Mutations in the Kvβ2 Binding Site for NADPH and Their Effects on Kv1.4*

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Kvβ2 enhances the rate of inactivation and level of expression of Kv1.4 currents. The crystal structure of Kvβ2 binds NADP*, and it has been suggested that Kvβ2 is an oxidoreductase enzyme (1). To investigate how this function might relate to channel modulation, we made point mutations in Kvβ2 in either the NADPH docking or putative catalytic sites. Using the yeast two-hybrid system, we found that these mutations did not disrupt the interaction of Kvβ2 with Kvα1 channels. To characterize the Kvβ2 mutants functionally, we coinjected wild-type or mutant Kvβ2 cRNAs and Kv1.4 cRNA in Xenopus laevis oocytes. Kvβ2 increased both the amplitude and rate of inactivation of Kv1.4 currents. The cellular content of Kv1.4 protein was unchanged on Western blot, but the amount in the plasmalemma was increased. Mutations in either the orientation or putative catalytic sites for NADPH abolished the expression-enhancing effect on Kv1.4 current. Western blots showed that both types of mutation reduced Kv1.4 protein. Like the wild-type Kvβ2, both types of mutation increased the rate of inactivation of Kv1.4, confirming the physical association of mutant Kvβ2 subunits with Kv1.4. Thus, mutations that should interfere with NADPH function uncouple the expression-enhancing effect of Kvβ2 on Kv1.4 currents from its effect on the rate of inactivation. These results suggest that the binding of NADPH and the putative oxidoreductase activity of Kvβ2 may play a role in the processing of Kv1.4.

Voltage-gated K+ (Kv) channels are integral membrane proteins that regulate the electrical properties of excitable cells. Channels in the Kv1 subfamily consist of hetero-oligomeric protein complexes comprising four pore-forming α subunits in permanent association with four modulatory β subunits (1). Three different Kvβ subunit genes (β1, β2, and β3) have been cloned from different tissues in several species (2–6). Kvβ subunits coassemble with Kvα subunits in the endoplasmic reticulum and enhance biosynthesis, maturation, and surface expression of voltage-gated K+ channel complexes. Kvβ2 increases Kv1.2 (7) and Kv1.4 (8) currents and also increases the cell surface dendrotoxin binding site of Kv1.2 (9). The N terminus of Kvβ1.x (β1.1, β1.2, and β1.3) acts as a ball peptide and rapidly inactivates open channels. This fast inactivation resembles the N-type inactivation produced by the N-terminal ball peptide of Kvα subunits. The Kvβ2 subunit while lacking the inactivation peptide accelerates the N-type inactivation of Kv1.4 and in addition increases Kv1.4 expression (8).

The conserved C-terminal core region of Kvβ subunits shares a remarkable structural homology to aldo-keto reductases (10). The crystal structure of the conserved core of Kvβ2 subunit depicts a 4-fold symmetrical TIM barrel structure with the bound cofactor NADP+ (1). The structure shows that residues Ser-188 and Arg-189 are important for the orientation of the nicotinamide ring of NADP+ and that Asp-85 and Tyr-90 are the putative catalytic site residues of Kvβ2. The phenolic moiety of Tyr-90 positioned near the C-4 of nicotinamide ring would be a proton donor for reduction of a putative substrate aldehyde or ketone. The Asp-85 residue is involved in extensive hydrogen bonding and together with Tyr-90 positions the catalytic site as for other aldo-keto reductases. Despite this structural information, it is not known whether Kvβ subunits function as oxidoreductases and, if so, what their physiological substrates may be.

In this study, we investigated the contribution of these residues to Kvβ2 function. We made site-directed point mutations in both the NADPH orientation and putative catalytic sites. The putative catalytic mutant (D85A, Y90F) is referred to as Kvβ2 Mut1 and the orientation mutant (S188A, R189L) is referred to as Kvβ2 Mut2. We tested the effects of these mutants on both the inactivation properties and the expression levels of Kv1.4 currents in Xenopus oocytes. We report that wild-type Kvβ2 increased Kv1.4 expression by enhancing its trafficking to the cell surface. Mutations at either site eliminated the ability of Kvβ2 to enhance Kv1.4 expression. However, mutant Kvβ2 subunits still interacted with Kv1.4 to increase the rate of inactivation. Thus, the dual effects of Kvβ2 regulation of Kv1.4 were shown to be independent by mutating residues involved in NADP* binding.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Site-directed mutations (D85A, Y90F mutant 1; S188A, R189L mutant 2) were made in Kvβ2 cDNA using the overlapping polymerase chain reaction method, from which the products were subcloned into the TOPO shuttle vector (Invitrogen) for sequencing. Wild-type and mutant Kvβ2 cDNAs were subcloned into the pCR3 vector (Invitrogen), which we modified to contain a poly(A)+ tail and a unique NruI site for linearizing the constructs prior to cRNA synthesis.

Analysis of Protein-Protein Interaction by the Yeast Two-Hybrid System—Protein-protein interactions were monitored with the yeast Matchmaker two-hybrid system (CLONTECH). Kvβ2 wild-type and Kvβ2 mutant cDNAs were subcloned into the yeast shuttle vector, pGBT9 (DNA binding domain vector). The N terminus of Kv1.4 (amino acids 1–305) was fused to the activation domain vector, pGAD424.

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Protein-protein interactions were tested in the yeast strain Y190 by cotransformation with pairs of pGBT9 and pGAD424 constructs as described previously (11). Cotransformants were selected on medium lacking tryptophan (trp”) and leucine (leu”) after growth for 2–3 days at 30 °C. Representative colonies were replated on trp” leu” medium to allow positive comparison of individual colonies. Transcription of the reporter gene, lacZ, was tested by a β-galactosidase filter assay.

In Vitro Transcription of RNA and Expression in Xenopus Oocytes—
cRNA was prepared using the T7 mMESSAGE mMACHINE kit (Ambion), and the concentrations were estimated on denaturing agarose gels stained with ethidium bromide by comparison with an RNA mass ladder. Xenopus oocytes were injected with 46 nl of cRNA solution. Final concentrations of cRNA were 2 ng/µl Kv1.4 and 50 ng/µl Kvβ2 or Kvβ2 mutants.

Electrophysiology—Whole cell current was measured at room temperature (20–22 °C) using the standard two-electrode voltage clamp technique. Electrodes were filled with 3 M KCl and had a resistance of 0.2–0.5 megohms when immersed in bath solution containing (in mM): 50 KOH, 55 NaOH, 0.5 CaCl2·2H2O, 100 methanesulfonic acid, 2 MgCl2·6H2O, and 10 HEPES, pH 7.3. All chemicals were purchased from Sigma. We used 50 µM K+ in bath solution to slow C-type inactivation and reduce its contribution to the N-type inactivation of Kv1.4. All currents were measured 6 days after injection. Data acquisition and analysis were performed using pCLAMP software (Axon Instruments). Data were low-pass-filtered at 2 kHz before digitization at 10 kHz. Data are reported as means ± S.E.

Xenopus Oocyte Fractionation and Western Blotting—Plasma membranes were prepared from Xenopus oocytes using a previously published procedure (12). Oocytes were homogenized (20 µl/oocyte) in buffer containing 0.25 mM sucrose, 10 mM HEPES, 1 mM EGTA, 2 mM MgCl2·6H2O, and 1 µg/ml phenylmethylsulfonyl fluoride with five strokes in a Dounce homogenizer using a loose-fitting pestle. Sheets of plasma membrane were allowed to settle by gravity for 15 min and then washed and resedimented three times. Washed plasma membranes were solubilized in a buffer containing 1% Triton X-100 prior to determination of protein concentration. The supernatant from the initial homogenization was spun at 3,000 × g for 10 min to remove debris, and the residual membranes were pelleted by centrifugation at 50,000 × g for 60 min. Protein concentrations were estimated by the BCA method. For Western blotting, proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted to polyvinylidene difluoride membranes, and blocked with 5% nonfat dry milk in phosphate-buffered saline plus 0.1% Tween 20. Blots were incubated with primary antibodies (monoclonal anti-Kv1.4, Upstate Biotechnology, Inc., 1:500; or polyclonal anti-Kvβ2, QCB, 1:50000) for 1 h at room temperature, washed three times (10 min/wash) with phosphate-buffered saline, 0.1% Tween 20, and incubated with secondary antibody (anti-mouse or anti-rabbit horseradish peroxidase conjugate, Amersham Pharmacia Biotech, 1:3000) for 1 h. After three washes in phosphate-buffered saline plus 0.1% Tween 20, the blots were developed with the ECL-Plus detection system (Amersham Pharmacia Biotech). Total membranes were prepared from oocytes by methods previously established in our laboratory (13). The oocytes were ground by 20 strokes in a Dounce homogenizer and spun at 3,000 × g for 10 min to remove debris, and the total membranes were pelleted by centrifugation at 50,000 × g for 60 min.

RESULTS

Interaction of Kv1.4 NTerminus with Kvβ2 Mutants—We first tested whether the mutations in the NADPH orientation and NADPH oxidoreductase catalytic sites affected interaction with Kv1.4 channels. Using the yeast two-hybrid assay, we found that neither set of mutations diminished the binding of Kvβ2 with the Kv1.4 N terminus, as evidenced by activation of the lacZ reporter gene (Fig. 1).

Effect of Kvβ2 Mutants on Kv1.4 Currents—Kvβ2 has been reported to increase both the amplitude and the rate of inactivation of Kv1.4 currents. We investigated the effects of Kvβ2 mutations on Kv1.4 currents by coexpressing cRNAs encoding Kv1.4 with wild-type or mutant Kvβ2 in Xenopus oocytes. Six days after injection, we observed that Kvβ2 increased Kv1.4 current amplitude by 1.6-fold at +80 mV (compare Fig. 2, panels A and B). The current amplitude measured at +80 mV was 15.35 ± 0.78 µA (n = 9) (Fig. 2B) in oocytes expressing a combination of Kv1.4 and Kvβ2 compared with 9.55 ± 0.88 µA (n = 7) for oocytes expressing Kv1.4 alone (Fig. 2A). The increase in current was manifest at all potentials above threshold (Fig. 3A). When Kvβ2 mutants were examined, the expression-enhancing effect of Kvβ2 on Kv1.4 was abolished. In fact, coexpression with Kvβ2 mutants produced a decrease in the amplitude of Kv1.4 current, and the decrease occurred at all potentials above threshold (Fig. 3A). At +80 mV, mutations in the NADPH oxidoreductase catalytic site (D85A, Y90F) decreased currents by 63.3% to 4.46 ± 0.24 nA (n = 5) (Fig. 2C), whereas mutations in the NADPH docking site (S188A, R189L) reduced currents by 36.6% to 6.05 ± 0.45 nA (n = 6) (Fig. 2D). The double mutant in which all four residues were simultaneously disrupted also showed a reduction in current ampi-
normalizing the currents at each potential to the maximal currents at
mV for 6s (followed by a 450-ms test pulse to

D is the slope factor.

alytic sites do not significantly alter the biophysical properties
A–D mV increments (current traces shown in Fig. 2,

vation time constants are significantly smaller when Kv

Kv1.4 with Kv β2 mutants were fitted with a single exponential function. The
inactivation time constants are significantly smaller when Kv β2 or wild-type
Kv β2 mutants are coexpressed with Kv1.4, indicating enhanced inac-
tivation; however, they are not significantly different from each other.

E, plot of recovery from inactivation. From a holding potential
of −90 mV, the oocyte was depolarized to +70 mV for 1 s and subsequently
repolarized to −90 mV for 6 s (n = 4). The recovery curve is the plot of the ratio of inactivated current during the
second pulse, normalized to the amount of inactivating current during the
first pulse versus the inter-pulse interval. Kv β2 slowed the recovery from
inactivation compared with Kv1.4 alone, whereas the Kv β2 mutants
produced an even slower recovery from inactivation.

tude to 7.24 ± 0.36 μA (n = 6), a decrease of 24% (data not shown).
The differences in mean current amplitude for Kv1.4

tions in the NADPH docking and oxidoreductase catalytic sites do not significantly alter the biophysical properties
of Kv β2. A, current-voltage relationship of currents recorded from
Xenopus oocytes. Whole cell currents were evoked from a holding potential of −80 mV by 125-ms depolarizing step pulses to +80 mV in 10 mV increments (current traces shown in Fig. 2, A–D). B, coexpression of Kv1.4 with Kv β2 or Kv β2 mutants did not alter the voltage dependence of activation. I / I max ratios were calculated by dividing the peak current at each potential with the maximal current at +80 mV and plotted as a function of potential. C, steady state inactivation levels of Kv1.4 in the presence and absence of Kv β2 and Kv β2 mutants were similar. Oocytes were held at −90 mV and pulsed to −10 mV in 10 mV increments for 1 s followed by a 450-ms test pulse to +70 mV and repolarization to −90 mV for 6 s (n = 4). Steady state inactivation curves were generated by normalizing the currents at each potential to the maximal currents at +70 mV. The data were fit to the Boltzman equation

B

FIG. 4. Kv β2 coexpression did not alter the total membrane
associated Kv1.4 protein in Xenopus oocytes, whereas Kv β2 mutations in the NADPH docking and putative oxidoreductase catalytic sites decreased Kv1.4 protein levels. Total membranes were isolated 6 days post-injection from Xenopus oocytes either injected with Kv1.4 (2 ng/μl) alone or coinjected with Kv β2 or Kv β2 mut1 or Kv β2 mut2 (all at 50 ng/μl). Proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted to polyvinylidene difluoride membranes, and probed with either monoclonal anti-Kv1.4 (1:5000, QCB; panel A) or monoclonal anti-Kv1.4 (1:500, Upstate Biotechnology, Inc.; panel B). Immunoreactive bands were visualized with ECL-Plus (Amersham Pharmacia Biotech). Kv1.4 appeared as three bands, a 78-kDa core-glycosylated band and a doublet at 105 kDa representing mature-glycosylated bands). Coexpression with wild-type Kv β2 did not increase the total Kv1.4 expression, whereas both of the Kv β2 mutants decreased total Kv1.4 protein levels. Kv β2 blot shows that the mutation in the catalytic site led to decreased β2 protein synthesis.

FIG. 5. Kv β2 enhanced the surface expression of Kv1.4, whereas Kv β2 mutations in the NADPH docking and oxidoreductase catalytic sites decreased Kv1.4 protein levels. Plasma membrane and internal membrane fractions were isolated 6 days post-injection from Xenopus oocytes injected with Kv1.4 (2 ng/μl) alone or coinjected with Kv β2, Kv β2 mut1, or Kv β2 mut2 (all at 50 ng/μl). Proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted to polyvinylidene difluoride membranes, and probed with either with monoclonal anti-Kv1.4 (1:500, Upstate Biotechnology, Inc; panels A and B) or polyclonal anti-Kv1.4 (1:5000, QCB; panels C and D). Immunoreactive bands were visualized with ECL-Plus (Amersham Pharmacia Biotech). Kv1.4 appeared as two bands in the internal membrane fraction (A), a 78-kDa core-glycosylated band and a 105-kDa mature-glycosylated band. Only the mature, glycosylated, 105-kDa band was seen in the plasma membrane fraction (B). Note that coexpression with wild-type Kv β2 increased Kv1.4 expression in the plasma membrane fraction, whereas both of the Kv β2 mutants decreased Kv1.4 protein levels in the internal as well as the plasma membrane fractions.

Yeast two-hybrid data indicated that the mutant Kv β2 subunits were still able to interact with the Kv1.4 N terminus (Fig. A, B). Panel A was a recombinant of yeast containing a pACT2 vector expressing wild-type Kv1.4, whereas panel B was the same vector expressing Kv β2 mut1. The yeast strains were transformed with a pGADT7 vector expressing the activation domain of mutant and wild-type Kv1.4, respectively. Only pGADT7-Kv1.4 interacted with pACT2-Kv β2, whereas both wild-type and mut1 interacted with pACT2-Kv1.4.
As a further test of association, we examined Kv1.4 currents for evidence of increased inactivation produced by association with Kvβ2 subunits. In an earlier study (8), we had shown that Kvβ2, although absent the inactivation peptide of Kvβ1.x, increased the rate of N-type inactivation of Kv1.4. Normalized current traces of Kv1.4 when coexpressed with either Kvβ2 or Kvβ2 mutants showed that wild-type and mutant Kvβ2 enhanced the intrinsic inactivation of Kv1.4 currents to a similar extent (Fig. 2F). Plots of inactivation time constants as a function of membrane potential were also similar (Fig. 3D). At +80 mV, the mean inactivation time constants for Kv1.4, Kv1.4 + β2, Kv1.4 + β2 mut1, and Kv1.4 + β2 mut2 were 49.55 ± 2.3, 25.52 ± 0.37, 21.67 ± 0.18, and 22.32 ± 0.28, respectively. Neither wild-type nor mutant Kvβ2 subunits altered the voltage dependence of activation (Fig. 3E) or steady state inactivation (Fig. 3C) of Kv1.4. Recovery from steady state inactivation, which was decreased when Kvβ2 was coexpressed with Kv1.4, was slowed further in the presence of the Kvβ2 mutants (Fig. 3E). At −90mV, the mean time for half-recovery from inactivation for Kv1.4, Kv1.4 + β2, Kv1.4 + β2 mut1, and Kv1.4 + β2 mut2 were 3.35, 3.42, 4.23, and 5.82 s, respectively. To eliminate the possibility of nonspecific effects of Kvβ2 mutants on Kv1.4 in oocytes, Kvβ2 wild-type and mutants were also coexpressed with Kv2.1, a Kvα subunit with which they do not interact. Neither Kvβ2 nor the Kvβ2 mutants had any effect on Kv2.1 current amplitude or kinetics (data not shown).

**Kvβ2 Enhances Trafficking of Kv1.4 to the Plasma Membrane**—Our electrophysiological data showed that the expression-enhancing effect of Kvβ2 on Kv1.4 was distinct from its effects on the biophysical properties of the channel. The enhancement of Kv1.4 expression by Kvβ2 could be caused by: 1) an increase in total Kv1.4 protein, 2) an increase in the number of functional channels at the cell surface without an increase in total Kv1.4 levels, or 3) an increase in opening probability and/or single channel conductance. The third possibility was considered unlikely because the conductance-voltage relationship of Kv1.4 was unaltered by wild-type and mutant Kvβ2s (Fig. 3B). To determine whether coexpression of Kvβ2 increased total Kv1.4 protein levels, we used Western blotting to examine membrane-enriched fractions from oocytes. Multiple bands of Kv1.4 were detected in oocytes injected with Kv1.4 cRNA: an immature or core-glycosylated ~78-kDa band; and a mature, glycosylated, 105-kDa band, which resolved as a doublet in some experiments (Fig. 4). Coexpression with Kvβ2 did not alter the amounts of Kv1.4 protein in the total membrane fraction. However, coexpression with either of the Kvβ2 mutants did show a reduction in total Kv1.4 protein.

Because Kvβ2 did not increase total Kv1.4 protein levels, we explored the possibility that Kvβ2 increased currents by enhancing trafficking of Kv1.4 channels to the plasma membrane by physically separating oocyte plasma membranes from internal membranes (i.e. endoplasmic reticulum and Golgi). Western blot analysis of Kv1.4 in these fractions showed an enhanced expression in the plasma membrane when coexpressed with Kvβ2 (Fig. 5, right). As expected, only the mature, fully glycosylated ~105-kDa band (which was not resolved into a clear doublet in this gel) was detected in the plasma membrane. In the internal membrane fraction, we found both the immature and mature Kv1.4 bands with no apparent change in expression in the presence or absence of Kvβ2 (Fig. 5, left). Consistent with decreased currents, coexpression with Kvβ2 mutants showed a reduction of Kv1.4 in both the plasma and internal membrane fractions (Fig. 5). Both wild-type and mutant Kvβ2 subunits were detected in both the internal and plasma membrane fractions, providing further evidence for the interaction of mutant Kvβ2 subunits with Kv1.4. Although equal amounts of cRNAs were injected for each Kvβ cDNA, we consistently observed a lower level of expression of Kvβ2 mutant 1. It is not known whether this finding reflects an increased degradation of mutant protein, decreased synthesis, or a combination of both.

**DISCUSSION**

Kvβ2 increased Kv1.4 current and its rate of inactivation (8). From the present results it appears that the increased Kv1.4 current may be due to a redistribution of Kv1.4 protein to the plasma membrane rather than an increase in the total amount of membrane-associated Kv1.4 protein. Mutating wild-type Kvβ2 at the orientation site for NADPH (Mut2) or the putative catalytic site for aldo-keto reductase activity (Mut1) abolished the ability of Kvβ2 to increase Kv1.4 current without changing the ability of Kvβ2 to increase inactivation. The latter result, together with the persistent yeast two-hybrid interactions, showed that the mutant Kvβ2 subunits were still permanently associated with Kv1.4 subunits. However, the Kvβ2 mutants produced a reduction in both the plasmalemmal and total membrane-associated Kv1.4 protein, which probably explains the reduction in Kv1.4 currents associated with their coexpression (Fig. 2F).

Our results do not show that NADPH binding by Kvβ2 has been abolished, nor do they show whether the effects of the mutations are specific for orientation of the nicotinamide ring or the putative catalytic site for aldo-keto reductase activity. Such studies are possible now that the crystal structure of Kvβ2 (1) and the assembly of Kvβ1.x with the T1 domain of Kv1.1 channels (14) have been resolved. Keeping these assumptions in mind, it may be possible that the aldo-keto reductase activity of Kvβ2 is important for the processing and trafficking of Kv1.4.

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