Voltage-gated Kv1 potassium channels consist of pore-forming α subunits and cytoplasmic Kvβ subunits. The latter play diverse roles in modulating the gating, stability, and trafficking of Kv1 channels. The crystallographic structure of the Kvβ2 subunit revealed surprising structural homology with aldo-keto reductases, including a triosephosphate isomerase barrel structure, conservation of key catalytic residues, and a bound NADP⁺ cofactor (Gulbis, J. M., Mann, S., and Mackinnon, R. (1999) Cell 90, 943–952). Each Kv1-associated Kvβ subunit (Kvβ1.1, Kvβ1.2, Kvβ2, and Kvβ3) shares striking amino acid conservation in key catalytic and cofactor binding residues. Here, by a combination of structural modeling and biochemical and cell biological analyses of structure-based mutations, we investigate the potential role for putative Kvβ subunit enzymatic activity in the trafficking of Kv1 channels. We found that all Kvβ subunits promote cell surface expression of coexpressed Kv1.2 α subunits in transfected COS-1 cells. Kvβ1.1 and Kvβ2 point mutants lacking a key catalytic tyrosine residue found in the active site of all aldo-keto reductases have wild-type trafficking characteristics. However, mutations in residues within the NADP⁺ binding pocket eliminated effects on Kv1.2 trafficking. In cultured hippocampal neurons, Kvβ subunit coexpression led to axonal targeting of Kv1.2, recapitulating the Kv1.2 localization observed in many brain neurons. Similar to the trafficking results in COS-1 cells, mutations within the cofactor binding pocket reduced axonal targeting of Kv1.2, whereas those in the catalytic tyrosine did not. Together, these data suggest that NADP⁺ binding and/or the integrity of the binding pocket structure, but not catalytic activity, of Kvβ subunits is required for intracellular trafficking of Kv1 channel complexes in mammalian neurons and for axonal targeting in neurons.

Voltage-dependent potassium channels of the Shaker or Kv1 family play a fundamental role in the mammalian nervous system by determining resting membrane potential, frequency of action potential firing, and neurotransmitter release (2). Mammalian Kv1 channels are composed of four voltage-sensing and pore-forming transmembrane Kv1 α subunits, and up to four cytoplasmic Kvβ subunits (3). A tremendous diversity in Kv channel structure arises from the combinatorial assembly of the products of 18 α subunit and 4 β subunit genes present in the mammalian genome (4). Moreover, different Kv1 α and Kvβ subunit combinations yield functionally distinct potassium channels (5). Different Kvβ subunits, namely Kvβ1.1, Kvβ1.2, Kvβ2, and Kvβ3, have distinct effects on channel function, most obviously on fast inactivation because of the “ball” inactivation peptide present on the N terminus of some but not all Kvβ subunits (6). The distinct N-terminal domains also mediate subtype-specific interaction with the cytoskeleton (7), which can further impact Kvβ subunit effects on inactivation (8).

Outside of these distinct N-terminal domains, the primary sequence of Kvβ subunits is highly conserved, such that all Kvβ subunits exhibit >85% amino acid identity over this ∼330-amino acid “core” region. Analysis of the deduced amino acid sequence of this core domain led to the proposal (9) that Kvβ subunits may be members of the aldo-keto reductase (AKR) famille enzyme family, which reduce aldehyde or ketone functional groups to primary or secondary alcohols. The crystal structure of the Kvβ2 core domain extended this analysis and revealed remarkable structural similarity to AKR family members (1). The core domain of Kvβ2 consists of a 4-fold symmetrical triosephosphate isomerase barrel structure with bound NADP⁺ cofactor. The essential features of catalysis by AKRs involve hydride transfer from the nicotinamide ring to a carbonyl carbon of the substrate, followed by proton transfer from a donor group on the enzyme to the carbonyl oxygen of the substrate. The Kvβ2 structure (1) predicts that Tyr-90 acts as the proton donor of Kvβ2 (Fig. 1) in analogy to the Tyr-48 residue of human aldose reductase (10). Residues Trp-57, Arg-189, Trp-243, Ser-244, and Arg-264 are important for catalytic activity and Kv1 channel inactivation may be coupled (11). One subsequent study has revealed that catalytic and cofactor binding site mutants are deficient in their ability to confer N-type inactivation to Kv1.5, suggesting that Kvβ AKR activity and Kv1 channel inactivation may be coupled (11). The vast majority of Kv1 channel complexes in mammalian brain have associated Kvβ2 subunits (12–14), which do not confer N-type inactivation on associated Kv1 channels (6). The interaction of Kv1 α and Kvβ subunit polypeptides is an early event in Kv1 channel biosynthesis, occurring in the endoplasmic reticulum (ER) (15, 16). This interaction is quite stable, and subsequent subunit exchange is not observed (15, 17). The

Received for publication, October 25, 2001, and in revised form, December 15, 2001 Published, JBC Papers in Press, December 17, 2001, DOI 10.1074/jbc.M110276200

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 277, No. 10, Issue of March 8, pp. 8298–8305, 2002
Printed in U.S.A.
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This paper is available online at http://www.jbc.org
interaction with Kvβ2 subunits results in increased stability of Kv1.2 α subunits and effects on cotranslational addition of N-linked oligosaccharides to Kv1.2 which are consistent with more efficient α subunit folding in the ER (15). Increased surface expression of Kv1 α subunits was also observed upon Kvβ2 coexpression, as revealed by staining intact cells with external antibodies (15, 18), 125I-labeled dendrotoxin binding (15), and clustering of cell surface channels by PSD-95 (19). Similar Kvβ2-mediated effects on surface expression were observed for Kv1.1-Kv1.2 heteromeric channels (20). Increases in the amplitude of potassium currents in Xenopus oocytes expressing certain Kv1 α subunits have also been observed upon Kvβ2 coexpression (21). These studies together imply that in addition to the role of Kvβ subunits as components of plasma membrane Kv channel complexes, Kvβ subunits may play a fundamental biosynthetic role in regulating intracellular trafficking and surface expression of Kv channels.

In this study, we first addressed whether all members of the Kvβ subunit gene family, each of which contains the highly conserved core region with key catalytic site and cofactor binding site residues, could affect Kv1.2 channel intracellular trafficking and surface expression as was observed previously for Kvβ2 (15). Despite dramatic differences in their effects on channel gating, each of the Kvβ subunits displayed robust trafficking effects. A recent study has shown that a double mutation (D85A/Y90F) in the Kvβ2 catalytic domain reduces the ability of Kvβ2 to promote plasma membrane trafficking of Kv1.4 in Xenopus oocytes (22). Here we address further the role for AKR activity in Kv1 channel trafficking by mutating only the side chain hydroxyl group of this critical catalytic tyrosine residue in Kvβ1.1 (Y124F) and Kvβ2 (Y90F), and using a variety of biochemical and cell biological assays to analyze the effects of this mutation on Kv1.2 trafficking in mammalian cells. We also investigate the role of key cofactor binding site residues in Kvβ-mediated trafficking of Kv1.2 through structure-based mutagenesis. Finally, we show that Kvβ subunits that enhance cell surface expression of Kv1.2 in COS-1 cells can also induce the targeting of Kv1.2 to axons in cultured hippocampal neurons, recapitulating the subcellular localization of Kv1.2 observed in mammalian central and peripheral neurons.

**EXPERIMENTAL PROCEDURES**

**Materials**—All materials not specifically identified were purchased from Sigma or Roche Molecular Biochemicals.

**Antibodies**—Rabbit polyclonal (Kv1.2C, pan-β, Kvβ2/N) and mouse monoclonal (anti-Kv1.2: K14/16, anti-pan-β: K25/70, anti-Kvβ2: K17/70, anti-Kvβ1.1: K9/40) antibodies against Kv1 and Kvβ subunit polypeptides have been described previously (15, 23–25).

**Generation of Kvβ Point Mutants**—Point mutants were generated using the QwikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as per the manufacturer’s instructions.

**Analyses of Transfected COS-1 Cells**—Cells were transfected with mammalian cDNA expression vectors for various Kv channel α and β subunit polypeptides (26) by the calcium phosphate precipitation method (27). Cells expressing various amounts of Kv1 α and Kvβ subunits were stained 48 h post-transfection using either a standard immunofluorescence staining protocol for permeabilized cells (27) or a surface immunofluorescence staining protocol for intact cells (15, 20). Cells were viewed under indirect immunofluorescence on a Zeiss Axioskop2 microscope. Surface versus total immunofluorescence staining was scored under narrow wavelength fluorescent and Texas Red filter sets. Surface expression index (SEI) values were determined as the percentage of Kv1.2-transfected cells with Kv1.2 surface staining and represent the mean ± S.D. determined from 100 transfected cells from each of three dishes. Fluorescent images of cells were captured into Adobe Photoshop from either a SPOT (Diagnostic Instruments, Sterling Heights, MI) or a Zeiss Axiocam (Oberkochen, Germany) cooled CCD 24-bit color digital camera mounted on a Zeiss Axioskop 2 microscope with a 40×, 1.3 numerical aperture plan neofluar objective, or a 63× 1.25 numerical aperture plan neofluar objective, using the software supplied with the cameras.

Confocal images were generated on a Zeiss LSM510 laser scanning confocal microscope system with an Axiovert microscope using a 63× 1.25 numerical aperture plan neofluar objective. Alexa 594 (red) immunofluorescence signals were obtained using a 560 nm long pass filter after excitation with a helium neon laser at 543 nm. Alexa 488 (green) immunofluorescence signals were obtained using a 505–530 band pass filter after excitation with an argon laser at 488 nm. Red and green signals were generated and collected individually on a frame-by-frame basis. Similar pinhole sizes and amplifier settings were used to obtain all images, and no further manipulation of image files was performed. Each individual image represents a three-dimensional projection of the entire cell derived from a z-series taken as an average of eight sweeps at 1024 × 1024 resolution and viewed en face relative to the apical surface. Projections were exported as Photoshop files for presentation. SDS-PAGE and immunoblotting analyses of cell lysates prepared from transfected cells were performed as described (15, 20). For proteinase K digestion, transfected cells were washed three times with ice-cold phosphate-buffered saline. Each 35-mm dish was incubated with 10 mM HEPES, 150 mM NaCl, and 2 mM CaCl2 (pH 7.4) with or without 200 μM EDTA. This was followed by three washes in ice-cold phosphate-buffered saline containing 6 mM phenylmethylsulfonyl fluoride and 25 mM EDTA. This was followed by three washes in ice-cold phosphate-buffered saline. Cell lysates were prepared and analyzed by immunoblot as described above.

**Primary Hippocampal Cultures**—Hippocampal cultures were prepared as described previously (29, 30) with modifications (31). Cover-
medium gently and incubated at 37 °C for 15 min at room temperature. The DNA-PLUS-LipofectAMINE reagent complexes were added to face up coverslips in individual wells of six-well tissue culture plates. Complexes were mixed into the medium gently and incubated at 37 °C with 5% CO₂ for 48 h. Cells expressing various amounts of Kv1.2 α and Kvβ subunits were stained 48 h post-transfection using a standard immunofluorescence protocol (20, 27, 31). Cells were viewed under indirect immunofluorescence on a Zeiss Axiophot microscope, as described above. Axonal versus total Kv1.2 staining was scored under narrow wavelength fluorescein and Texas Red filter sets. Axonal Expression Index values were determined as the percentage of transfected cells with Kv1.2 staining of fine caliber MAP2(−) processes (axons) and represent the mean ± S.D. determined from 50 transfected cells from each of three dishes.

RESULTS

All Kvβ Subunits Promote Surface Expression of Kv1.2 α Subunits—All Kvβ subunits have a highly conserved ~330-amino acid core domain that follows divergent N termini of 30–70 amino acids. Previous studies have shown that coexpression with either Kvβ1.1 or Kvβ2 increased 125I-dendrotoxin binding in cells expressing Kv1.2 (15) and that the autonomous highly conserved core domain is sufficient to mediate increases in current amplitude of coexpressed Kv1.4 α subunits (21). We addressed whether each wild-type Kvβ subunit harboring this core domain would display the effects on Kv1.2 intracellular trafficking and surface expression observed previously for Kvβ2 (15). Rat Kvβ1.1 and Kvβ2 (26, 32), human Kvβ1.2 (33), and rat Kvβ3 (34) were coexpressed with the rat Kv1.2 α subunit in COS-1 cells. We performed double immunofluorescence staining using antibodies specific for Kv1.2 extracellular (prior to detergent permeabilization) and intracellular (after detergent permeabilization) domains to determine the relative amount of Kv1.2 surface expression in the transfected cells. In the absence of any Kvβ subunit coexpression, the majority of Kv1.2-expressing cells exhibited robust perinuclear Kv1.2 staining, which we have previously shown corresponds to ER-localized Kv1.2 (20), and no detectable surface staining (Fig. 2A, top panel). A minority of the transfected COS-1 cells expressing Kv1.2 alone had Kv1.2 surface staining (Fig. 2B). Upon coexpression with Kvβ1.1 the vast majority of cells (Fig. 2B) now exhibited robust Kv1.2 surface staining (Fig. 2A, bottom panel). In fact, regardless of the Kvβ subunit used, significant increases in the number of cells expressing Kv1.2 on the cell surface were observed upon Kvβ subunit coexpression (Fig. 2B). Thus, despite disparate effects of these different Kvβ subunits on Kv1 channel inactivation (32–34), each Kvβ subunit affects intracellular trafficking and surface expression of coexpressed Kv1.2 channels.

The deduced amino acid sequence of the Kv1.2 polypeptide contains a single N-linked glycosylation site located in the extracellular loop between transmembrane segments S1 and S2 (4). Differences in processing of N-linked oligosaccharide chains at this site can be used as an independent biochemical marker of intracellular trafficking via shifts in apparent molecular mass on SDS-PAGE gels (20, 35). Lower (~60 kDa) molecular mass forms of Kv1.2 carry simple high mannose chains and correspond to ER pools, whereas higher (~86 kDa) molecular mass forms, similar to Kv1.2 in rat brain membranes, carry sialylated complex chains and correspond to Golgi and plasma membrane pools (20). We found the bulk of Kv1.2 present in COS-1 cells expressing Kv1.2 alone in the lower molecular mass ER form (Fig. 2C) (20). However, coexpression with Kvβ2 yields a dramatic increase in amount of Kv1.2 in the higher molecular mass post-ER form, similar to the Kv1.2 found in rat brain (Fig. 2C). Coexpression with other Kvβ subunits yielded similar increases in the amount of Kv1.2 that had undergone post-ER processing of the N-linked oligosaccharide chain (data not shown). Together, these cell biological and biochemical analyses reveal that coexpression with
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Fig. 3. Kvβ2 catalytic site mutants reveal distinct effects on Kv1.2 surface expression. A, COS-1 cells were cotransfected with Kv1.2 and either RBG4 (−), wild-type Kvβ2, Kvβ2 Y90F, or Kvβ2 Y90A at 1:4 cDNA ratios. Transfected cells were analysed for Kv1.2 SEI as described in the Fig. 2 legend. * indicates $p < 0.02$ versus Kv1.2– and RBG4-transfected cells. B, COS-1 cells were transfected as in A and were treated with externally applied proteinase K (+) or buffer alone (−) as indicated above the lanes. Cell lysates were analyzed for Kv1.2 by SDS-PAGE and immunoblotting. The arrow labeled S indicates the immunoblot position of surface Kv1.2, and the arrow labeled I indicates the immunoblot position of intracellular Kv1.2. * indicates the immunochemical mass standards.

Kvβ subunits increases the cell surface expression of Kv1.2 α subunits with a concomitant decrease in retained ER pools.

Effect of Kvβ2 Catalytic Site Mutants on Kv1.2 Surface Expression—Previous studies on AKR family members have revealed a critical role for the hydroxyl group of a specific catalytic site tyrosine in proton transfer to the substrate carbonyl oxygen (10). The crystal structure of Kvβ2 (1) defines Tyr-90 as this residue in Kvβ2 (Fig. 1). Mutation of the analogous tyrosine (Tyr-48) in human aldose reductase to phenylalanine (Y48F), which preserves both the spatial fit and hydrophobicity of the tyrosine residue yet eliminates the possibility of any general acid catalysis by removing the tyrosine hydroxyl group, led to a completely inactive enzyme (10). To determine the role of the potential Kvβ subunit enzymatic activity in Kv1 channel trafficking experimentally, we made the analogous tyrosine to phenylalanine mutation (Y90F) in Kvβ2. A less subtle Kvβ2 Y90A mutation was also made.

When coexpressed with Kv1.2 in COS-1 cells at a fixed α/β subunit cDNA ratio of 1:4, the Kvβ Y90F mutant was indistinguishable from wild-type Kvβ2 in its effects on promoting increased surface expression of Kv1.2 (Fig. 3A). In contrast, the Kvβ2 Y90A mutant failed to induce increased surface expression of Kv1.2 and was not significantly different from no Kvβ subunit addition (Fig. 3A). In these experiments the steady-state expression level of wild-type Kvβ2 and the two different mutant proteins was similar (data not shown). Moreover, dose-response curves show that the respective phenotypes of the wild-type and mutant Kvβ2 isoforms shown in Fig. 3A are maintained over an 8-fold range (1:1–1:8) of α/β subunit cDNA ratios (data not shown). As such, the lack of activity of Kvβ2 Y90A is not simply the result of differences in expression level.

To confirm and extend these cell biological assays, quantitative biochemical assays of Kv1.2 cell surface expression were performed. Cells expressing Kv1.2 with and without coexpressed wild-type and mutant Kvβ2 subunits were treated with proteinase K, a relatively nonspecific protease that when externally applied cleaves the ectodomain of cell surface proteins (28). Cell extracts prepared from proteinase K-treated and control cultures were then analyzed by SDS-PAGE and immunoblotting. We have established previously the utility of this assay in analyses of trafficking and surface expression of Kv1α subunits (20, 36). As shown in Fig. 3B and discussed above, lysates prepared from untreated cells expressing Kv1.2 and wild-type Kvβ2 have enhanced levels of the higher apparent molecular mass (sialylated Golgi/plasma membrane) form of Kv1.2, relative to cells expressing Kv1.2 alone. Lysates from cells expressing Kvβ2 Y90F have levels of this post-ER form of Kv1.2 similar to those found in cultures coexpressing wild-type Kvβ2, whereas extracts from cells coexpressing Kvβ2 Y90A were similar to those without Kvβ subunit coexpression (Fig. 3B). In each case treatment of the cultures with externally applied proteinase K eliminated the bulk of the 86-kDa form of Kv1.2 (Fig. 3B), verifying the cell surface localization of this polypeptide pool. Digestion with sialidase confirmed that the higher apparent molecular mass pool in each sample carried sialic acid (data not shown) because of the processing of the N-linked oligosaccharide chains on this Golgi/plasma membrane pool of Kv1.2. Together these data show that the Kvβ2 Y90F mutation, which, based on previous studies, should eliminate any AKR activity of Kvβ2, is phenotypically identical to wild-type Kvβ2 in its effects on Kv1.2 trafficking, whereas Kvβ2 Y90A exhibits little or no trafficking activity.

When expressed in COS-1 cells, both wild-type Kvβ2 (7) and the Kvβ2 Y90F mutant exhibit a diffuse cytoplasmic localization (Fig. 4A). However, we found that the Kvβ2 Y90A mutant is present as perinuclear clusters suggestive of a misfolded phenotype (Fig. 4B). Moreover, the altered localization of Kvβ2 Y90A is conferred to coexpressed Kv1.2 (Fig. 4B). These results suggest that the Kvβ2 Y90A mutant retains its ability to interact with Kv1.2 but that this interaction does not allow for enhanced surface expression of Kv1.2. To determine directly whether the lack of ability of Kvβ2 Y90A to promote Kv1.2 surface expression was not simply the result of a lack of association, we performed coimmunoprecipitation experiments. As shown in Fig. 4C, each of the wild-type and mutant Kvβ2 subunits is found associated with Kv1.2, although the interaction of Kv1.2 with the Kvβ2 Y90A mutant is slightly less robust. Thus, the lack of ability of Kvβ2 Y90A to promote the surface expression of Kv1.2 is not simply the result of a lack of interaction.

Effect of Cofactor Binding Site Mutations on Kv1.2 Surface Expression—The other domain important in the potential AKR activity of Kvβ subunits is the binding site for the NADP+/NADPH cofactor (Fig. 1). NADPH cofactor would serve as the potential electron donor for substrate reduction. NADP+ was present in the crystallographic structure of bacterially expressed Kvβ2 (1), and residues lining the cofactor binding pocket are 100% conserved between Kvβ1.1 and Kvβ2 (32). We addressed the role of cofactor binding in Kvβ subunit function by analyzing Kvβ1.1 mutants carrying structure-based point mutations in the cofactor binding region (Fig. 1) for their ability to promote surface expression of Kv1.2. Mutations were made...
in 10 different residues lining the cofactor binding site, and in each case more than one substitution was made for each residue. When expressed in COS-1 cells, the subcellular distribution of each of these mutants was similar to wild-type Kvβ1.1 and consisted of staining associated with the actin cytoskeleton (7) and additional perinuclear staining (Fig. 5A). Similar loss of activity was observed upon mutation of Kvβ1.1 Tyr-124 (Trp-121 in Kvβ2) (8). These results suggest that mutation at a critical hydroxyl group from a key catalytic tyrosine residue does not affect the trafficking activity of either Kvβ1.1 or Kvβ2, although more drastic alterations of the side chain do reduce activity.

Effects of Kvβ Subunits on Kv1.2 Trafficking in Hippocampal Neurons—Kv1.2 α subunits are found associated with Kvβ2 subunits on axons and terminal fields in the mammalian central and peripheral nervous systems (12, 14, 38). We next used transfection of recombinant potassium channel subunits into low density cultures of embryonic rat hippocampal neurons to address the role of Kvβ2 subunits in axonal targeting of Kv1.2. These cultures do not express detectable Kvβ2 or Kvβ2 staining at the age of culture used for these experiments (20). Hippocampal neurons transfected with Kv1.2 alone express robust Kvβ2 staining associated with the soma and very proximal portions of the dendrites (Fig. 6A), suggesting that as in COS-1 cells, the majority of Kv1.2 is found in the ER. A similar staining pattern was obtained with antibodies specific for BiP, a resident ER protein (not shown). Hippocampal neurons transfected with wild-type Kvβ2 alone express robust Kvβ2 staining in the soma, dendrites, and in the MAP2(−) fine caliber axon (Fig. 6B).
Given the lack of axonal staining of introduced Kv1.2, we next addressed whether Kvβ2 subunit coexpression could induce the appropriate trafficking of Kv1.2 to axons. Hippocampal neurons cotransfected with Kv1.2 and Kvβ2 subunit cDNAs and subjected to double staining for the appropriate subunits, or for the dendritic marker MAP2 to distinguish MAP2(+) dendrites from MAP2(-) axons. Arrows in panels B and C point to fine caliber MAP2(-) processes = axons. A, neurons transfected with Kv1.2 alone (Kv1.2, green; MAP2, red). B, neurons transfected with Kvβ2 alone (Kvβ2, green; MAP2, red). C, neurons transfected with Kv1.2 plus wild-type Kvβ2 (Kv1.2, red; Kvβ2, green). D, neurons transfected with Kv1.2 plus Kvβ2 Y90A (Kv1.2, red; Kvβ2, green). The inset shows a higher magnification of the cell body to highlight Kvβ2 Y90A clusters. E, bar graph displaying the percentage of Kv1.2-transfected neurons that exhibit axonal Kv1.2 staining = axonal expression index (AEI, for details, see “Experimental Procedures”).

Our previous studies showed that both Kvβ1.1 and Kvβ2 coexpression increased surface dendrotoxin binding activity of Kv1.2-expressing COS-1 cells (15). Here we show that all identified Kv1-associated Kvβ subunits can promote increased surface expression of Kv1.2. The precise mechanism by which this is achieved remains to be elucidated. However, biochemical analyses presented here show that the relative proportions of Kv1.2 carrying the ER (high mannose) and post-ER (complex) forms of N-linked oligosaccharide chains are altered dramatically upon Kvβ subunit coexpression. This indicates that the effects of Kvβ subunits in increasing Kv1.2 surface expression are most probably the result of changes in the subcellular distribution of Kv1.2, as opposed to uniformly increasing expression levels with a resultant increase in cell surface levels.

Similar results have been observed for Kvβ2 effects on Kv1.4 surface expression in Xenopus oocytes (22). Whether the enhanced surface expression of Kv1.2 results from more efficient export of Kv1.2 from the ER or enhanced stability of cell surface Kv1.2 is not known. A recent study described an autonomously acting ER export signal (FCYENE) that, when appended to the cytoplasmic C terminus of Kv1.2, increased Kv1.2 surface expression (18). Interestingly, this appended ER export signal oc-
cluded the ability of Kvβ2 to promote Kv1.2 surface expression further (18), suggesting that the mechanism that underlies the Kvβ2-induced increases in Kv1.2 surface expression is the enhanced ER export of Kv1.2.

Previous studies of the enzyme kinetics and crystal structure of human aldose reductase (10) revealed a complete loss of enzymatic activity upon mutation of the tyrosine residue (Tyr-48) structurally homologous to Kvβ2 Tyr-90 and Kvβ1.1 Tyr-124. These and other studies of AKRs (45, 46) have shown that the tyrosine hydroxyl group, whose pKₐ is perturbed through association with a neighboring lysine and aspartate, acts as the proton donor during the protonation of the carbonyl oxygen group of the substrate. Thus, eliminating the critical side chain hydroxyl group in the Kvβ2 Y90F and Kvβ1.1 Y124F mutants, although retaining the overall hydrophobicity and size of the residue, should eliminate catalysis in Kvβs as it does in other AKRs. The complete lack of effects of the Kvβ2 Y90F and Kvβ1.1 Y124F mutations on any measurable aspect of Kv1.2 trafficking in COS-1 cells, and on axonal targeting in neurons, argues strongly against a role for AKR catalytic activity in these Kvβ subunit-mediated events. It should also be noted that the Kvβ1.1 Y124F mutation also had no effect on the ability of Kvβ1.1 to confer N-type inactivation to Kv1.5 (11). However, a catalytic site double mutation in Kvβ2 (D85A/Y90F) led to an observed loss of the Kvβ2-mediated enhancement of Kv1.4 plasma membrane trafficking in Xenopus oocytes (22). We have shown that Kv1.2 and Kv1.4 have inherently distinct trafficking characteristics in mammalian cells (20) because of differences in the sequence of a trafficking determinant located near the external mouth of the channel pore (36). Whether the differences between the findings of Peri et al. (22) and those presented here are the result of these inherent differences between Kv1.2 and Kv1.4 trafficking, differences between the oocyte and mammalian expression backgrounds, or unintentional effects of the Y90F/D85A double mutation on Kvβ2 structure, with a subsequent loss of Kvβ2 trafficking effects, is not known.

Given our results on the apparent lack of a requirement of catalytic activity for Kvβ-mediated trafficking effects, our observations on the dramatic and consistent effects of mutations in the NADPH cofactor binding site are somewhat surprising. The crystal structure of bacterially expressed Kvβ2 contained tightly bound NADP⁺ that presumably bound in vivo and remained tightly bound throughout the extensive purification and crystallization procedures (1). Having NADP⁺/NADPH bound may be critical to the initial folding and/or stability of the Kvβ subunit structure such that subunits without cofactor misfold and are degraded. A recent study showed that bound cofactor was critical to the proper folding of 2,5-diketo-6-D-glucronic acid reductase A, another member of the AKR family, with regions of the enzyme undergoing coordinated conformational changes of up to 8 Å when synthesized in the absence of cofactor (47). Fluorescence measurements have revealed that bacterially expressed Kvβ2 exhibits high affinity binding to both NADPH and NADP⁺ (48). A mutation in Kvβ2 analogous to our Kvβ1.1 cofactor binding mutation R298G (Kvβ2 R264G) resulted in a complete loss of NADPH binding (48). Interestingly, mutations at Kvβ2 residues analogous to Kvβ1.1 Arg-223, Trp-277, and Cys-282 did not affect NADPH binding (48), although we observed a loss of Kvβ1.1-induced trafficking effects. In only one case was the same mutation (W277A) analyzed, thus some of these differences may result from the differences in specific side chain substitution used in the two different studies. However, it is also possible that mutations in this highly conserved core domain induce structural alterations independent of cofactor binding which lead to loss of Kvβ1.1-mediated trafficking. Future analyses of NADP⁺/NADPH binding in our mutants which display altered trafficking of Kvβ subunit-associated complexes may help resolve these intriguing discrepancies.

The lack of effects of the discrete catalytic site mutations on the trafficking function of Kvβ subunits raises questions as to why each of the detailed structural features of AKR family members is present in the Kvβ2 structure, and why all of the important domains are so highly conserved in all Kvβ subunits. Certainly catalysis could be coupled to another aspect of Kvβ subunit function, such as modulation of N-type inactivation (11), although this does not explain the conservation of AKR structure in Kvβ2. Another possibility is that Kvβ subunits are “moonlighting” as bona fide AKRs in contexts outside their role as potassium channel subunits. This would be analogous to the enzymatic activity of gephyrin, a protein that associates with and clusters glycine receptor ion channels in neurons, but which in non-neuronal cells functions as an enzyme in molybdenum cofactor biosynthesis (49). A number of studies have suggested that to conserve genome size the same protein may be used for completely unrelated functions in different cells, depending on cell-specific protein-protein interactions affecting subcellular localization or quaternary structure (50). Because Kvβ subunits have a more widespread tissue expression than do Kv α subunits (NCBI Unigene), the catalytic activity of Kvβ2 may only be relevant in cells lacking Kv α subunits and where Kvβs function autonomously. Any physiological role of the putative catalytic activity of Kvβ subunits remains to be elucidated.

Our findings that Kvβ subunits mediate the trafficking of Kv1.2 to axons of cultured hippocampal neurons suggest a new role for cytoplasmic Kvβ subunits in Kv channel function. Immunoelectron microscopy studies (51) and experiments utilizing circumscribed excitotoxic lesions (14) have shown that Kv1.2-containing Kv1 channels are associated predominantly with axons and axon terminals in the mammalian nervous system. Moreover, these axonal Kv1 channels are associated extensively with Kvβ subunits, especially Kvβ1.1 and Kvβ2 (12, 14). Because Kvβ2 coexpression leads to axonal targeting of Kv1.2 not observed in cells expressing Kv1.2 alone, it is interesting to speculate that axonal targeting signals may exist on Kvβ2 which are critical to Kv1 channel localization in neurons. Although determinants of polarized and clustered localization of dendritic Kv2.1 potassium channels in both polarized epithelial Madin-Darby canine kidney cells (52) and cultured hippocampal neurons (31) have been characterized, similar studies of signals involved in the localization of axonal potassium channels have not been performed. A previous study of the localization of recombinant Kv1.4 α subunits expressed in Madin-Darby canine kidney cells revealed a basolateral localization (53). Based on the analogous membrane hypothesis (54), axonal Kv1.4 should assume an apical localization in Madin-Darby canine kidney cells, thus the predicted localization was not obtained. Another study using biologic gene transfer to express recombinant Kv1.4 in high density postnatal hippocampal cultures also found an aberrant somatodendritic localization for Kv1.4 (55). It should be noted that both of these studies were performed in the absence of Kvβ subunit coexpression. Future studies in cells coexpressing Kv1 α and Kvβ subunits may allow for a better understanding of the respective role of these component subunits in determining the subcellular localization of axonal potassium channels in neurons.

Acknowledgments—We thank Joan Speh and Dr. Gail Mandel for the use of the confocal microscope and Dr. JoAnne Engebrecht for a critical reading of the manuscript.
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J. Biol. Chem. 2002, 277:8298-8305.
doi: 10.1074/jbc.M110276200 originally published online December 17, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110276200

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