Exaggerated Mg\(^{2+}\) Inhibition of Kir2.1 as a Consequence of Reduced PIP\(_2\) Sensitivity in Andersen Syndrome

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ABSTRACT

Andersen syndrome is an autosomal dominant disorder characterized by cardiac arrhythmias, periodic paralysis and dysmorphic features. Many Andersen syndrome cases have been associated with loss-of-function mutations in the inward rectifier K\(^+\) channel Kir2.1 encoded by KCNJ2. Using engineered concatenated tetrameric channels we determined the mechanism for dominant loss-of-function associated with a trafficking-competent missense mutation, Kir2.1-T74A. This mutation alters a conserved threonine residue in an N-terminal domain analogous to the slide helix identified in the structure of a bacterial inward rectifier. Incorporation of a single mutant subunit in channel tetramers was sufficient to cause a selective impairment of whole-cell outward current, but no difference in the level of inward current compared with wild-type (WT) tetramers. The presence of two mutant subunits resulted in greatly reduced outward and impaired inward currents. Experiments using excised inside-out membrane patches revealed that tetramers with one mutant subunit exhibited increased Mg\(^{2+}\) inhibition. Additional experiments demonstrated that concatenated tetramers containing one T74A subunit had reduced PIP\(_2\) sensitivity, and that outward current carried by mutant tetramers could be restored by addition of PIP\(_2\) in the absence of Mg\(^{2+}\). Our results are consistent with the involvement of the Kir2.1 N-terminus in PIP\(_2\) modulation of channel activity and support the existence of an inverse relationship between PIP\(_2\) sensitivity and Mg\(^{2+}\) inhibition of Kir2.1 channels. Our data also indicate that a single mutant subunit is sufficient to explain dominant-negative behavior of Kir2.1-T74A in Andersen syndrome.

INTRODUCTION

KCNJ2 encodes Kir2.1, an inward rectifier potassium (K\(^+\)) channel expressed in brain, heart and skeletal muscle.\(^{1,2}\) Loss-of-function KCNJ2 mutations have been associated with Andersen syndrome, a genetic disorder with autosomal dominant inheritance characterized by periodic paralysis, cardiac arrhythmias, and dysmorphic features.\(^{3,4}\) Dominant-negative effects, in which the mutant KCNJ2 allele inhibits the function of the wild-type (WT) allele, occur in this disease. The involvement of Kir2.1 channels in Andersen syndrome has emphasized the physiological importance of sarcolemmal inward rectifiers in regulating resting membrane potential and action potential duration in myocytes.\(^{3}\) However, our understanding of the fundamental molecular and biophysical mechanisms responsible for Kir2.1 dysfunction and an explanation for dominant-negative mutant channel behavior in this disease are incomplete.

Kir2.1 is classified as an inward rectifier K\(^+\) channel because potassium ions are conducted more readily in the inward than outward direction. The inward rectification exhibited by this class of K\(^+\) channels is the result of voltage-dependent pore block by intracellular cations such as Mg\(^{2+}\) and polyamines (i.e., spermine, spermidine and putrescine).\(^{6-9}\) In the absence of intracellular blockers, inward rectification is almost completely eliminated, and the small amount of residual inward rectification is probably mediated by organic molecules present in electrophysiological recording solutions.\(^{10,11}\) However, some mutations confer intrinsic rectification properties to Kir2.1.\(^{12}\)

In addition to Mg\(^{2+}\) and polyamines, phospholipids are important cellular modulators of Kir2.1 and other inward rectifier K\(^+\) channels.\(^{13}\) For example, phosphatidylinositol bisphosphate (PIP\(_2\)) modulates the activity of Kir2.1 channels and this interaction appears necessary for Kir2.1 function, especially in heterologous expression systems.\(^{14}\) Several amino acid residues in the cytoplasmic carboxyl- and amino-terminal domains...
of Kir2.1 are involved in the channel’s interaction with PIP₂.¹³,¹⁵-¹⁷
Interestingly, some Kir2.1 mutations associated with Andersen syndrome, mostly located within the carboxyl-terminus, have been demonstrated to decrease PIP₂ affinity.¹⁵ These findings demonstrate that the modulation of Kir2.1 by PIP₂ is physiologically and pathophysiologically important.

We previously identified and performed limited functional characterization of an Andersen syndrome mutation (Kir2.1-T74A) that exhibits normal trafficking to the plasma membrane but is nonfunctional.¹⁸ Based on the structure of the bacterial inward rectifier KirBac1.1, the T74A mutation is located within the slide helix, a region implicated in channel gating.¹⁹ We designed experiments with the goal of understanding the mechanism for the loss-of-function phenotype exhibited by this mutation and to determine the number of mutant subunits required to cause channel dysfunction. Our data indicate that the loss of activity associated with Kir2.1-T74A is a consequence of decreased PIP₂ sensitivity and an associated exaggerated inhibitory effect of intracellular Mg²⁺. We further demonstrated that a single mutant subunit is sufficient to impair Kir2.1 function and this contributes to our understanding of the dominant inheritance of Andersen syndrome.

MATERIALS AND METHODS

Construction of concatenated Kir2.1 tetramers. Concatenated Kir2.1 tetramers were coexpressed in the bicistronic plasmid vector, pRES2-EGFP (BD Biosciences-Clontech, Mountain View, CA, USA) for heterologous expression in cultured mammalian cells. The coding regions of four subunits were joined through addition of a 5 amino acid linker with sequence EHAAA. The first residue of the linker replaced the stop codon of subunits in positions 1–3 of the tetramer, while the native stop codon of the fourth position subunit was not altered. Additional silent mutations were incorporated by site-directed mutagenesis in the N- and C-termini of each of the 4 subunits to engineer unique restriction sites enabling directional assembly. Silent mutations also allowed for the design of sequencing primers specific for each position of the tetramer. All mutations and linker additions were accomplished using recombinant PCR (primer sequences available upon request). Mutagenesis of Kir2.1-T74A was previously described.¹⁸

Whole-cell recordings. HEK-293 cells were transiently transfected with 1 μg of WT-WT-WT-WT plasmid cDNA. Forty-eight hours after transfection, either wortmannin (EMD Biosciences Inc., La Jolla, CA, USA) or LY294002 (Cayman Chemical Company, Ann Arbor, MI, USA), dissolved in DMSO were added to the tissue culture media at a final concentration of 50 μM and 100 μM respectively. After incubation for two hours with either wortmannin, LY294002 or DMSO (control), whole-cell currents were measured as described above.

Inside-out macro-patch recording. CHO-K1 cells were transiently transfected with 1 μg of WT- Kir2.1 tetramer (WT-WT-WT-WT) cDNA or cDNA from tetramers containing a Kir2.1-T74A subunit in either the first (T74A-WT-WT-WT) or last (WT-WT-T74A-WT) position. For these experiments we selected CHO-K1 cells because smaller endogenous currents facilitated the analysis of Kir2.1 currents obtained from excised patches. Forty-eight hours post transfection, macro-patches were excised into Mg²⁺/polyamine free bath solution and perfused for several minutes until rectification was eliminated as determined by intermittent monitoring of current-voltage relationships. The pipette solution contained (in mM): 140 KCl, 2 CaCl₂, 5 HEPES, pH 7.4. The Mg²⁺/polyamine free bath solution contained (in mM): 145 KCl, 5 EDTA, 7.2 K₂HPO₄, 2.8 KH₂PO₄, pH 7.4. Current rundown under these conditions was minimal and the currents were stable for several minutes. Current traces were filtered at 2 kHz and acquired at 10 kHz. Whole-cell currents were measured from -100 mV to 100 mV in 10 mV increments from a holding potential of 0 mV. Voltage-dependence of Mg²⁺ inhibition was assessed by fitting the data to a Boltzmann function to determine V₁/₂, the membrane potential for half-maximal inhibition and the slope factor.

Mg²⁺ dose response. Free Mg²⁺ concentration in solutions was calculated as described above. In order to obtain the desired free Mg²⁺ concentration, MgCl₂ was added to the bath solution and the pH was readjusted with KOH. After excision of inside-out patches and loss of rectification, the Mg²⁺ containing solution was perfused into the chamber. After application of each Mg²⁺ concentration, the patch was washed with Mg²⁺-free solution for a time sufficient to eliminate rectification (typically 2–3 min). To obtain K₅ values for Mg²⁺ inhibition, the data obtained at -60 mV were fitted with a Hill equation of the form y = I₁ / [1 + (x/K₅)ⁿ], where x refers to current, y indicates the free Mg²⁺ concentration and n is the Hill coefficient. The data obtained at 40 mV were fitted with the sum of two Hill equations, y = I₁ / [1 + (x/K₅)ⁿ] + I₂ / [1 + (x/K₆)ⁿ].²²
Single-channel analysis. CHO-K1 cells were transfected with 0.4 µg of WT-Kir2.1 tetramer (WT-WT-WT-WT) cDNA or the tetramer containing a Kir2.1-T74A subunit in the last position (WT-WT-WT-T74A). Inside-out patches were obtained as described above. Only patches with less than 6 channels were used for analysis. Pipettes were made as described before and coated with Sylgard® (Dow Corning Corp. Midland, MI, USA.). Currents were filtered at 2 kHz and acquired at 20 kHz. Amplitude histograms were generated using Clampfit 9.0.1 (Molecular Devices) and fitted with a Gaussian function to calculate the single-channel amplitude. In order to calculate $N_{Po}$, the steady state current from each macro-patch was divided by the single-channel amplitude.

**PIP2 dose response.** L-α-phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] dissolved in chloroform (Avanti Polar Lipids, Inc., Alabaster, AL, USA) was dried under a stream of N2, hydrated with Mg2+-free bath solution, and sonicated for 5 minutes (Bransonic 1510 bath sonicator, Branson, Danbury, CT, USA). For inside-out patches obtained from CHO-K1 cells expressing either WT-WT-WT-WT or WT-WT-T74A, we added 1 mM Mg2+ to the bath solution to reduce baseline channel activity ( rundown), as described before. After rundown, inside-out patches were washed with Mg2+-free bath solution to remove any traces of Mg2+. Patches were then perfused with Mg2+-free bath solutions containing PIP2 concentrations ranging from 0.03 to 30 µM. The current in the patch at -100 mV was then measured 2 minutes after exposure to each PIP2 concentration and normalized to the baseline current level measured after complete channel rundown and before addition of PIP2.

**RESULTS**

We previously demonstrated that Kir2.1-T74A channels are expressed at the plasma membrane but are not functional. In addition, when coexpressed with WT-Kir2.1, Kir2.1-T74A channels exhibited a dominant-negative suppression of WT currents. To further explore the mechanism for the loss-of-function and dominant-negative effects associated with the T74A mutation, we engineered a concatenated Kir2.1 tetramer to express channels with a fixed proportion of WT and mutant subunits. Western blot analysis of the concatenated tetramer heterologously expressed in HEK-293 cells demonstrated a band of approximately 200 kDa, four times larger than the Kir2.1 monomer (see Supplemental data, Fig. S1). Whole-cell and single-channel properties measured for the concatenated WT-Kir2.1 tetramer (WT-WT-WT-WT) were indistinguishable from channels expressed from a monomeric construct (see Supplemental data, Fig. S1).

As illustrated by Figure 1, incorporation of two T74A subunits in either terminal (T74A-WT-WT-T74A) or alternating positions (T74A-WT-T74A-WT and T74A-WT-WT-WT) in the Kir2.1 tetramer resulted in a strong reduction of Kir2.1 activity when compared to WT-WT-WT-WT. The peak inward current densities for T74A-WT-WT-T74A, T74A-WT-T74A-WT and WT-T74A-WT-T74A channels were -31.0 ± 4.0 pA/pF ($N = 7$), -32.2 ± 10.0 pA/pF ($N = 5$) and -38.5 ± 7.6 pA/pF ($N = 7$) respectively (see Supplemental data, Fig. S2). All values were approximately 15% of the current density measured in cells expressing WT-WT-WT-WT (-252.6 ± 34.7 pA/pF; $N = 7$). By contrast, tetramers with only one mutant subunit exhibited peak inward current densities that were not statistically different from WT-WT-WT-WT channels (T74A-WT-WT-WT, -207.1 ± 18.5 pA/pF; $N = 7$; WT-WT-T74A, -229.4 ± 16.1 pA/pF; $N = 6$). In
agreement with a previous report placing the mutant subunit in these different position within the tetramer did not influence the effect of the mutation.

Incorporation of a single T74A subunit nearly abolished outward currents without significantly affecting inward currents (Fig. 1B). Figure 1C illustrates that the rectification index (see legend for explanation) obtained for WT-WT-WT-T74A (34.2 ± 4.6, N = 6) and T74A-WT-WT-WT (33.8 ± 9.7, N = 7) are reduced by 67.3% and 67.6%, respectively (p < 0.001 for both), when compared to WT-WT-WT-WT (104.6 ± 8.1 mV, N = 25; NS). These observations suggest that a dominant-negative effect caused by Kir2.1-T74A requires incorporation of only one mutant subunit into the channel complex. Furthermore, because Kir2.1 outward currents are affected by the mechanism of inward rectification, we initially suspected that the T74A mutation might affect this process. An alteration of inward rectification could occur as a result of stronger pore block by polyamines or Mg2+. Alternatively, the T74A mutation could confer the apparent change in rectification by another mechanism.

Kir2.1-T74A exhibits exaggerated Mg2+ inhibition. If the T74A mutation conferred intrinsic rectification properties to Kir2.1, then we would expect mutant channels to exhibit inward rectification even in the absence of intracellular blockers. To test this hypothesis, we measured the activity of WT and mutant concatenated tetramers using excised inside-out patches. As illustrated by Figure 2, in the absence of Mg2+ and polyamines, the level of peak outward current normalized to peak inward current in patches expressing WT-WT-WT-WT or WT-WT-WT-T74A is similar. Addition of 1 mM Mg2+ to the cytoplasmic face of inside-out patches blocked outward currents from both WT and mutant tetramers restoring rectification (Fig. 2A and B). These data suggest that the mutant subunit does not confer intrinsic rectification properties to T74A-WT-WT-WT or WT-WT-WT-T74A. However, channels containing a mutant subunit exhibited an unexpected stronger inhibition of inward currents by Mg2+ (Fig. 2B) in inside-out patch recordings.

To further analyze this effect of Mg2+ on mutant channels, we compared the voltage-dependence of Mg2+ block for WT-WT-WT-WT and WT-WT-WT-T74A channels using inside-out macro-patches (Fig. 3A). Based on fits of the data with a Boltzmann function, V1/2 values for WT-WT-WT-T74A (17.4 ± 1.9 mV) and T74A-WT-WT-WT (13.5 ± 4.2 mV) were not significantly different.
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Figure 3. Inhibition of Kir2.1-T74A concatemers by Mg²⁺ and spermine. (A) Voltage dependence of Mg²⁺ block for WT-WT-WT-WT (squares, $V_{1/2} = 15.9 \pm 1.5$ mV), WT-WT-T74A (triangles, $V_{1/2} = 17.4 \pm 1.9$ mV) and T74A-WT-WT-WT (data not plotted, $V_{1/2} = 13.5 \pm 4.2$ mV). (B) Voltage dependence of spermine block for WT-WT-WT-WT (squares, $V_{1/2} = -4.1 \pm 1.6$ mV) and WT-WT-T74A (triangles, $V_{1/2} = -4.3 \pm 4.9$ mV). For every voltage, the currents recorded from inside-out macro-patches in the presence of 1 mM Mg²⁺ or 1 µM spermine were normalized to the currents recorded before application of the respective blocker. The data were fitted with a Boltzmann function to obtain $V_{1/2}$ ($N = 6–9$). Similar responses were observed for 300 µM spermine (data not shown). Slope factors were not significantly different among the three channel constructs. *, $p < 0.005$.

Figure 4. Dose response for Mg²⁺ inhibition. Levels of inhibition at different Mg²⁺ concentration are illustrated for WT-WT-WT-WT (squares) and WT-WT-T74A (triangles) at -60 mV (open symbols) and 40 mV (closed symbols). Data for WT-Kir2.1 expressed from monomeric channels are illustrated by the gray dotted line. The currents recorded in the presence of Mg²⁺ were normalized to the currents recorded before application of the corresponding Mg²⁺ concentration. Data obtained at -60 mV were fitted with the Hill equation, whereas the sum of two Hill equations was used to fit data obtained at 40 mV ($N = 4–12$). *, $p < 0.005$.

Consistent with single-site binding, and therefore, data obtained at 40 mV were fitted with the sum of two Hill functions with high and low affinity binding sites as previously described.²³ Wild-type Kir2.1 expressed from a monomeric construct exhibited Mg²⁺ sensitivity that was indistinguishable from that of WT-WT-WT-WT (gray dotted line in Fig. 3A), and there were no apparent differences in Mg²⁺ affinity at 40 mV between WT-WT-WT-WT ($K_{d1} = 7.5$ µM, $K_{d2} = 3.6$ mM, $N = 12$) and WT-WT-T74A ($K_{d1} = 6.7$ µM, $K_{d2} = 2.8$ mM, $N = 15$). By contrast, WT-WT-T74A channels have a greater affinity for Mg²⁺ at -60 mV ($K_{d} = 2.0$ mM, $N = 15$) than WT-WT-WT-WT channels ($K_{d} = 13.5$ mM, $N = 12$).

To further understand the mechanism of Mg²⁺ inhibition, we performed single-channel recordings. Figure 5A depicts WT-WT-WT-WT and WT-WT-T74A single-channel activity in the absence or presence of 1 mM Mg²⁺. Given that the current magnitude (I) in a macro-patch is determined by the following equation: $I = NP_oi$, where $N$ is the number of channels in the excised patch, $P_o$ represents the open probability, and $i$ is the single-channel amplitude, we considered which of these channel parameters most likely explains the effect of Mg²⁺. As illustrated in Figure 5B, in the absence of Mg²⁺, single-channel conductances are not significantly different between WT-WT-WT-WT ($30.5 \pm 0.9$ pS, $N = 5$) and WT-WT-WT-T74A ($29.9 \pm 2.6$ pS, $N = 6$). Furthermore, addition of 1 mM Mg²⁺ to the intracellular side of the patch did not alter the single-channel conductance for WT-WT-WT-WT ($32.7 \pm 1.3$ pS, $N = 3$) or WT-WT-WT-T74A ($31.4 \pm 1.3$ pS, $N = 5$) channels. The number of channels in an excised patch is expected to remain constant because the patch is isolated from the intracellular machinery responsible for protein trafficking. In the absence of a reduction in single-channel amplitude (Fig. 5B) and the assumed steady-state number of channels in the patch, we inferred that Mg²⁺ is acting by decreasing channel open probability. Figure 5C illustrates
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that addition of 1 mM Mg2+ appears to cause a stronger reduction of $N_p$ for WT-WT-WT-T74A compared with WT-WT-WT-WT.

Kir2.1-T74A exhibits reduced PIP2 sensitivity. An inverse relationship between Mg2+ sensitivity and PIP2 affinity has been described for several channels including Kir2.1. For example, PIP2 has been shown to modulate the effect of Mg2+ on Kir2.1, Kir2.3 and several TRP channels.24-26 Given this observed relationship between PIP2 and Mg2+ sensitivity, we hypothesized that the exaggerated Mg2+ inhibition exhibited by Kir2.1-T74A might reflect a change in PIP2 sensitivity. To test our hypothesis, we measured the ability of PIP2 to activate WT and mutant concatenated Kir2.1 channels following rundown. Figure 6 illustrates PIP2 dose responses for WT-WT-WT-WT (EC50 = 0.05 μM) and WT-WT-WT-T74A (EC50 = 1.5 μM) channels. As indicated by the higher EC50 value, WT-WT-WT-T74A channels are 30-fold less sensitive to PIP2 activation than WT-WT-WT-WT channels.

We next tested whether removal of Mg2+ and addition of PIP2 would rescue the functional defect associated with the T74A mutation under whole-cell conditions. We observed a small increase in the rectification index of WT-WT-WT-T74A following addition of 50 μM PIP2 (39.3 ± 9.2%, N = 8) or by removal of Mg2+ (65.0 ± 11.1%, N = 10) from the pipette solution (Fig. 7A).

However, addition of 50 μM PIP2 to Mg2+-free pipette solution further increased the rectification index of WT-WT-WT-T74A (87.0 ± 9.29%, N = 6) to a level that was not significantly different from WT-WT-WT-WT (109.1 ± 14.7%, N = 8) (see Supplemental data, Fig. S3A). We then tested whether increasing endogenous PIP2 levels by addition of fluoride and vanadate (FVPP solution), which inhibit lipid phosphatases14 would rescue the defect associated with the T74A mutation. FVPP solution in the presence of 1 mM Mg2+ caused no significant change in the rectification index of WT-WT-WT-T74A (76.3 ± 12.9%, N = 9) channels. However, when Mg2+-free FVPP pipette solution was used, the rectification index of WT-WT-WT-T74A channels (105.5 ± 15.8%, N = 11; p < 0.05) increased to levels indistinguishable from WT-WT-WT-WT (see Supplemental data Fig. S3B). From these data we suggest that reduced PIP2 sensitivity may unmask Mg2+ inhibition in WT-WT-WT-T74A channels.

Figure 6. Effects of PIP2 on WT-Kir2.1 and Kir2.1-T74 concatemers. Dose response for PIP2 activation of WT-WT-WT-WT (squares, EC50 = 0.05 μM) and WT-WT-WT-T74A (triangles, EC50 = 1.5 μM) channels (N = 3–5).

Figure 5. Single-channel properties of WT-Kir2.1 and Kir2.1-T74 concatemers. (A) Representative single-channel traces for WT-WT-WT-WT (top) and WT-WT-WT-T74A (bottom) recorded before and after addition of 1 mM Mg2+ to the patch. (B) Single-channel amplitude was plotted against test voltage and the data fitted to a linear function to calculate the single-channel conductance (n = 3–6). (C) The currents at -100, -80 and -60 mV obtained from inside-out macro-patches were divided by the single-channel amplitude at the corresponding voltage to calculate $N_p$. To calculate % reduction in $N_p$, the value for $N_p$ determined in the presence of 1 mM Mg2+ was divided by the value measured before application of Mg2+, then this ratio was subtracted from 1 and multiplied by 100 (n = 6-9). *, p < 0.001.
Effects of reduced PIP$_2$ on WT-Kir2.1. These findings led us to hypothesize that a reduction of endogenous PIP$_2$ levels might also increase the degree of inward rectification for WT-Kir2.1 channels. To test this hypothesis we treated cells expressing WT-WT-WT-WT channels with either 50 μM wortmannin, 100 μM LY294002 or DMSO (control). Micromolar concentrations of wortmannin or LY294002 inhibit phosphatidylinositol-4-kinase (PI 4-kinase) activity, decreasing PIP$_2$ synthesis and reducing cell PIP$_2$ levels.\textsuperscript{27,28} As shown in Figure 7B, treatment with wortmannin (54.3 ± 11.3%, \(N = 9\)) or LY294002 (42.4 ± 11.3%, \(N = 8\)) reduced the rectification index of WT-WT-WT-WT channels compared to the DMSO treated control (94.1 ± 12.4%, \(N = 10\)). By contrast, the magnitude observed that a single mutant subunit is sufficient to impair Kir2.1 function in a manner that selectively reduces current conducted by the channel at membrane voltages positive to \(E_K\). Because the terminal phase of cardiomyocyte action potential repolarization occurs within this voltage range,\textsuperscript{35} we speculate that this selective defect in outward current exhibited by mutant channels contributes directly to the cardiac phenotype in this disorder. The observation that only a single mutant subunit incorporated into a channel tetramer is sufficient to render the channel dysfunctional helps to explain the dominant-negative behavior of this Andersen syndrome mutation. In an individual heterozygous for Kir2.1-T74A where one could assume random aggregation of mutant and WT subunits of inward currents was not significantly different between wortmannin, LY294002 and DMSO treated cells (data not shown). Together, our results indicate that either a reduction in PIP$_2$ levels or a reduction in the sensitivity for PIP$_2$, increase the degree of inward rectification for Kir2.1 by a Mg$^{2+}$-dependent mechanism.

**DISCUSSION**

Mutations in inward rectifying potassium channels have been associated with a variety of inherited disorders that affect glucose homeostasis, renal potassium handling or striated muscle excitability.\textsuperscript{4,29-31} Inward rectifying K$^+$ channels are critical for setting the resting membrane potential in excitable membranes and are specifically responsible for the terminal phase of action potential repolarization in cardiac myocytes.\textsuperscript{32,33} In Andersen syndrome, mutations in Kir2.1 cause abnormal cardiomyocyte repolarization leading to increased risk of reentrant arrhythmias\textsuperscript{34,34} while the specific reasons for altered skeletal muscle excitability and dysmorphic development are less clear. Understanding the fundamental molecular mechanisms responsible for Kir2.1 channel dysfunction in Andersen syndrome is expected to guide the development of new treatment strategies.

**Mechanisms for Kir2.1-T74A dominant loss-of-function.** In this study, we determined the mechanisms responsible for loss-of-function observed for a trafficking-competent Kir2.1 mutant, T74A, associated with Andersen syndrome. We
into tetramers, only a small fraction of assembled tetramers would be composed of just WT subunits while the majority of channels would have at least one mutant subunit, have impaired function and contribute to the disease.

Our data indicated that the T74A mutation impairs Kir2.1 sensitivity to PIP$_2$ similar to other Andersen syndrome mutations, but the functional impairment caused by incorporation of a single mutant subunit is not explained entirely by reduced PIP$_2$ sensitivity as evidenced by the incomplete rescue of outward current with exogenous PIP$_2$ (Fig. 7A). We also reported evidence that tetramers with one mutant subunit have an exaggerated inhibition by intracellular Mg$^{2+}$ and that the rectification index is fully restored by the combination of exogenous PIP$_2$ and the absence of Mg$^{2+}$ (Fig. 7A). Therefore, the reduction in PIP$_2$ sensitivity exhibited by tetramers with one mutant subunit is compounded by an exaggerated channel inhibition by intracellular Mg$^{2+}$. The effect is greater when there are two mutant subunits, and we can speculate that this occurs because of a more critical reduction in PIP$_2$ sensitivity. However, the dramatically impaired function of these channels makes this hypothesis difficult to test.

Possible mechanisms to explain Mg$^{2+}$ inhibition. In addition to decreasing Kir2.1 affinity for PIP$_2$, the T74A mutation caused Kir2.1 channels to exhibit an exaggerated inhibition by intracellular Mg$^{2+}$. We can speculate on possible direct and indirect mechanisms to explain this effect. One potential explanation for the relationship between PIP$_2$ sensitivity and Mg$^{2+}$ inhibition of Kir2.1 channels could be charge screening of the lipid mediator. Masking of PIP$_2$ negative charges by Mg$^{2+}$ cations has been proposed as a mechanism for modulation of TRPM7 channel activity. Screening of PIP$_2$ charges by Mg$^{2+}$ could decrease the effective levels of PIP$_2$ available for interaction with Kir2.1. Decreased PIP$_2$ availability provides a plausible explanation for the reduced channel activity observed in the presence of Mg$^{2+}$.

Another potential indirect explanation for the relationship between PIP$_2$ sensitivity and Mg$^{2+}$ inhibition involves the activity of phospholipase-C (PLC). Activation of PLC stimulates the hydrolysis of PIP$_2$ into inositol triphosphate and diacylglycerol, reducing intracellular PIP$_2$ levels. Furthermore, receptor-mediated activation of this enzyme has been demonstrated to decrease the activity of several inward rectifier K$^+$ channels including Kir2.1. Magnesium is a known activator of PLC, and therefore, differences in Mg$^{2+}$ sensitivity between WT and T74A containing channels might reflect different sensitivities to the Mg$^{2+}$-induced PIP$_2$ hydrolysis. Similarly, a Mg$^{2+}$-dependent phosphatidylinositol phosphatase could also explain our findings.

A relationship between Mg$^{2+}$ inhibition and PIP$_2$ has also been demonstrated for TRPV5 channels. Magnesium ions inhibit TRPV5 channels by blocking the pore and through an additional mechanism that may result from conformational changes in the channel. Interestingly, reducing PIP$_2$ levels made TRPV5 channels more sensitive to Mg$^{2+}$ inhibition without affecting pore block. A similar mechanism may be mimicked by reduced PIP$_2$ sensitivity as we have observed for WT-WT-WT-T74A channels. We speculate that different gating transitions are influenced by PIP$_2$ and Mg$^{2+}$. For example, PIP$_2$ activates channels presumably by increasing the probability of transitions from a closed to open conformation ($C \rightarrow O$), and pore-block by Mg$^{2+}$ involves an independent transition to a blocked state from the open conformation ($O \rightarrow B$). In addition, Mg$^{2+}$ appears to also promote reduced channel activity by another mechanism that we propose involves an increased rate of channel closure ($O \rightarrow C$), essentially the reverse of the PIP$_2$ effect.

The relative effects of PIP$_2$ and Mg$^{2+}$ may differ among Kir channels. Chuang and colleagues reported that activation of a muscarinic acetylcholine receptor (m1-AchR), which stimulates PIP$_2$ hydrolysis, inhibited the inward rectifier K$^+$ channel Kir2.3. The effects of m1-AchR activation correlated with Mg$^{2+}$-induced Kir2.3 inactivation caused by a mechanism different from pore block, whereas WT-Kir2.1 was less sensitive to this inhibition. Modulation of Kir2.3 by m1-AchR and Mg$^{2+}$ reduced outward currents to a greater extent than inward currents, increasing the degree of rectification. Because Kir2.1 has a stronger affinity for PIP$_2$ than Kir2.3, we speculate that the different sensitivity of Kir2.1 and Kir2.3 to the m1-AchR induced Mg$^{2+}$ inhibition is a consequence of differences in PIP$_2$ sensitivity. Our results provide evidence that mutant Kir2.1 channels with reduced PIP$_2$ sensitivity (such as channels with the T74A mutation), are more sensitive to the inhibitory effects of Mg$^{2+}$. Perhaps reduced PIP$_2$ sensitivity would render Kir2.1 channels more sensitive to m1-AchR modulation as shown for Kir2.3, although this hypothesis remains to be explored.

Structure-function implications. Several amino acids have been shown to be involved in Kir2.1 interactions with PIP$_2$, and most of these residues are located within the C-terminus. Negatively charged phosphate head groups in PIP$_2$ have been proposed to enable its interaction with cationic residues in the channel protein resulting in a more stable open state. Interestingly, the T74A mutation occurs in the N-terminus, specifically in a region designated as the slide helix based on the crystal structure of the bacterial inward rectifier (KirBac1.1). Similarly, a mutation in this region, R67W, has also been shown to affect PIP$_2$ interactions and cause Andersen syndrome. Similarly, a mutation in Kir6.2 slide helix (F55L) decreases sensitivity to PIP$_2$, reduces open probability and has been associated with congenital hyperinsulinemia. Our results imply that threonine-74 within the slide helix participates in the interaction of PIP$_2$ with Kir2.1 but we did not further explore whether this occurs through a direct or allosteric mechanism.

Summary. In summary, we have elucidated the mechanism for the dominant loss-of-function associated with the Kir2.1-T74A mutation as reduced PIP$_2$ sensitivity accompanied by increased sensitivity to Mg$^{2+}$ inhibition. Our results support the idea that decreased sensitivity to PIP$_2$ is a common molecular mechanism underlying Andersen syndrome. In addition, our results indicate that incorporation of one mutant subunit into the channel complex is sufficient to impair outward current carried by Kir2.1 complexes and this contributes to our understanding of dominant-negative effects of mutants in Andersen syndrome.

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