Genetic Analysis of the Mammalian K⁺ Channel β Subunit Kvβ2 (Kcnab2)*

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Kvβ2 binds to K⁺ channel α subunits from at least two different families (Kv1 and Kv4) and is a member of the aldo-ketoreductase (AKR) superfamily. Proposed functions for this protein in vivo include a chaperone-like role in Kv1 α subunit biogenesis and catalytic activity as an AKR oxidoreductase. To investigate the in vivo function of Kvβ2, Kvβ2-null and point mutant (Y90F) mice were generated through gene targeting in embryonic stem cells. In Kvβ2-null mice, Kv1.1 and Kv1.2 localize normally in cerebellar basket terminals and the juxtaparanodal region of myelinated nerves. Moreover, normal glycosylation patterns are observed for Kv1.1 and Kv1.2 in whole brain lysates. Thus, loss of the chaperone-like activity does not appear to account for the phenotype of Kvβ2-null mice, which include reduced life spans, occasional seizures, and cold swim-induced tremors similar to that observed in Kv1-null mice. Mice expressing Kvβ2, mutated at a site (Y90F) that abolishes AKR-like catalytic activity in other family members, have no overt phenotype. We conclude that Kvβ2 contributes to regulation of excitability in vivo, although not directly through either chaperone-like or typical AKR catalytic activity. Rather, Kvβ2 relies upon as yet unidentified mechanisms in the regulation of K⁺ channel and/or oxidoreductive functions.

Kvβ2 was isolated biochemically through its association with the Kv1 family of K⁺ channels in a 4:4 stoichiometry (1) and later found to coassemble with Kv4 channels (2, 3). It is expressed abundantly in the nervous system and also in T-lymphocytes, where it appears to play a role in K⁺ channel activation in at least some mammalian species (4). Subsequent cloning of the Kvβ2 homologs, Kvβ1and Kvβ3, revealed a conserved core region and variable N-terminal domains that may contain a ball-and-chain motif conferring rapid inactivation to even noninactivating Kv1 α subunits (5–7). The observed alterations in K⁺ channel α subunit inactivation are likely to be a primary function of the Kvβ1 and Kvβ3 gene products. Mice rendered deficient in Kvβ1 by gene targeting display reduced K⁺ current inactivation, frequency-dependent spike broadening, and slow after-hyperpolarization in hippocampal CA1 pyramidal neurons (8).

The Kvβ2 gene product, by far the most abundantly expressed Kv1-associated subunit protein (1, 9), unlike Kvβ1 and Kvβ3, lacks the ball-and-chain domain and does not confer rapid inactivation to noninactivating Kv1 α subunits. In heterologous expression systems it does, however, increase the rate of rapid inactivation of endogenously inactivating Kv1.4 currents (10). Kvβ2 also induces small shifts in the activation threshold of Kv1.5 currents (11) and alters inactivation rates when coassembled in combination with Kvβ1 subunits (10, 12, 13). In addition to the relatively restricted effects observed on K⁺ channel gating, Kvβ2 has also been reported to increase the amplitudes of Kv1.4 and Kv1.3 currents in Xenopus oocytes (4, 10). In cultured mammalian cells the absence of Kvβ2 results in unglycosylated forms of Kv1.1 and Kv1.2 that are not efficiently transported to the membrane surface, leading Shi et al. (2) to propose that Kvβ2 facilitates the glycosylation of Kv1 α subunits in the endoplasmic reticulum, thereby promoting the trafficking of Kvαβ complexes from the endoplasmic reticulum to the surface cell membrane. Whether Kvβ2 is required for trafficking of these Kvα species in vivo is unknown.

Given their homology to other AKR1 superfamily members, including the conservation of nucleotide-cofactor binding and catalytic residues, additional physiological functions for Kvβ2 have been proposed relating to potential oxidoreductive and/or catalytic roles (14, 15). Interestingly, coexpression of Kvβ2 in cultured mammalian cells confers oxygen sensitivity to Kv4.2 but not Kv1 channels (16). Subsequently, the structural and potential functional similarities between Kvβ2 and other AKR superfamily members, including cofactor binding, was confirmed through x-ray crystallographic resolution (17). Kvβ2 binds NADPH with an affinity comparable with that found for other AKR enzymes but with only 10-fold lower affinity for NADH; NADPH and NADH bind more tightly than NADP⁺ and NAD⁺, respectively, indicating that Kvβ2 would be more likely to function as a reductase than an oxidase (18); cofactor binding is not a prerequisite for multimer formation. It therefore remains possible that Kvβ2 is an oxidoreductive enzyme or acts as a metabolic sensor with regard to cofactor binding and interaction with at least some Kvα subunits. Despite much research into the expression of Kvβ2 in heterologous systems, little is known regarding the function of Kvβ2 in vivo. The human Kvβ2 locus (KCNAB2) maps to chromosome 1p36, where it has been implicated in complex syndromes that include seizures (19, 20).

To investigate the possible functions of Kvβ2 in the intact mammalian nervous system, we used gene targeting in embryo-
ontic stem cells to generate Kvβ2-null or Y90F point mutant (which should specifically inhibit typical AKR catalytic activity) mice. We conclude that Kvβ2 plays an important role in modulating excitability in vivo but that this role does not rely upon Kvβ2 enhancement of Kv1 α subunit trafficking and/or targeting. We also find that the primary physiological role of Kvβ2 does not appear to take place through typical AKR-like oxidoreductive catalysis.

**Experimental Procedures**

**Construction of the Kvβ2-null Targeting Vector**

The Kvβ2-null targeting vector was constructed from a genomic clone isolated from a 129/SvEv genomic library (Stratagene number 949305) as diagrammed in Fig. 1A. Exon/intron boundaries of mouse Kvβ2 as determined by our sequencing do not correspond with those previously reported for 129/SvJ mice (21), but do correspond with those reported for exons 5–9 of the human Kvβ2 gene locus (GenBankTM accession number NT_019265.6). The targeting vector contained a 2.5-kb 5’ arm of homology (XhoI–NsiI fragment) and a 3 kb 3’ arm of homology (SalI–BglII fragment). The internal NsiI–SalI fragment containing genomic DNA corresponding to exons 7–9 of human Kvβ2 was deleted such that any potential transcript would have a minimal deletion of 176 nucleotides of coding sequence; PGKneobpA (22) was inserted in place of the deleted fragment. MC1-thymidine kinase was attached at the 3’ end of homology containing the point mutation and the 4.4-kb 3’ arm of homology (see Fig. 1B).

**Creation of Targeted Embryonic Stem Cells and Kvβ2-null and Y90F Mice**

AB-1 ES cells (obtained from Allan Bradley) were electroporated with 10 μg of linearized targeting vector and treated with G418 (350 μg/ml) and FIAU (200 nM) for selection of double resistant colonies. Southern analyses were performed as described (23). For the ES cells transfected with the Kvβ2-null targeting vector, DNA was digested with BglII (for 5’ probe) or HindIII (for neo probe). For the ES cells transfected with the Kvβ2-Y90F targeting vector, DNA was digested with BamHI (for the 3’ probe) or AccI and AccI/SalI (for the neo probe). Excision of the PGKneo insert was induced through transient transfection of CMV-Cre recombinase (pBBS185; Invitrogen), and recombinant clones were identified through Southern blot analysis utilizing the same 0.8-kb AvrII/SalI 3’ probe by comparison of BamHI versus BamHI/SalI digestion as well as by PCR using primers corresponding to genomic DNA sequences 5’ of exon 5 and 3’ of the XhoI site in intron 5.

Targeted ES cells were injected into C57BL/6J (B6) blastocysts (University of Wisconsin Transgenic Core Facility) to generate chimeric founder mice. Founder mice were bred to inbred B6 mice to generate F1 heterozygotes, which were then bred together to generate mutant mice on a mixed B6×129 genetic background. The Kvβ2-null and Y90F point mutant mice described in this report have been given the strain designations Kenab22null and Kenab22null, respectively.

**Survival Analysis**

All Kvβ2-null, Y90F, and heterozygous mice that were generated through the F2 and F3 generations (in mixed B6×129 backgrounds) were included in the survival analysis. Mice that were alive as of an arbitrary end date (January 29, 2001) or were removed for various reasons (for example, use in experiments) were treated as censored data. Kaplan–Meier survival curves were generated for both groups and compared using the logrank test.

**Myeloclonus Score Following Cold Water Swim**

Mice were placed in a tank of 17 °C water for 2 min. The mice were then placed in a dry cage for observation by a scorer blinded to the genotype. The myeloclonus score is the number of whole body jerks observed during the first 2 min post-swim. Kvβ2-null mice and wild type littermates were 42–58 days old, Kvβ2-Y90F and wild type littermates were 50–53 days old, and Kv1.1-null mice (24) and wild type littermates were 30–41 days old. All mice scored for myeloclonus were on an inbred 129 background.

**Antibodies**

**Primary Antibodies**—For Anti-Kv1.1, the polyclonal antibody was generated in rabbit against residues 416–495 of rat brain Kv1.1 (Alomone Labs; catalog number APC-009), and the monoclonal antibody was generated in mouse against residues 458–476 of rat brain Kv1.1 (Upstate Biotechnology Inc.; 1:200, catalog number 05-407) (25). For anti-Kv1.2, the polyclonal antibody was generated in rabbit against residues 417–499 of rat brain Kv1.2 (Alomone Labs; catalog number APC-010), and the monoclonal antibody was generated in mouse against residues 428–499 of rat heart Kv1.2 (88 kDa) (Upstate Biotechnology Inc.; 1:100, catalog number 05-408) (2). For anti-Kv1.4, the monoclonal antibody was generated in mouse against residues 13–37 of rat heart Kv1.4 (96 kDa) (Upstate Biotechnology Inc.; 1:100, catalog number 05-409) (26). For anti-Kvβ1, the monoclonal antibody to Kvβ1 (K9/40.1) was a gift from James Trimmer (SUNY Stony Brook) (25). For anti-Kvβ2, the monoclonal antibody to Kvβ2 (K1/70) was a gift from James Trimmer (SUNY Stony Brook) (25).

**Secondary Antibodies**—For immunofluorescence, fluorescein isothiocyanate-conjugated goat anti-mouse (Jackson ImmunoResearch) and fluorescent isothiocyanate-conjugated goat anti-rabbit (Invitrogen) were used. For ECL, horseradish peroxidase-conjugated goat anti-mouse IgG + IgM (Pierce; 1:2000) and horseradish peroxidase-conjugated rabbit anti-mouse (Pierce; catalog number 31450) were used.

**Immunofluorescence**

**Sciatic Nerve**—Briefly, the sciatic nerves were immersion fixed with 4% paraformaldehyde in phosphate-buffered saline at 4 °C and then teased into single fibers on glass slides and processed for Kv1.1, Kv1.2, and Kvβ2 immunofluorescence as previously described (27). Confocal images of immunofluorescence were captured using a Bio-Rad confocal microscope (MRC 1024). The published images were obtained by plane projection from 4–10 consecutive z-section images with 0.2-μm z-increments. During image capturing, the settings on the microscope (background, gain, and axis) were adjusted so that the peak readings at the juxtaparanode were always lower than 256 (the maximum value) to minimize saturation. Spatial distribution of the Kv1.1 and Kv1.2 immunofluorescence intensity along single fibers at the nodal region was quantitatively measured along a fixed length (60 μm) using MetaMorph software. The line scan function was used with a setting of 6 × 60 pixels (300 points/μm).

**Cerebellum**—All mice were anesthetized deeply with inr, and transcardially perfused with 60 ml of chilled fixative containing 15% picric acid, 4% paraformaldehyde in 0.1 M phosphate-buffered saline buffer. The brain was removed and immersed in the same fixative for another 3–4 h at 4 °C. After several washes, the fixed brain was cryo-protected in 30% sucrose solution in 0.1 M phosphate-buffered saline overnight. Frozen sagittal sections of the cerebellum were cut at 50 μm and permeabilized in blocking solution for 2 h with 5–10% goat serum, 0.4% Triton X-100, and 0.02% sodium azide in 0.1 M phosphate-buffered saline. The subsequent staining and imaging were similar to that described above for sciatic nerves.

**Western Blot Analysis of Kvα Subunits**

Age-matched littermate or C57BL/6J mice were used as wild type controls. For Western blot analysis of Kvα subunits, whole brains were removed, washed several times with ice-cold lysis buffer (150 mM NaCl, 10 mM Tris, pH 8.0, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA) and then homogenized with 10–15 strokes in a glass homogenizer in buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 2 μM aprotinin, 2 μg/ml antipain, and 10 μg of benzamide as protease inhibitors. The extracts were sonicated for 1 min and then spun in a microcentrifuge for 4 min at 4 °C. The supernatant was quickly mixed with 5× sample buffer (250 mM Tris, pH 6.8, 500 mM dithiothreitol, 10% (w/v) SDS, 0.5%, bromphenol blue, 50%, glycerol) in a 4:1 ratio and boiled for 2 min. The protein concentration in each sample was quantified by a Bradford assay (Bio-Rad), and the volume of each sample was adjusted so that all were at 20 μg/ml.
Gene Targeting of Kvβ2 in Mice

The proteins were separated on a 9% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes (Bio-Rad; 0.45 μm). The membranes were blocked in TBS-Tween (20 mM Tris, pH 7.6, 150 mM NaCl, 0.1% (v/v) Tween 20) and then incubated in primary antibodies (monoclonal anti-Kv1.1, Kv1.2, and Kv1.4, used at 1:1000, 1:500, and 1:1000, respectively). The membranes were then washed four times for 5 min before incubation with horseradish peroxidase-conjugated secondary antibodies (Pierce; 1:3000 dilution). After washing, the membranes were developed with enhanced chemiluminescence (Pierce) for 1 min and then exposed to film. To verify equal loading of lanes, the membranes were stained following ECL with Ponceau S for 5 min, air-dried, and then wrapped in plastic and mounted in a PhosphorImager cassette. The upper and lower bands, which correspond to the mature and core glycosylated forms (see below), were analyzed together with background subtraction. The ratio of upper band to lower band in each sample was calculated directly.

**Western Blot Analysis of Kvα Subunits**

For Western blot analysis of Kvα subunits, whole brains were homogenized in 0.3 ml of 10 mM sodium phosphate, pH 7.4, 10 mM sodium fluoride, 0.1% (v/v) aprotinin, and 1 μg/ml pepstatin. The homogenate was centrifuged at 3000 × g for 10 min to remove nuclei and cellular debris, and the supernatant was used for Western blot analysis. Protein content was assayed by the Bradford assay. The samples of supernatant (10 μl, containing 50 μg of protein) were boiled in SDS sample buffer containing 63 mM Tris-HCl, pH 6.8, 1% SDS, 1% β-mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue for 10 min. The proteins were then separated on a 10% polyacrylamide gel and transferred onto polyvinylidene difluoride plus membrane. The membranes were then washed four times for 5 min before incubation with horseradish peroxidase-conjugated secondary antibodies (Pierce; 1:3000 dilution). After washing, the membranes were developed with enhanced chemiluminescence (Pierce) for 1 min and then exposed to film. To verify equal loading of lanes, the membranes were stained following ECL with Ponceau S for 5 min, air-dried, and then wrapped in plastic and mounted in a PhosphorImager cassette. The upper and lower bands, which correspond to the mature and core glycosylated forms (see below), were analyzed together with background subtraction. The ratio of upper band to lower band in each sample was calculated directly.

**Generation of Kvβ2-null Mice—Kvβ2-null mice were generated by gene targeting in embryonic stem cells (Fig. 1A). The targeting vector was designed to introduce a deletion toward the beginning of the constitutive “core” region of the protein that includes essential β-sheet structural elements (14, 17) such that any β2 gene products would be structurally compromised, unlikely to fold, and rapidly degraded. Following electroporation of the targeting vector into ES cells, 80 clones were isolated that were doubly resistant to G418 and FIAU. Southern blot analysis revealed that 11 of these contained the disrupted Kcnab2 locus (data not shown). One clone (K59) was used to generate chimeric founder mice and transmission of the mutant allele to heterozygous offspring, which were interbred to produce homozygous mutant mice.**

These crosses yielded the expected Mendelian ratios of wild type, heterozygous, and homozygous mutant mice (+/+ = 32, +/− = 78, −/− = 32; χ² = 1.38, df = 2, p = 0.50). The data presented below demonstrated that the mutant mice express no detectable Kvβ2 protein in the peripheral or central nervous systems.

**Kvβ2-null Mutants Exhibit Occasional Seizures, Shortened Life Spans, and Cold Swim-induced Tremors—Kvβ2-null mice are viable, live to adulthood, and are fertile but display occasional seizures. However, the life expectancy of Kvβ2-null mice is significantly shortened, with median survival of 255 days compared with heterozygous mice, which typically live to at least 400 days (Fig. 2; p = 0.0001; Kvβ2 +/−, n = 58; Kvβ2 −/−, n = 63).

Kvβ2-null mice also exhibit a cold swim-induced tremor. Mice were forced to swim in a tank of water for 2 min at 17 °C and then observed on a dry platform, where they displayed whole body tremors indicative of hyperexcitability (Table I). This phenotype was not observed after swimming in 37 °C water and was never observed in age-matched wild type controls. Interestingly, similar but somewhat stronger phenotypes were observed in Kv1.1-null (α subunit) mutants with respect to cold swim-induced tremors, reduced life span, and spontaneous seizures (24, 27). The phenotypic effects are therefore consistent with the possibility that Kvβ2-null mice have reduced expression of Kv1.1 or other Kv1 proteins caused by abnormal biogenesis or trafficking, as has been observed in heterologous expression systems (2). If so, one would expect that the absence of Kvβ2 would result in significant reductions in Kv1 α subunit expression levels, given that Kv1.1 heterozygotes display no tremors following exposure to cold water and exhibit normal life spans (data not shown), despite substantial reductions in mRNA (24) and protein (see Fig. 7).

**Kv1.1/Kv1.2 Trafficking and Biogenesis in Kvβ2-null Mutants—If Kvβ2 proteins play an important role in the early biogenesis and trafficking of Kv1 channels in vivo, in Kvβ2-null mice the channels should become trapped in the endoplasmic reticulum and exhibit a lack of Golgi-processed glycosylation (2). To assess the processing of Kv1 α subunits, we analyzed glycosylation patterns of Kv1.1 and Kv1.2 in Western blots of brains from wild type and Kvβ2-null mice. Both the wild type and Kvβ2-null mice show high (−88 kDa) and low (−60 kDa) molecular mass species for Kv1.2 (Fig. 3, left lanes) and Kv1.1 (data not shown).

The two bands observed for Kv1.2 are thought to represent different glycosylated forms; the low and high molecular bands correspond to the core-glycosylated and mature-glycosylated forms of the α subunit, respectively (2, 28). To verify this classification, we tested the susceptibility of these bands to two widely used glycosidases; EndoH and PNGase F. EndoH preferentially cuts the high mannose type glycans from asparagine found in core-glycosylated forms, whereas PNGase cleaves all asparagine-linked glycans. With EndoH, the high molecular mass band was unaffected, whereas the lower molecular mass band was reduced−3 kDa (Fig. 3). These results are consistent with previous reports that core glycosylation leads to 2−3-kDa increases in molecular mass (28). In contrast, PNGase treatment reduced the high molecular mass band by −20 kDa, consistent with it being the mature glycosylated form.

In Kvβ2-null mice the glycosylation patterns of Kv1.2 subunits in Kvβ2-null mice appeared comparable in size and relative proportions to those from wild type mice, and no bands were found at the size predicted for uncleaved Kv1.2 subunits trapped in the endoplasmic reticulum (2). Similar results were observed for the glycosylation patterns of Kv1.1 and Kv1.4 (data not shown). Thus, in contrast with results observed
in cultured cell lines, mouse Kv1α subunits are efficiently glycosylated in the absence of Kvβ2 in vivo.

Quantitative Analysis of Western Blots—To quantify the proportion of Kv1α subunits that are glycosylated in the mutant mice, we performed two types of quantitative Western blot analysis from whole brain extracts. Fig. 4A represents serial dilutions from brains of a representative pair of wild type and Kvβ2-null mice at p45, probed for Kv1.2 and detected by chemiluminescence, indicating little or no difference in the intensity of the high molecular mass band. Similar results were obtained in blots probed for Kv1.1 (data not shown). In a second type of analysis, we probed blots for Kv1.2 using 125I-labeled protein A with PhosphorImager detection and compared the ratio of the high versus low molecular mass bands in each sample from four independent sets of age-matched wild type and Kvβ2-null mice (Fig. 4B). Because each ratio (high versus low molecular mass bands) is calculated against an internal control (the low molecular mass band), these values would not be subject to minor differences in protein loading between lanes. Although the ratios for mutants ranged from 72 to 104% that of their age-matched wild type control in these four samples, the differences were not statistically significant (paired t test, \( t = 1.933, df = 3, p = 0.149 \)). These results confirmed that there is little or no change in the proportion of Kvα subunits that achieve mature glycosylation in the absence of Kvβ2.

Kvβ2/Kv1.1/Kv1.2 Expression in Cerebellum and Myelinated Nerves—The loss of Kvβ2 expression in vivo appears to have little effect on the biogenesis of Kv1 channels, and yet it has
significant phenotypic consequences for the animal. To verify the absence of Kvβ2 protein in the null animals, we evaluated central and peripheral nervous system tissues from Kvβ2-null mice using a monoclonal antibody directed against the N-terminal region of the protein. We focused on two regions of the nervous system where colocalization of Kv1.1/Kv1.2/Kvβ2 has been extensively studied: the terminals of cerebellar basket cells and the juxtaparanodes of myelinated nerves (29). As shown in Fig. 5, immunofluorescent staining for Kvβ2 in either cerebellum or myelinated nerve fibers control mice reliably shows prominent labeling of basket cell terminals or axons juxtaparanodes, respectively. However, only background fluorescence is detected in the Kvβ2-null mice. Moreover, neither full-length nor truncated Kvβ2 protein products were observed in Western blot analyses of whole brain lysates from the null mice (data not shown).

We subsequently examined Kv1α expression and distribution in cerebellar cortex by immunofluorescent staining. Consistent with our results from Western blot analysis of whole-brain lysates, which showed apparently normal glycosylation, Kv1.2 in the cerebellar cortex was properly concentrated, and at approximately comparable levels, in the basket cell terminals of Kvβ2-null mice (Fig. 6). Similar results were obtained for Kv1.1 (data not shown). Furthermore, no labeling of basket cell bodies was observed in the molecular layer as might have been expected if Kv1α subunits became trapped within the endoplasmic reticulum.

We further investigated the expression and targeting of Kv1α subunits in peripheral myelinated fibers by comparing the axial distribution of Kv1.1 along the paranode-node-paranode region of single fibers using quantitative fluorescence. Initially, we assayed the sensitivity of our method by analyzing paranodes from Kv1.1 heterozygous mice. In such mice, Northern blot analysis indicates a reduction of brain mRNA to 54% of wild type levels (24), and electrophysiological analysis indicates a reduction of Kv1.1 protein level in sciatic nerves (30). Fig. 7A represents fluorescence profiles, with valleys centered at the node (where Kv1.1 is absent) flanked by two peaks at the juxtaparanodes (where Kv1.1 density is highest), for the spatial distribution of Kv1.1 in single fibers from Kv1.1 heterozygote and wild type fibers. The averaged peak juxtaparanodal intensity from three Kv1.1 heterozygote is about 67% that of wild type, indicating that this method is capable of detecting reduction in Kv1 protein levels at the juxtaparanodes.

Similar comparison of Kv1.2 (Fig. 7B) and Kv1.1 (Fig. 7C) immunoreactivity in sciatic nerves from wild type and Kvβ2-null mice indicated that the expression and distribution of these α subunits were not appreciably affected by the absence of Kvβ2. For Kv1.1 and Kv1.2, the averaged ratio of the peak paranodal intensity of Kvβ2-null mice to wild type was 1.02 (three pairs of mice) and 0.954 (two pairs of mice), respectively. Taken together, the glycosylation, trafficking, surface expression, and clustering of Kv1 channels in vivo was not significantly affected by the absence of the Kvβ2 gene product.

Although Kvβ2 is by far the most abundantly expressed Kv1 β subunit in brain (1, 9), we examined whether up-regulation of related β subunits such as Kvβ1 might compensate for the loss of Kvβ2 through Western blot analysis of the Kvβ1 protein on whole brain extracts from wild type and Kvβ2-null mice. No evident up-regulation of Kvβ1 was observed (data not shown).

Electrophysiological Effects of Native and Mutant Y90F Kvβ2 in Xenopus Oocytes—To investigate the potential enzymatic role of Kvβ2 in vivo, we sought to create a mutation of the protein that would abolish enzymatic activity without altering the stability or potential nucleotide binding properties of the protein. Mutation of the Tyr90 residue, which is homologous to the tyrosine of other AKR family members where it plays a direct role in catalysis (31), to Phe90 was introduced into the
human Kv2 cDNA. Kv2-Y90F was subsequently coexpressed with human Kv1.4 channels in *Xenopus* oocytes as an assay for its stability and ability to interact with Kv1 channels (10). Equivalent mutations in several other AKR members to any amino acid residue other than histidine completely abolish enzymatic activity while having little effect on nucleotide binding or stability (31, 32). Both native and mutant Y90F subunit proteins accelerate the inactivation and increase the current amplitudes of Kv1.4 currents (Fig. 8) similar to that reported previously (10). This result indicates that the wild type β2 protein increases the rate of Kv1.4 inactivation and current amplitudes, either through nonenzymatic or atypical AKR enzymatic processes. It also suggests that the Y90F β2 protein is stable. Although the mutation would be expected to inhibit any inherent AKR-like enzymatic function, the protein should be able to carry out any other physiological functions including atypical AKR activity (see “Discussion”) and cofactor binding.

**Generation of Kv2 Y90F Mice**—To test the role of putative oxidoreductive catalysis by the Kv2 subunit in vivo, mice containing the Y90F point mutation of the Kcnab2 gene were generated in two steps. Following electroporation of the targeting vector (Fig. 1B) into ES cells, 90 clones were isolated that were doubly resistant to G418 and FIAU. Seven of these clones contained the mutated Kcnab2 locus (data not shown). One clone (K91) was subsequently expanded and transiently transfected with a Cre recombinase expression vector; subsequent Southern blot analysis identified 17 of 96 subclones having undergone excision and loss of PGKneo (data not shown). One subclone (K113) was chosen to generate chimeric founder mice, which subsequently demonstrated germline transmission of the mutant allele. Mating F1 heterozygotes yielded the expected Mendelian ratios of wild type, heterozygous, and homozygous mutant mice (Table I and Fig. 2; for life span analysis, Y90F/Kcnab2+/− and Y90F/Y90F−/−.

**Y90F Kv2 Mice Do Not Show Phenotypic Alterations Observed in Kv2-null Mutants**—The Y90F mutant mice were not observed to undergo spontaneous seizures and were similar to wild type for each pair was calculated and subjected to paired sample statistical analysis as described under “Results.” Approximately 50 μg of protein was loaded in each lane.

![Quantitative comparison of Kv1.2 expression in wild type and Kvβ2-null brains. A, Western blot analysis was carried out on brain extracts from wild type and Kvβ2-null littermates, with a representative pair shown here at p45. The samples were adjusted so that protein concentrations were the same and then subjected to serial dilutions before loading on the gel. After development with ECL and exposure to film, the membrane was stained with Ponceau S to reveal total protein and comparable loading between wild type and mutant lanes. 16 μg of total protein was loaded in the 1:1 lane. B, Western blot analysis using 125I detection was carried out on brain extracts from four pairs of age-matched wild type and mutant mice. The nitrocellulose membrane was coincubated with monoclonal antibodies to both Kv1.2 and the 145-kDa neurofilament protein (as an internal control for loading), then incubated with a rabbit anti-mouse antiserum, and then finally incubated with 125I-labeled protein A. After detection by PhosphorImager, the image was analyzed by Quantity-One (Bio-Rad). The ratio of the high molecular mass to low molecular mass bands for Kv1.2 were calculated for each mouse (after background subtraction), and then the ratio of mutant to wild type for each pair was calculated and subjected to paired sample statistical analysis as described under “Results.” Approximately 50 μg of protein was loaded in each lane.

| Age | Kvβ2 genotype | dilution |
|-----|----------------|----------|
| P43 | +/- 1:1 +/+    |          |
| P45 | +/- 1:2 +/+    |          |
| P31 | +/- 1:4 +/+    |          |
| P35 | +/- 1:8 +/+    |          |
|     | +/- 1:16 +/+   |          |
|     | +/- 1:1 +/+    |          |
|     | +/- 1:2 +/+    |          |
|     | +/- 1:4 +/+    |          |
|     | +/- 1:8 +/+    |          |
|     | +/- 1:16 +/+   |          |

![Gene Targeting of Kvβ2 in Mice](13224)
Y90F/Y90F

These results strongly indicate that mutant phenotypes associated with the Kv2-null mutant mice involve loss of a nonenzymatic function or that Kvβ2 exhibits atypical AKR catalytic activity.

DISCUSSION

In this paper, we describe the generation of Kvβ2-null and Kvβ2-Y90F mutant mice to examine the in vivo function of Kvβ2, a major auxiliary subunit of Kv1α channels in the mammalian nervous system. Kvβ2-null mice display cold swim-induced tremors, occasional seizures, and reduced life spans similar to that observed in Kv1.1-null mice. We therefore examined whether the absence of Kvβ2 might result in a failure of Kv1.2 subunit expression and distribution in vivo. Normal localization is seen in the wild-type controls, but no detectable Kvβ2 protein is seen in the null mutants. Scale bar, 15 μm.

FIG. 5. Absence of Kvβ2 proteins in Kvβ2-null cerebellum and sciatic nerves. Cerebellar slices (top row, 3-month-old mice) or teased sciatic nerve fibers (bottom row, 4-month-old mice) were stained with monoclonal anti-Kvβ2 antibody in wild type (left panels) and Kvβ2-null (right panels) mice. Normal localization is seen in the wild type controls, but no detectable Kvβ2 protein is seen in the null mutants. Scale bar, 15 μm.

FIG. 6. Kv1.2 immunofluorescence in normal and Kvβ2-null cerebellum. Cerebellar slices stained with polyclonal anti-Kv1.2 antibody and visualized with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. Normal localization of Kv1.2 to the basket cell terminals is observed, with no abnormal concentration in the basket cell bodies. Age of mice: wild type, P26; Kvβ2-null, P28. Scale bar, 15 μm. g, granule cell layer; m, molecular cell layer.

Y90F/Y90F (n = 77). These results strongly indicate that mutant phenotypes associated with the Kvβ2-null mutant mice involve loss of a nonenzymatic function or that Kvβ2 exhibits atypical AKR catalytic activity.

FIG. 7. Quantitative expression and distribution of Kv1.1/Kv1.2 in sciatic nerves. Kv1.1 and Kv1.2 immunofluorescence centered around the node of Ranvier (arrow) measured with line scan (MetaMorph) from single fibers for the various genotypes shown. A, quantification of Kv1.1 subunit expression and distribution in P28 wild type (n = 146 single fibers from three mice) and Kv1.1 heterozygous (n = 127 single fibers from three mice) littermates detect the predicted reduction in Kv1.1 in fibers of Kv1.1 heterozygotes. B, Kv1.2 expression and distribution in P42 wild type (n = 62 single fibers from two mice) and Kvβ2-null (n = 83 single fibers from two mice) littermates. C, Kv1.1 expression and distribution in P42 wild type (n = 150 from three mice) and Kvβ2-null (n = 149 from three mice) littermates. Error bars indicate the standard error.
roughly equal intensity, with a significant amount of Kv1.2 immunoreactivity retained in an intracellular compartment (likely to be endoplasmic reticulum). When coexpressed with Kvβ2, most of the Kv1.2 was in the glycosylated 66-kDa band, and stronger Kv1.2 immunoreactivity was observed at the cell surface. Shi et al. (2) therefore proposed that the primary physiological role of Kvβ2 is to facilitate the folding, glycosylation, and trafficking of Kv1 proteins to the cell membrane. More recently, Campomanes et al. (35) found that Kvβ subunits promoted axonal targeting of transfected Kv1.2 in primary neuronal cultures.

In contrast to the studies carried out in cultured cells, the absence of Kvβ2 in vivo does not lead to a detectable retention of Kv1.1/Kv1.2 immunoreactivity in the cell bodies of the cerebellar basket cells. Furthermore, our immunofluorescence studies show that both the clusters of Kv1.1/Kv1.2 proteins at the basket cell terminal and the juxtaparanodal regions of myelinated axons are not affected in the Kvβ2-null mutants. In addition, our Western blot analysis failed to reveal accumulation of unglycosylated species for Kv1.1 and Kv1.2 in Kvβ2-null mutants, suggesting that the Kv1α subunits are glycosylated efficiently in vivo without Kvβ2. Our results are most consistent with the data of Nagaya and Papazian (36), who found that the β2 subunit did not increase the rate or extent of Shaker protein maturation in transfected mammalian cells.

We considered two ways in which a chaperone-like role of Kvβ2 might have eluded our detection. First, other Kvβ subunits such as Kvβ1 may compensate for the loss of Kvβ2. However, this appears unlikely because Kvβ1 is normally absent in the basket cell terminals and the juxtaparanodal regions of myelinated fibers, and we detected no significant up-regulation of Kvβ1 in Western blot analysis of whole brain lysates from Kvβ2-null mice. Although a third Kvβ subunit has been identified in rat brain, little is known regarding its cell specificity, level of expression, and ability to associate with or assist in trafficking of Kv1α subunits in vivo (6). Second, our biogenesis analysis was performed on mice between the ages of P30–P40, and given the reduced life span of mutant mice older than 5 months, we cannot exclude the possibility that a significant biogenesis defect emerges in older mice.

Kv′ Channel Subunit Composition—Most individual neurons expressing Kv1 channels appear to express multiple family members, and some Kv1α subunits may facilitate efficient surface expression of heteromultimeric Kv1 channels in the absence of Kvβ2 (37). For example, homotetramers of Kv1.2 and Kv1.4, but not Kv1.1, are expressed efficiently on the surface of mammalian cell lines; Kv1.1/Kv1.2 or Kv1.1/Kv1.4 heterotetramers are expressed more efficiently than Kv1.1 is by itself. Intrinsic differences in the trafficking of specific Kv1α subunits may be regulated to some degree by Kvβ2, and its absence might therefore lead to changes in the composition of native multimeric Kv1 channels and contribute to the phenotypic effects observed in Kvβ2-null mice.

Loss of Kvβ2 might also affect the formation of channels between Kv1α subunits and those from different families, such as eag (38) or Kv4 (3). Alternatively, the absence of Kvβ2 may change the ability of Kv4 to interact with other known binding partners, including the KChIP family of proteins (39). Studies of Kv channels in the Drosophila mutant hyperkinetic (which lack K′ channel β subunits) have shown altered sensitivity to K′ channel blockers (40), consistent with a change in Kvα subunit composition of native channels in the mutant. Preliminary studies of the neuromuscular junction in our Kvβ2-null mutant mice revealed a dramatically heightened sensitivity to TEA2, whether this altered pharmacology reflects a change in the Kvα composition of native K′ channels in the Kvβ2-null mice remains to be established.

Kv′ Channel Kinetics—Another possible mechanism that could contribute to the phenotype of Kvβ2-null mice is alteration of K′ channel kinetics caused by modulation of K′ channel inactivation. Despite the absence of the ball-and-chain motif to rapidly inactivate α subunits (the N terminus of Kvβ2 is shorter than those of Kvβ1 and Kvβ3), Kvβ2 has been shown to modulate α subunit kinetics directly, as has also been shown for Kvβ subunits in Drosophila (15). Kvβ2 accelerates Kv1.4 inactivation (10, 41), accelerates Kv1.5 activation, and shifts the Kv1.1 and Kv1.5 activation threshold (11). Kvβ2 could also modulate α subunit inactivation indirectly by competing with other β subunits that bind to α subunits. For instance, Kvβ2 alters the kinetic effects of Kv1 channel inactivation induced by Kvβ1 in coexpression experiments (10, 12, 13). Deletion of Kvβ2 might favor saturation of Kvβ1 binding to some individual α subunits, resulting in faster inactivation of a proportion of K′ currents. Faster inactivation could lead to prolongation of the action potential, faster firing frequencies, and hyperexcitability.

Do Kvβ Subunits Have an Enzymatic Function?—Kvβ1 subunits are members of an aldo-ketoreductase enzyme superfamily that catalyze the oxidation/reduction of a wide range of physiologic substrates (14, 15, 31, 42), and crystallographic and binding studies reveal a tightly bound NADPH cofactor (17, 18). Although sequence homology, structural resolution, and critical residue conservation have all strongly suggested Kvβ2 as a potential catalytic AKR, no specific enzyme activity has been directly identified for the Kvβ2 protein to date. The enzymatic activity of Kvβ2, if it exists, may be difficult to investigate for a number of reasons, including the identity of a potential substrate and the hypothesis that the catalytic activity of Kvβ2 may be induced by the activity of functional (and therefore intact) Kvα subunits (14, 17). It is difficult if not impossible to assay a large number of potential substrates biochemically given the potential necessity for reconstitution of...
Kvα/β complexes with active electrophysiological stimulation. We therefore specifically chose to address this question using genetic methods and focusing on what is widely accepted as the essential residue for AKRs, Tyr. It is not redundant and that compensation from other Kv genes, if present, is limited and cannot provide full functional restoration. No apparent defects could be detected in biogenesis and trafficking of Kv1.1/Kv1.2 in the Kvβ-null mouse in the cerebellar basket cells and the myelinated fibers, nor were any alterations detected in glycosylation of Kv1 channels in whole brain lysates. These results suggest that the abnormal excitability phenotype in Kvβ-null mouse results from alterations in K⁺ channel functions that are still not well characterized and differ from the chaperone-like effects reported in heterologous systems (2, 10, 28, 44, 49).

However, alterations in the composition and/or physiological properties of native Kv channel α/β complexes may contribute to the observed phenotype of Kvβ-null mice. In addition, the lack of similar phenotypic effects in Kvβ-Y90F mice indicate that typical AKR oxido-reductive catalytic activity is unlikely to be the primary physiological role of Kvβ gene products.

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