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NADPH Binding to β -Subunit Regulates Inactivation of Voltage-Gated K⁺ Channels

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Abstract

Ancillary β -subunits regulate the voltage-dependence and the kinetics of Kv currents. The Kv β proteins bind pyridine nucleotides with high affinity but the role of cofactor binding in regulating Kv currents remains unclear. We found that recombinant rat Kv β 1.3 binds NADPH ($K_d = 1.8 \pm 0.02$ M) and NADP⁺ ($K_d = 5.5 \pm 0.9$ M). Site-specific modifications at Tyr-307 and Arg-316 decreased NADPH binding; whereas, K_d NADPH was unaffected by the R241L mutation. COS-7 cells transfected with Kv1.5 cDNA displayed non-inactivating currents. Co-transfection with Kv β 1.3 accelerated Kv activation and inactivation and induced a hyperpolarizing shift in voltage-dependence of activation. Kv β -mediated inactivation of Kv currents was prevented by the Y307F and R316E mutations but not by the R241L substitution. Additionally, the R316E mutation weakened Kv α - β interaction. Inactivation of Kv currents by Kv β :R316E was restored when excess NADPH was included in the patch pipette. These observations suggest that NADPH binding is essential for optimal interaction between Kv α and β subunits and for Kv β -induced inactivation of Kv currents.

Introduction

The activity of the voltage-gated potassium (Kv) channels generates the membrane potential of excitable cells. These channels modulate the frequency of the action potential and they regulate cardiac pacemaking and neurotransmitter release [1;2]. In non-excitable cells Kv channels participate in volume regulation and secretion [2]. A characteristic feature of these channels is their sensitivity to changes in oxygen concentration and cell metabolism. In small resistance pulmonary arterioles, Kv channels are inactivated by hypoxia, which causes membrane depolarization and calcium entry leading to pulmonary vasoconstriction [3–5]. Although, the mechanisms by which Kv channels sense changes in oxygen and metabolism remain unclear, studies suggest that this sensitivity could in part be attributed to the presence of auxiliary β -subunits which associate with native Kv channels [1;6;7].

The β -subunits of the Kv channels (Kv β 1 – 4) are cytosolic proteins that bind to the cytoplasmic T1 domain of the membrane spanning Kv1 and Kv4 proteins [6;7]. Co-assembly with Kv β promotes surface expression of Kv heteromeric complexes, and facilitates intracellular trafficking of Kv1 channel complexes [6;7]. The Kv β proteins introduce inactivation into non-inactivating currents, e.g., those due to Kv1.1 and 1.5, and they accelerate inactivation of self-inactivating Kv currents such as those generated by Kv1.4 [8]. On the basis of amino acid sequence analogy, the Kv β proteins have been assigned to the aldo-keto reductase (AKR)

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superfamily [9]. We had reported previously that Kv β 2 binds NADPH with high affinity [10], suggesting that NADPH may be its intrinsic ligand. In addition, we found that NAD(P)H supports, while NAD(P)⁺ abolishes Kv β 1.3-mediated Kv1.5 inactivation [11]. These observations indicate that Kv currents could be differentially regulated by oxidized and reduced pyridine coenzymes and that this might represent a unique mechanism for sensing changes in oxidative metabolism. Nevertheless, it remains unclear whether pyridine nucleotide binding to Kv β active site affects Kv currents directly or if coenzyme binding is even required for β -mediated changes in the kinetics and the voltage-dependence and inactivation of Kv channels. To test this, we generated site-directed mutants of Kv β and we examined how changes in NADPH binding affinity affect the inactivating properties of Kv β . Our results show that decreasing NADPH binding diminishes the ability of Kv β to inactivate Kv α currents. Regulation of Kv inactivation by NADPH binding to Kv β may be an important aspect of the pyridine nucleotide sensing ability of the α - β couple.

Materials and Methods

Expression and Purification of Kv β 1.3

The full-length cDNAs encoding rat Kv α 1.5 in a pNKS2 vector and human Kv β 1.3 in pBluescript (KS+) vector were kindly provided by Dr. M.M. Tamkun, Colorado State University, Fort Collins, CO. Site-directed mutants were generated by using QuikChange mutagenesis kit as described earlier [10]. The mutations were confirmed by DNA sequencing. The wild-type (WT) and mutant Kv β 1.3 were expressed in *E. Coli* (BL-21) with N-terminal His Tag using pET-28 system (Novagen) and purified over Ni-affinity columns [10].

Transfection of COS-7 cells

For transient transfection, full length cDNAs for Kv α 1.5, Kv β 1.3, and corresponding mutant was inserted into NotI/BamHI sites of pIRES-hrGFP-1a vector (Stratagene) containing green fluorescence protein (GFP) as a reporter gene. The constructs were confirmed by DNA sequencing analysis and were transfected into COS-7 cells using Lipofectamine reagent (Invitrogen).

Coenzyme binding studies

Binding of pyridine nucleotides to Kv β was determined by fluorescence titration [10]. Protein fluorescence was measured before and after the addition of pyridine nucleotides using an excitation wavelength of 280 nm and an emission wavelength of 340 nm. The absorbance correction used in the curve-fitting procedure was verified by titrating solutions of tryptophan (of equal absorbance as the protein) with NADPH or NADP⁺.

Patch clamp studies

Whole-cell currents were recorded as reported previously [11]. The COS-7 cells were placed in a 2.5 ml recording chamber and superfused with normal Tyrode's solution containing (in mM): NaCl 135, MgCl₂ 1.1, CaCl₂ 1.8, KCl 5.4, HEPES 10, glucose 10, pH 7.4 adjusted with NaOH at room temperature (21 to 23° C). Voltage-clamp experiments were performed in the whole-cell configuration of the patch-clamp method by use of an Axopatch-200 patch-clamp amplifier (Axon Instruments, Foster City, CA), using fire-polished borosilicate glass pipettes with resistance of 2–4 M Ω when filled with the normal pipette solution containing (in mM): K-aspartate 100, KCl 30, MgCl₂ 1, HEPES 5, EGTA 5, Mg-ATP 5, Na₂-creatine phosphate 5, pH adjusted to 7.2 with KOH [11];[12].

Immunolocalization

The expression of Kv β 1.3 protein was examined using a polyclonal antibody (rabbit anti-human Kv β 1.3) raised against full-length Kv β 1.3. Cells were permeabilized and stained with Kv β 1.3 antibody and Texas red (TR)-conjugated anti-rabbit IgG as a secondary antibody and images were acquired using a confocal microscope [13].

Statistical Analysis

Data are reported as mean $\pm$ SEM. Data were analyzed using paired or unpaired *t*-test or ANOVA followed by Bonferroni correction for all pairwise comparisons. *P* < 0.05 was considered significant.

Results

NADPH binding studies

Our previous studies show that Kv β 2 binds pyridine nucleotides [10]. The Kv β 2, however, does not impart inactivation to Kv currents. Therefore, to study functional changes in inactivation, we used Kv β 1.3, which has been shown to inactivate Kv1.5 currents [11;14]. The Kv β 1.3 gene was expressed in bacteria and the recombinant protein was purified to homogeneity. Titration of dialyzed WT Kv β 1.3 with NADPH quenched the intrinsic fluorescence of the protein. Fluorescence quenching was saturated at high nucleotide concentrations. The dissociation constant calculated using a saturation isotherm indicated high affinity binding of the protein to NADPH (K_d = 1.8 M; Table 1, Fig. 2). The binding of NADPH to Kv β 1.3 appeared to be localized exclusively to the AKR domain, since deletion of the N-terminus did not result in a significant change in the affinity of the protein for NADPH.

To alter the NADPH affinity, site-specific mutants were prepared (Fig. 1). Because the crystal structure of Kv β 1.3 is not available, the mutants were generated based the Kv β 2 structure [15]. The AKR domains of the two proteins display >90 % homology and the NADPH binding site is conserved [7]. To study the role of NADPH binding, residues interacting with both the nicotinamide and ribose ring of the pyridine nucleotide were targeted. Arg-241 (R189 in Kv β 2) which interacts with the carbonyl of the amide side chain of the nicotinamide ring (Fig. 1) was replaced with leucine to perturb charge interaction between O₇N of NADP⁺ and the protein. To alter the binding of the ribose phosphate and the pyrophosphate backbone of NADPH with the protein Tyr-307 (Y255 in Kv β 2) were replaced with phenylalanine. Arg-316 was replaced by glutamine to disrupt the H-bond established between free OH of the adenine ring ribose and OP₄R of the 2' phosphate and Arg-264 in the Kv β 2 structure (Fig. 1). As shown in Table 1, the R241L mutation did affect NADPH binding, whereas K_d^{NADPH} was increased from 1.8 to 13 M by the Y307F mutation. The most dramatic effect was observed with the R316E substitution. With this protein, no significant quenching was observed even after the addition of 50 M NADPH, (Fig. 2A) indicating that K_d^{NADPH} may be > 50 M. The $K_d^{\text{NADP}^+}$ of WT Kv β 1.3 was 5.5M which is ~3 fold higher than its K_d^{NADPH} . The R241L mutation increased $K_d^{\text{NADP}^+}$, but no significant change in the [K_d^{NADPH}]/[$K_d^{\text{NADP}^+}$] ratio was observed, indicating that this mutation affects NADPH and NADP⁺ binding in an identical fashion. The Y307F and R316E site of Kv β 1.3 dramatically decreased NADP⁺ binding ($K_d^{\text{NADP}^+}$ >25 M)

Immunolocalization

To examine how mutations at the NADPH binding site affect the cellular distribution of Kv β , confocal images of cells transfected with Kv1.5 and mutant forms of Kv β 1.3 were obtained. The Kv β proteins were identified by Texas Red linked antibodies and cell transfections were performed with GFP. When the cells were transfected with the Kv1.5 vector

alone, no immunoreactivity to TR was observed, whereas GFP was expressed diffusely in the cytosol. In cells transfected with Kv β 1.3 vector alone, the distribution of the Kv β 1.3 protein was similar to that of GFP suggesting that the two proteins were distributed throughout the cytosol. When the β 1.3WT gene was co-transfected with Kv1.5, the immunoreactivity to TR was restricted to the edges of the cell, indicating that Kv1.5 sequesters Kv β 1.3 to the plasma membrane. A similar distribution pattern was observed when Kv β :R241L or Y307F was expressed with Kv1.5, indicating that these mutations do not significantly affect the interaction of Kv β with Kv1.5 (Fig. 3). However, in cells co-transfected with Kv β :R316E and Kv1.5, a more diffuse localization pattern was observed in which the Kv β protein was associated with the membrane and also seen in the cytosol, indicating that inhibition of NADPH binding (by the R316E mutation) weakens the interaction of Kv β with the membrane bound Kv proteins (Fig 3).

Patch-clamp studies

In cells transfected with Kv1.5, whole-cell outward currents showed ~4.5 % inactivation after 800 msec of depolarization (Fig. 4A; Table 2). For the same duration of depolarization, currents recorded from cells co-transfected with Kv β 1.3 showed nearly 42 % inactivation (Fig 4B; Table 2). The monophasic slow decline in Kv α current ($\tau_{\text{slow inactivation}}=2400$ msec) was converted to a biphasic decay with $\tau_{\text{fast inactivation}}=9$ and $\tau_{\text{slow inactivation}}=350$ msec. The half activation potential shifted from -5 to -17 mV indicating effective interaction between Kv α and β proteins. The half inactivation potential was -16.7 mV. These data are consistent with previous descriptions [11;14] and suggest that Kv α and β proteins co-assemble effectively in COS-7 cells and that Kv β inactivates Kv1.5 currents.

Cells transfected with Kv1.5 and Kv β :R241L displayed currents similar to those transfected with Kv1.5 and WT Kv β (Fig. 4E) with no significant change in % inactivation, even though $\tau_{\text{fast inactivation}}$ was decreased without a significant change in $\tau_{\text{slow inactivation}}$. The Y307F mutation also did not significantly affect the voltage-dependence of inactivation of Kv currents, however, the τ_{fast} was abolished and % inactivation was significantly reduced (from 42 % to 16%; Table 2), indicating that even though the interaction of Kv β with Kv was not affected, inactivation was decreased. Cells expressing Kv β :R316E and Kv1.5 (Fig. 4C) showed a complete loss of fast inactivation and a significant decrease in slow inactivation ($\tau_{\text{slow}}=800$ msec). Total inactivation was markedly reduced (from 41 to 12 %) and the half inactivation potential was shifted to depolarizing potentials from -17 to -3 mV, suggesting that the drastic decrease in K_d^{NADPH} caused by the R316E mutation not only prevents inactivation but also decreases the interaction of Kv β with Kv proteins. This is consistent with confocal immunohistochemical analysis showing a more diffuse distribution of Kv β :R316E as compared with the WT protein.

Effects of NADPH on Kv β :R316E mutant

Since the R316E mutation increased K_d^{NADPH} , the depolarizing shift in $V_{1/2}$ inactivation and the loss of inactivation due to this mutation may be related to a decrease in the affinity of the Kv β for NADPH. We reasoned that if the decrease in NADPH affinity is the sole reason for loss of inactivation, an increase in intracellular NADPH concentration should restore inactivation. To test this, whole cell currents were recorded with pipettes containing NADPH (0.3 mM) in their internal solution (Fig. 4D). Inclusion of NADPH in the patch pipette did not affect inactivation of currents recorded from cells transfected with Kv1.5 and WT Kv β 1.3 [11]. However, currents recorded from cells transfected with Kv1.5-Kv β :R316E with the NADPH-containing pipettes showed 30 % inactivation (Fig. 4D), which could be attributed to partial restoration of fast inactivation ($\tau_{\text{fast}}=92$ msec). No change in τ_{slow} was observed (Table 2). Significantly, $V_{1/2}$ of inactivation was indistinguishable for WT Kv β (-16 mV). The change in $V_{1/2}$ of activation caused by the R316E mutation was also prevented by NADPH

(Supplemental figure 1A). Addition of NADPH did not affect the current recorded with Kv β :R241L (Table 2). Taken together, these observations suggest that changes caused by the R316E mutation could be partially restored by elevating intracellular NADPH, and consequently loss of inactivation and changes in the voltage-dependence of Kv current due to R316E mutation could be related directly to a decrease in NADPH binding rather than to some other non-specific manifestation of the mutation.

Discussion

The results of this study show that NADPH binding to Kv β is a critical determinant of Kv α - β interaction as well as the inactivation of Kv currents by Kv β . While changes in α - β interactions due to mutation at the Kv β active site have been reported before [16–18] the current report is the first to link changes in K_d^{NADPH} to Kv inactivation. Measurements of K_d^{NADPH} and α - β interaction, and the rescue experiments with NADPH allowed us to unambiguously identify NADPH-dependent changes from those due to changes in α - β interactions caused by structural modification of the Kv β AKR domain. As reported for Kv β 2 [10], we found that Kv β 1.3 displays high affinity for NADPH, indicating that NADPH binding may be a general feature of Kv β -subunits and that these proteins are capable of high affinity interaction with pyridine coenzyme via their conserved AKR domains. However, in comparison with Kv β 2, the NADPH binding affinity of Kv β 1.3 was much lower, suggesting that different Kv β proteins respond to different NADPH concentrations. We speculate that this diversity may allow native Kv channels to respond to a wide range of nucleotide concentration depending upon the nature of the Kv β subunit bound to the protein.

The findings of this study support the notion that NADPH binding is required for optimal binding of Kv β to Kv1.5 and for imparting inactivation to Kv current. A permissive role of NADPH in Kv β -mediated Kv inactivation is consistent with the observation that mutations that decrease NADPH binding affinity of the protein cause a proportionate decrease in inactivation. Thus, the R241L mutation which had minimal effects on NADPH binding caused only small changes in current inactivation, whereas, the R316E substitution, which drastically reduced NADPH binding, dramatically reduced inactivation. The Y307F mutation displayed intermediate results. Results obtained with Y307F showing selective loss of fast inactivation without changes in the voltage-dependence of Kv currents suggest that NADPH binding specifically regulates the inactivation of Kv currents by Kv β (independent of its role in Kv α - β binding) by affecting the movement of the inactivating ball-and-chain inactivating peptide tethered to the N-terminus of Kv β . In addition, NADPH binding may also be controlling α - β interactions, because more a severe disruption of NADPH binding by the R316E mutation prevented inactivation as well as Kv β -mediated depolarizing shift in the voltage dependence of Kv current. This is consistent with diffuse distribution of R316E but not WT Kv β in Kv1.5-transfected cells. That both changes in the voltage-dependence of Kv current and the extent of inactivation could be rescued by NADPH provides firm evidence that these changes are specifically due to alterations in NADPH binding and not due to non-specific changes in protein folding caused by structural modification to the active site. Hence, taken together, these data support the possibility that NADPH binding is essential for optimal binding of Kv β to the T1 domain of Kv channels as well as for functional changes in Kv β conformation required to impart inactivation to Kv current.

The essential role of NADPH binding in Kv inactivation suggested by our data may be of physiological significance. Because of its high affinity binding, NADPH appears to be the intrinsic ligand of Kv β and changes in the cellular concentrations of NADPH (which imparts inactivation) and NADP⁺ (which removes inactivation) [11] may be metabolic regulators of Kv activity. Levels of NADPH/NADP⁺ are sensitive to the metabolic and redox state of the cell [19;20] and hence could entrain Kv currents to fluctuations in intermediary metabolism,

oxygen concentration or the redox poise of the cell. The values of $K_d^{\text{NADP(H)}}$ determined by the current study support the possibility that changes in cellular concentration of NADPH could affect NADPH bound to Kv β and hence Kv inactivation. For example, using the competition binding equation [10] and the values of K_d^{NADPH} and $K_d^{\text{NADP}^+}$ listed in Table 1, we estimate that basally in the perfused rabbit heart (reported NADPH=230M; NADP⁺=38 M [21]) 94% of the Kv β will be bound to NADPH. Hence changes in NADP⁺ concentration could decrease inactivation (e.g, a two-fold increase in NADP⁺ will decrease NADPH binding to 87%). Further modulation of NADPH binding to Kv β may be due to changes in NAD⁺. Although $K_d^{\text{NAD}^+}$ was not measured for Kv β 1.3, values obtained with Kv β 2 suggest that in the physiological range, NAD⁺ would decrease NADPH binding to Kv β to 75% of its unperturbed value. Thus modulation in NADP⁺ and NAD⁺ contents could affect NADPH binding to Kv β and thereby Kv inactivation. It should also be pointed out that these estimates are based on total nucleotide content. Because a large fraction of the pyridine nucleotides remains bound, it is difficult to measure the free nucleotide concentrations. Although the exact intracellular concentration of free nucleotides near Kv β and of K_d^{NADPH} (which could change upon binding to Kv channels, phosphorylation, etc) remains unknown and the basal ratio of reduced and oxidized pyridine nucleotide may vary from one cell to another, we expect that physiological changes in the ratios of reduced and oxidized pyridine nucleotides could affect the inactivation of Kv current by modulating the complement of Kv β subunits expressed in that cell. Finally, it is also possible that Kv inactivation is regulated by NADPH-NADP⁺ exchange due to the catalytic activity of the protein. In this model, reduction of an endogenous (but currently unknown) carbonyl substrate would change NADPH to NADP⁺ and therefore change inactivation per catalytic cycle [22;23]. Further studies are required to test these intriguing possibilities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Structure of NADP⁺ bound to Kvβ

The figure shows stick diagram of NADP⁺ bound to Kvβ2. The R316E, Y307F and R241L mutations are shown and numbered according to the Kvβ2 sequence. The corresponding number in the Kvβ1.3 sequence is given in the parenthesis. Also shown is the ribbon structure of the KvαT1 domain from residues 52–60 which interact with the Kvβ2 protein. The model was drawn in Rasmol using coordinates from the PDB accession number 1QRQ.

Fig 2.

Fluorimetric titration of recombinant Kvβ1.3 subunit in 0.2M potassium phosphate buffer pH7.4. The protein buffer mixture was equilibrated for 15 min at room temperature and titration was performed by addition of NADPH or NADP⁺ solution to the mixture and fluorescence quenching was monitored at excitation 290 and emission 340nm. The graph shows the normalized fluorescence vs. pyridine nucleotide concentration for Kvβ1.3 WT and mutants. The binding isotherm was fitted with equation and the dissociation rate constants (K_d) was obtained after analysis. Panel (A) shows fluorescence titration data for NADPH binding, Panel (B) shows NADP⁺ binding and Panel (C) displays NADPH or NADP⁺ binding to Kvβ:Y307F mutant.

Fig 3. Co-localization of Kv $\alpha 1.5$ with Kv β -subunit

Representative confocal images of COS-7 cells transfected either with $\alpha 1.5$ alone or $\beta 1.3$ WT alone or co-transfected with Kv $\alpha 1.5$ + Kv β WT or + β :R316E or + β :R241L or β :Y307F. Green fluorescence (GFP; upper panel) shows transfected cells and visualizes the cytosol and the dimensions of the cell. Photomicrographs in the lower panel shows cells stained with antibody against Kv $\beta 1.3$ and visualized with secondary antibody conjugated with Texas red (TR).

Fig 4. Representative recordings of outward currents from COS-7 cells expressing Kv proteins
 Whole cell currents were recorded from COS-7 cells transfected with Kvα1.5WT alone (A), Kvα1.5 + Kvβ1.3WT (B), Kvα1.5 + Kvβ1.3:R316E (C), Kvα1.5 + Kvβ1.3:R241L (E). NADPH rescues loss of Kv inactivation due to site mutation at the NADPH binding site of Kvβ:R316E. Panel (D) and (F) shows the effect of 0.3mM NADPH on currents in cells transfected with Kvβ1.3:R316E + Kvα1.5 or Kvβ1.3:R241L + Kvα1.5 respectively.

Table 1**Binding of NADPH or NADP⁺ to Kvβ1.3 and its site-directed mutants**

Recombinant full-length and N (1–73) human Kvβ1.3, R241L, Y307F, and R316E or mutant protein were purified from bacteria. The K_d values of the proteins were determined in 0.2 M potassium phosphate, pH 7.4.

Data are mean $\pm$ S.E.M. of 3–5 separate determinations.

Protein	K_d^{NADPH} (M)	$K_d^{\text{NADP}^+}$ (M)
Kvβ1.3 WT	1.8 $\pm$ 0.02	5.5 $\pm$ 0.09
Δ NKvβ1.3	2.4 $\pm$ 0.28	5.8 $\pm$ 0.12
Δ NKvβ1.3R241L	2.2 $\pm$ 0.14	6.2 $\pm$ 0.13
Δ NKvβ1.3Y307F	12.9 $\pm$ 1.1	> 25 μ M [*]
Δ NKvβ1.3R316E	> 50 μ M [*]	> 50 μ M [*]

* Minimal quenching of protein fluorescence observed after the addition of NADPH or NADP⁺ precluded estimates of K_d values.

Table 2
Changes in the characteristics of Kv current due to site-directed mutations at the NADPH binding site

	Mutant Type	% Inactivation	V _{1/2} inactivation (mV)	Mean inactivation Decay Constants (ms)		V _{1/2} activation (mV)
				τ _{fast}	τ _{slow}	
1.	Kv1.5	4.5±0.8*	#	-	2041±724*	-4.9±1.4*
2.	+β1.3 WT	41.6±3.2	-16.7±1.0	8.9±2.1	348.6±50.7	-17±0.5
3.	+β: R241L	36.1±3.0	-25±0.4*	63±14*	387.1±38	-24±1.2
4.	+β: Y307F	16.3±2.1*	-15.9±1.5	-	428.7 ±44.4	-18.3±0.9
5.	+β: R316E	12.1±1.1*	-3.1±1.3*	-	791.6 ±109*	-11±1.3*
6.	+β: R241L +NADPH	32±3	-18.7±0.5	92±38	539 ± 87	-21.3±1.0
7.	+β: R316E + NADPH	30.3±4.8	-16.3±1	94±22*	849±221*	-23±1.1

due to minimal inactivation, the voltage-dependence of inactivation could not be reliably estimated
* p≤0.05 as compared to Kvβ1.3 WT using one way ANOVA (Bonferroni t-test) n=5–9 cells each