels and K_d values between 10 and 500 μM have been reported [14,19,24]. Families of GIRK5-Δ25, GIRK5-Δ25(K157E) and GIRK5-Δ25(N129E) current traces were recorded in 118 mM of [K+]o at variable concentrations of external Ba^{2+} (Fig. 6). The current amplitude at the onset and at the end of the pulse became smaller with increasing [Ba^{2+}]o (Fig. 6). This blockade could be readily reversed by removal of Ba^{2+} from the external solution (not shown).

In several Kir channels, Ba^{2+} blocks the steady-state current rather than the peak current, and this blockade is a voltage-dependent process [15,20,25]. In GIRK5-Δ25 and GIRK5 mutants, Ba^{2+} affected more importantly the instantaneous current amplitude rather than the current at the end of the pulse (‘late current’) (Figs. 6 and 7, Table 2).

<table>
<thead>
<tr>
<th></th>
<th>−160 mV (μM)</th>
<th>−80 mV (μM)</th>
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<tbody>
<tr>
<td>I_{inst}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIRK5-Δ25</td>
<td>111.35 ± 7.13</td>
<td>107.62 ± 6.16</td>
</tr>
<tr>
<td>GIRK5-Δ25(K157E)</td>
<td>43.83 ± 3.74</td>
<td>39.32 ± 4.0</td>
</tr>
<tr>
<td>GIRK5-Δ25(N129E)</td>
<td>1.74 ± 0.77</td>
<td>2.01 ± 1.36</td>
</tr>
<tr>
<td>I_{late}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIRK5-Δ25</td>
<td>188.45 ± 12.78</td>
<td>330.23 ± 26.45</td>
</tr>
<tr>
<td>GIRK5-Δ25(K157E)</td>
<td>95.75 ± 3.40</td>
<td>119.06 ± 6.01</td>
</tr>
<tr>
<td>GIRK5-Δ25(N129E)</td>
<td>5.65 ± 1.95</td>
<td>7.20 ± 1.92</td>
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K_d values (μM) were estimated at −160 and −80 mV for the instantaneous (I_{inst}) and late currents (750 ms) from Fig. 7 (mean ± S.E.).
positive amino acid residue lysine K157 in the second external loop of GIRK5-Δ25 to glutamate (E).

Current-voltage relationships were determined in 118 mM K\(^+\) containing Ba\(^{2+}\) from 0.001 to 20 mM (n = 5; Fig. 7). The blocked fractions (I/I\(_{\text{max}}\)) for the instantaneous and late currents were measured at all voltages. GIRK5-Δ25 showed a \(K_d = 102 \pm 2\) μM and a \(K_d = 227 \pm 9\) μM for Ba\(^{2+}\) blockade at the instantaneous and late current, respectively.

We obtained a change of about two orders of magnitude in the \(K_d\) for Ba\(^{2+}\) with the GIRK5-Δ25(N129E) mutant (\(K_d = 1.8 \pm 0.5\) μM). By contrast, the GIRK5-Δ25(K157E) mutant was only 2-fold more sensitive to Ba\(^{2+}\) blockade (\(K_d = 41.7 \pm 3.5\); Fig. 7, Table 2). Therefore, the amino acid residue position in the vestibule of a Kir channel is crucial in a possible electrostatic interaction with Ba\(^{2+}\).

Table 2 shows the \(K_d\) values for instantaneous and steady-state (late) currents, confirming the voltage independence of Ba\(^{2+}\) blockade for GIRK5-Δ25 and the charge mutant channels (Fig. 7).

4. Discussion

An outstanding result of this work is that the GIRK5 gene with a shorter N-terminal end, the GIRK5-Δ25, gives rise to functional channels, in contrast with the lack of functionality observed with the GIRK5 cRNA injected alone or with m2 and Gβγ cRNAs in \(X.\ laevis\) oocytes [10]. This result is in accordance with the functionality also observed with the GIRK3.2d with a shorter N-terminal end [9].

The high basal activity of GIRK5-Δ25 channels in the oocytes is intriguing. GIRK1/GIRK4 heteromultimers and some GIRK2 isoforms express significant basal K\(^+\) currents without receptor stimulation or G-protein activation [6,9,22]. This activity has been related to the direct action of intracellular sodium ions, requiring ATP hydrolysis. The ATP dependence of GIRK channel activity is mediated via phosphoinositid diphosphates (PIP\(_2\)). The presence of PIP\(_2\) is essential not only for the channel gating by sodium ions but also for efficient Gβγ signaling [21,22]. Since channels work efficiently even at high expression levels in the oocytes, the endogenous pool of any of these gating molecules does not appear to be a limiting factor for GIRK5-Δ25 functionality. We have also observed functional GIRK5-Δ25 homomultimers in an insect line but GTP-γS is required to allow for channel activity (not shown).

Inward rectifier potassium channels display only a small inactivation [15]. The inactivation of GIRK5-Δ25 currents had a slow time course. So far two inactivation mechanisms have been described for voltage-dependent potassium channels: a fast process referred as ‘the ball and chain’ (N-type [23]) and a slow decaying current event that strongly depends on the channel pore structure (C-type [24]). Further studies should be performed to investigate the GIRK5-Δ25 inactivation.

Ba\(^{2+}\) blockade of the GIRK5-Δ25 instantaneous and late currents was voltage-independent (Fig. 7, Table 2), implying the existence of an external binding site. In other Kir channels, extracellular Ba\(^{2+}\) enhances dramatically the rate of current decay by entering and blocking their pores [14,16,24]. The presence of a Ba\(^{2+}\) binding site located outside the pore of the GIRK5-Δ25 channels was tentatively confirmed by the higher affinity determined for the two charge mutants: GIRK5-Δ25(N129E) and GIRK5-Δ25(K157E). Furthermore, the 100-fold higher sensitivity of mutant GIRK5-Δ25(N129E) for Ba\(^{2+}\) suggests that the first channel external loop contributes to the binding site for this blocker.

Important conclusions are derived from this work. First, GIRK5 channels lacking the first 25 amino acids at the N-terminal end produce functional homomultimers with an appreciable basal activity suggesting a high sensitivity of this truncated channel to the endogenous activators (i.e. Gβγ proteins, Na\(^+\), Mg-ATP and PIP\(_2\)). Second, in contrast to other potassium channels, GIRK5-Δ25 homomultimers present a prolonged inactivation and a voltage-independent Ba\(^{2+}\) blockade. The higher Ba\(^{2+}\) affinity of the mutant charge GIRK5-Δ25(N129E) at the first loop of the channel vestibule supports the presence of an external Ba\(^{2+}\) site in GIRK5 channels.

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