THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 18, Issue of May 2, pp. 11728–11735, 1997
Printed in U.S.A.
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Kvβ2 Inhibits the Kvβ1-mediated Inactivation of K^+ Channels in Transfected Mammalian Cells*

(Received for publication, January 13, 1997, and in revised form, February 14, 1997)

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Cloned auxiliary β-subunits (*e.g.* Kvβ1) modulate the kinetic properties of the pore-forming α-subunits of a subset of Shaker-like potassium channels. Coexpression of the α-subunit and Kvβ2, however, induces little change in channel properties. Since more than one β-subunit has been found in individual K^+ channel complexes and expression patterns of different β-subunits overlap *in vivo*, it is important to test the possible physical and/or functional interaction(s) between different β-subunits. In this report, we show that both Kvβ2 and Kvβ1 recognize the same region on the pore-forming α-subunits of the Kv1 Shaker-like potassium channels. In the absence of α-subunits the Kvβ2 polypeptide interacts with additional β-subunit(s) to form either a homomultimer with Kvβ2 or a heteromultimer with Kvβ1. When coexpressing α-subunits and Kvβ1 in the presence of Kvβ2, we find that Kvβ2 is capable of inhibiting the Kvβ1-mediated inactivation. Using deletion analysis, we have localized the minimal interaction region that is sufficient for Kvβ2 to associate with both α-subunits and Kvβ1. This mapped minimal interaction region is necessary and sufficient for inhibiting the Kvβ1-mediated inactivation, consistent with the notion that the inhibitory activity of Kvβ2 results from the coassembly of Kvβ2 with compatible α-subunits and possibly with Kvβ1. Together, these results provide biochemical evidence that Kvβ2 may profoundly alter the inactivation activity of another β-subunit by either differential subunit assembly or by competing for binding sites on α-subunits, which indicates that Kvβ2 is capable of serving as an important determinant in regulating the kinetic properties of K^+ currents.

The heterogeneity of voltage-sensitive potassium currents present in excitable and nonexcitable cells is essential for diverse biological functions (1–4). In addition to the large number of genes encoding the channel subunits and posttranslational modulations of channel protein, the diversity of potassium channels is further enhanced by the mix-and-match assembly of different subunits (5, 6). Within the large family of Shaker-like potassium channels, the selective subunit assembly includes heteromultimer formation of distinct pore-forming α-subunits and/or assembly of different kinds of subunits such as the α-subunits and hydrophilic cytoplasmic β-subunit(s) (7, 8). Together, these give rise to the vast heterogeneity of K^+ currents. Changes in expression of a given subunit may alter the composition of heteromultimers *in vivo*, which would allow a cell to tune its K^+ current system(s) during development and in response to changes in the cellular environment.

There are more than 60 cloned genes encoding functional Shaker-like α-subunits, which have been divided into several subfamilies. Among them, subunits in the Kv1 to Kv5 subfamily are capable of functional homomeric channels in heterologous systems, such as *Xenopus* oocytes (9–13). Four α-subunits within a given subfamily can form a functional channel either as a homotetramer or a heterotetramer (14, 15). In the case of auxiliary subunits in animals, four genes encoding β-subunits for Shaker-like potassium channels have been well characterized: Kvβ1, Kvβ2, Kvβ3 (which has now been suggested to be a splice variant of Kvβ1), and Hk (7, 8, 16–20). These β-subunits share at least 85% amino acid sequence identity in their COOH-terminal core regions, but differ significantly in length and sequence of the remaining NH_2-terminal regions. Despite the remarkable sequence similarity among different β-subunits, their functional effects are quite different. For example, coexpression of β-subunits with certain α-subunits in *Xenopus* oocytes induces pronounced alterations in channel kinetic properties, most noticeably acceleration of fast inactivation by either Kvβ1 or Kvβ3 (8, 17, 18–20). Kvβ2, on the other hand, binds to α-subunits, such as Kv1.2 (or RCK5) (21). However, it has little effect on inactivation of α-subunits such as Kv1.2 (RCK5) (7, 8, 22, 23). Recent data have shown that Kvβ2 is capable of increasing the surface expression of certain K^+ channels in transfected cells (24).

Biochemical evidence has indicated that there are more than one β-subunit present in each K^+ channel complex (21). Given that cloned β-subunits have different modulatory effects on α-subunits, it would be interesting to test whether different β-subunits can interact with each other, which could be an important mechanism to increase the diversity of potassium currents. To test this hypothesis, we have used the yeast two-hybrid system to study the interaction specificity of Kvβ2 with various α- and β-subunits. The functional consequences of heteromeric α-β and β-β interactions were evaluated by electrophysiological analyses.

MATERIALS AND METHODS

Vector Construction and Expression—Plasmid vector construction was performed according to standard recombinant DNA techniques (26). The vectors that express partial cDNA fragments were constructed by a high fidelity polymerase chain reaction cloning strategy according to the procedures described by Li *et al.* (27). The oligonucleotides used are listed in Table I. In yeast, the expression of different fusion proteins of α-subunits, Kvβ1, and Kvβ2 was carried out by inserting the corresponding cDNA fragments into the *Sma*I/*Not*I, *Sal*I/*Not*I, or *Bgl*II/*Not*I sites of pPC97 and pPC86 vectors (28–31). Construction of tagged

* This work was supported in part by grants from the National Institutes of Health, the Council for Tobacco Research, Inc., and by an American Heart Association-Pfizer award (to M. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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TABLE I
Sequences of oligonucleotide primers

Oligonucleotides	Sequences[a]	Gene	Amino acid position
ML1005:	GCACCATGGAGTCGACAATGTATCCGGAATCAACCACG	Kvβ2	1
ML1006:	CAGGCGGCCGCTTAGGATCTATAGTCCTTTTTGC	Kvβ2	367
ML1015:	CAGGTCGACTGAATTCTACAGGAATCTGGGC	Kvβ2	39
ML1021:	CAGGAATTCAAGATCTCAATGCAAGTCTCCATAGC	Kvβ1	1
ML1022:	CAGAGATCTCATATAGGAATCTTGGG	Kvβ1	73
ML1023:	CAGAGATCTTGGTGATTCTGGGGAGC	Kvβ1	131
ML1024:	CAGAGATCTACAGCAACACCCCCATGG	Kvβ1	196
ML1025:	CAGGCGGCCGCTAATATTTTCCTGAAATAATTCC	Kvβ1	289
ML1026:	CAGGCGGCCGCTACAGGCACCATGCCACAGC	Kvβ1	346
ML1027:	CAGGCGGCCGCTAGCCTTAGGATCTATAGTCC	Kvβ1	401
ML1031:	CAGGAATTCGAAGCAATGCAAGTCTCCATAGCC	Kvβ1	1
ML1032:	CAGGAATTCGGATCTATAGTCCTTTTTGC	Kvβ1	401
ML1044:	CAGGAATTCGGCCCCATGTATCCGGAATCAACC	Kvβ2	1
ML1045:	CAGGAATTCGGATCTATAGTCCTTTTTGC	Kvβ2	367
ML1069:	CAGGAATTCGGCCCCATGTACAGGAATCTGGGCAAATC	Kvβ2	39
ML1079:	CAGGAATTCGCCCTCGTTCCTCAGG	Kvβ2	316

[a] The underlined sequence represents coding sequence or complementary to coding sequence of the indicated gene.

Kvβ1, Kvβ2, and Kvβ2 mutants was carried out by fusing the coding fragment with a peptide which represents a heart muscle kinase recognition sequence and the 12CA5 monoclonal epitope (PYDVPDYASL), at the end of the coding sequences before the stop codon (28). Transient expression and immunodetection of potassium channel subunits were performed according to our published protocol (28, 51).

Methods for the Yeast Two-hybrid System—The procedures were performed according to our published protocol (28, 29) using HF7c yeast strain (*MATα ura3–52 his-200 ade 2–101 lys2–801 trp1–901 leu2–3, 112 gal4–542 gal80–538 LYS2::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3 URA3::GAL4* $_{17mer(x3)}$-*Cycl*$_{TATA}$-*lacZ*) as host cells (25).

Whole-cell Patch Clamp Recording—Whole-cell voltage clamp recordings were carried out according to the published protocol (28, 33). The liquid junction potential was calculated to be 7.2 mV using JPCalc software (34) and corrected from the holding potential. Typically, the cell was held at −87 mV, and the holding voltage was then jumped from this potential up to a test potential of +53 mV in 10-mV increments for 300 ms. Current data were filtered at 1 kHz, digitized at 100-μs intervals. Data analysis was done using clampfit software (pCLAMP6, Axon Instrument, Foster City, CA).

Statistical Analysis—A standard formula to compare two proportions was used to determine the statistical significance of different pairs of sample sets (35). We have used $\tau = 64$ ms as the cutoff to separate populations with or without fast inactivation. In this analysis, the one-sided z test statistics were calculated using the following formula $z = \theta 1\text{-}\theta 2 /\{\theta_p(1\text{-}\theta_p)[1/n_1 + (1/n_2)]\}^{0.5}$, where θ_1 and θ_2 are sample proportions that showed fast inactivation of each given group, θ_p is the weighted average of the sample proportions, and n_1 and n_2 are sample sizes.

RESULTS

Differential Modulation on α-Subunits by Kvβ1 and Kvβ2—The interaction between α-subunits and Kvβ1 has been studied in more detail. In particular, the α-β complex is assembled, at least in part, by the association of the conserved core regions in Kvβ1 with NAB_{Kv1} of α-subunits, a critical assembly motif located in the hydrophilic NH_2-terminal domains (28, 29, 36). The formation of an α-β complex presumably recruits the inactivation particle of Kvβ1 close to the "receptor" site, thereby either accelerating the rate of inactivation of α-subunits of Kv1.4 (RCK4) and ShB (or H4) or inducing inactivation of compatible α-subunits which lack intrinsic fast inactivation, such as ShBΔ(6–46) (28, 36). Because the formation of the α-Kvβ1 complexes is subfamily-specific, *i.e.* Kvβ1 binds only to the NH_2-terminal domains of Kv1 α-subunits (28), this allows Kvβ1 to selectively modulate a subset of α-subunits.

To investigate whether Kvβ2 alters the electrophysiological properties of α-subunits in transfected mammalian cells, we have constructed two plasmids that express either Kvβ1 or Kvβ2 with the 12CA5 monoclonal antibody tag fused at the COOH terminus of each coding sequence (see "Materials and Methods"). Both plasmids use the Kvβ1 5′-untranslated sequence. Thus, the two expression vectors are identical except for the amino acid coding sequence. Experiments utilizing these constructs permit better comparison of Kvβ1 and Kvβ2 expression and their ability to modulate α-subunits. By transient transfection in COS cells, we functionally expressed ShBΔ(6–46), a mutated ShB potassium channel that lacks the inactivation gate (37, 38). The Kvβ2 effects on this α-subunit were studied by whole-cell voltage clamp recording. Fig. 1*A* shows a series of traces obtained by stepping up from a holding potential of −87 mV to a final test potential of +33 mV in 20-mV increments. The recorded cells were transfected with ShBΔ(6–46) alone (*upper panel*), ShBΔ(6–46) in the presence of either Kvβ1 (*middle panel*), or Kvβ2 (*bottom panel*). In contrast to the ShBΔ(6–46) + Kvβ1 cotransfection in which we observed the Kvβ1-mediated inactivation (Fig. 1*A, middle panel*), expression of ShBΔ(6–46) in the presence of Kvβ2 resulted in no detectable changes of the fast inactivation properties (Fig. 1*A, bottom panel*). When traces were averaged within the group of ShBΔ(6–46) + Kvβ2 ($n = 12$) or ShBΔ(6–46) alone ($n = 17$), we observed little variations of inactivation properties between these two groups of recorded cells (Fig. 1*B*). To examine the protein expression of Kvβ2 in the experiments, total cell lysates from the transfected cells were separated by SDS-polyacrylamide gel electrophoresis. The expression of Kvβ1 and Kvβ2 was detected by immunoblot using the 12CA5 monoclonal antibody (mAb12CA5). Indeed, Kvβ2 was found to express in the transfected COS cells and exhibited higher expression as compared with that of Kvβ1 (Fig. 1*C, lanes 1* and *2*). The higher expression of Kvβ2 has been reproducible in multiple transfection experiments.[1] The strong mAb12CA5 binding signal indicates that the failure of Kvβ2 to modulate N-type fast inactivation was not due to the lower protein expression of Kvβ2. Thus, Kvβ2 differs from Kvβ1 and by itself fails to induce the fast inactivation of the ShB α-subunit.

The Subfamily-specific Association of Kvβ2 with Kv1 α-Subunits—Biochemical characterization supports direct physical interaction between Kvβ2 and Kv1.2 (RCK5) (7, 21) as well as Kv1.4 (RCK4) (39). However, the existing results for Kvβ2 binding specificity and region(s) involved are not conclusive (Ref. 23, also see "Discussion"). In addition, there is no information on whether Kvβ2 binds to ShB. To test the association between Kvβ2 and Kv1 α-subunits, we expressed various regions of α-subunits and Kvβ2 in yeast and used the yeast two-hybrid system to study the potential interaction(s) (30, 31). In the case of Kvβ1, it has been found that the NH_2-terminal

[1] M. Bezanilla, J. Xu, and M. Li, unpublished results.

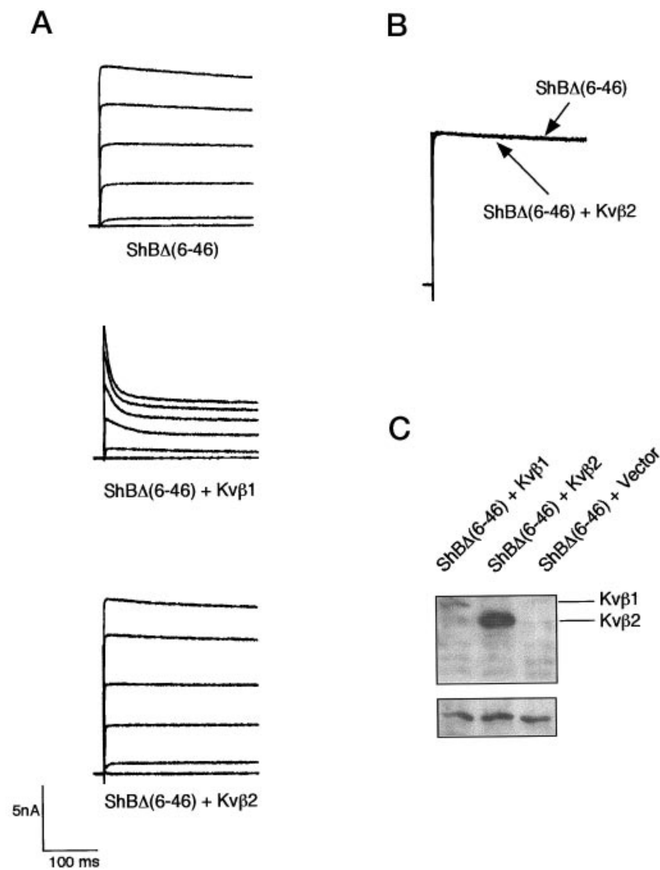

FIG. 1. **Differential modulation of Kv1 α-subunits by Kvβ1 and Kvβ2.** *A*, K^+ currents recorded by whole-cell voltage clamp. COS cells transfected with different combinations of plasmids: ShBΔ(6–46) only (n = 17, *top*), ShBΔ(6–46) + Kvβ1(n = 15, *middle*), or ShBΔ(6–46) + Kvβ2 (n = 12, *bottom*). Plasmid inputs in transfections were 3 μg for ShBΔ(6–46) and 18 μg for β-subunits. A plasmid (2 μg) encoding the CD4 antigen is included in all COS transfections for whole-cell voltage clamp recording. Typical current responses were obtained by stepping up holding potential from −87 mV to a final test potential of +33 mV in 20-mV increments, and one cell from each group is shown. *Scale bars*, 5 nA and 100 ms. *B*, averaged K^+ current responses of ShBΔ(6–46) in the presence or absence of Kvβ2. Current responses to a voltage step from −87 mV to +33 mV were normalized according to the peak current and averaged within the group. The control trace shows the averaged current response recorded from 17 ShBΔ(6–46)-transfected COS cells. The second trace (indicated by the *arrow*) illustrates the averaged current response obtained from 12 COS cells transfected with ShBΔ(6–46) + Kvβ2. The time scale is identical to that in *A*. *C*, expression of Kvβ1 and Kvβ2 polypeptides in COS cells detected by immunoblot analysis. An aliquot of the transfected COS cells from each of the above transfections was collected. Total cell lysates were prepared according to a standard protocol (Ref. 28; see "Materials and Methods"). Protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. The 12CA5-tagged Kvβ1 and Kvβ2 polypeptides (as indicated on the side) were detected by an affinity-purified 12CA5 monoclonal antibody. The *lower panel* is a non-12CA5 signal endogenous to COS cells, indicating that comparable amounts of protein were loaded in each lane.

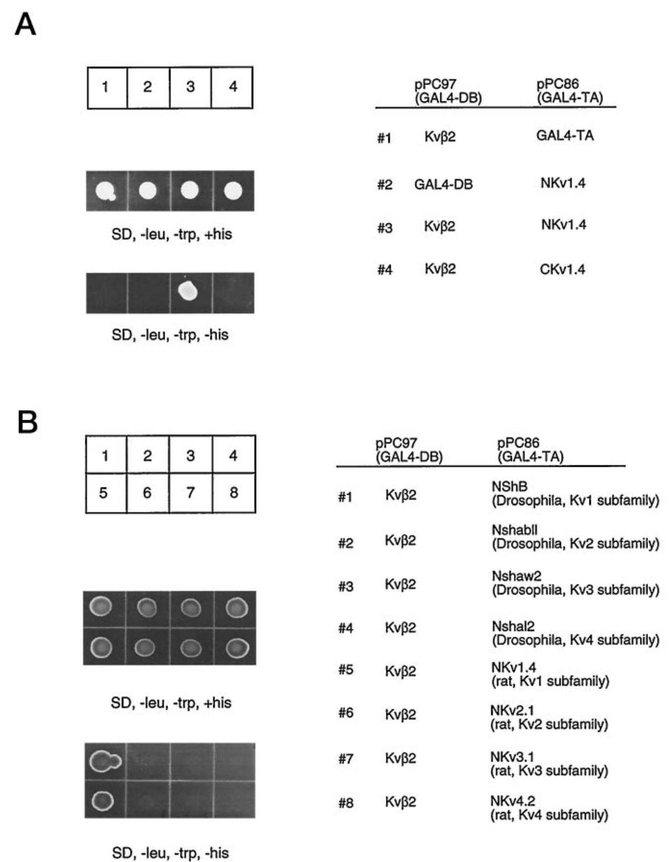

FIG. 2. **Subfamily-specific interaction of Kvβ2 with the NH_2-terminal domains of the Kv1 α-subunits.** HF7c yeast cells were transformed by different pairwise combinations of the two-hybrid constructs that express fusion proteins of either the DNA binding domain of GAL4 (*DB, pPC86*) or the transcription activation domain of GAL4 (*TA, pPC97*) (Ref. 29 and see "Materials and Methods"). The transformants carrying the two different fusion proteins were first selected by dextrose synthetic drop-out medium with no supplement of leucine and tryptophan (*SD, −leu, −trp, +his*) to ensure that in different combinations the transformants have both pPC97 and pPC86 plasmids. Identical numbers of cells in each combination were also dotted on the same medium without histidine (*SD, −leu, −trp, −his*) to test the protein interaction mediated growth. The transformants were allowed to grow at 30 °C for 48 to 72 h. *A*, interaction of Kvβ2 with the NH_2-terminal domain of Kv1.4. Four plasmid combinations are listed on the *right*. The vector construction of the Kv1.4 fragments has been described in Xu *et al.* (29). Their growth on the SD, −leu, −trp, +his medium is shown in the *middle panel* on the *left*. Their growth on the SD, −leu, −trp, −his medium is shown in the *lower panel* on the *left*. *B*, subfamily-specific interaction of Kvβ2 with the NH_2-terminal domains of K^+ channels. The NH_2-terminal domains of eight K^+ channel α-subunits of four subfamilies were subcloned (29) and subjected to the yeast two-hybrid analysis. The subcloned coding sequences are: NShB (aa 1–227), Nshabll (aa 1–435), Nshaw2 (aa 3–174), Nshal2 (aa 38–185), NKv1.4 (aa 1–310), NKv2.1 (aa 1–182), NKv3.1 (aa 1–180), and NKv4.2 (aa 1–183). The eight plasmid combinations are listed on the *right*. Their growth on the SD, −leu, −trp, +his medium is shown in the *middle panel* on the *left*. Their growth on the SD, −leu, −trp, −his medium is shown in the *lower panel* on the *left*.

domains of the Kv1 α-subunits are involved in the α-Kvβ1 interaction (28, 36). Because Kvβ1 and Kvβ2 share considerable sequence homology, we first tested the potential interaction of Kvβ2 with the cytoplasmic regions of the Kv1.4, an α-subunit which has been found to interact with Kvβ2 (39). The truncated cytoplasmic fragments, *i.e.* the NH_2-terminal domain (aa[2] 1–306) and COOH-terminal domain (aa 566–651), were expressed individually with Kvβ2 as GAL4 fusion proteins. If Kvβ2 interacts with one or both truncated Kv1.4 fragments, the resultant interaction(s) should confer the ability of the yeast transformants to grow on synthetic medium lacking histidine. Fig. 2*A* shows that when Kvβ2 was expressed alone either as a fusion protein of the GAL4 DNA binding domain (GAL4-DB) or that of the GAL4 transcription activation domain (GAL4-TA), the yeast transformants grew on double selection medium supplemented with histidine, indicating that they carry both plasmids (Fig. 2*A*, *numbers 1* and *2, middle left panel*). When the same number of transformants were tested to grow on the triple selection medium lacking histidine, they showed no growth (Fig. 2*A, numbers 1* and *2, lower left panel*).

[2] The abbreviation used is: aa, amino acids.

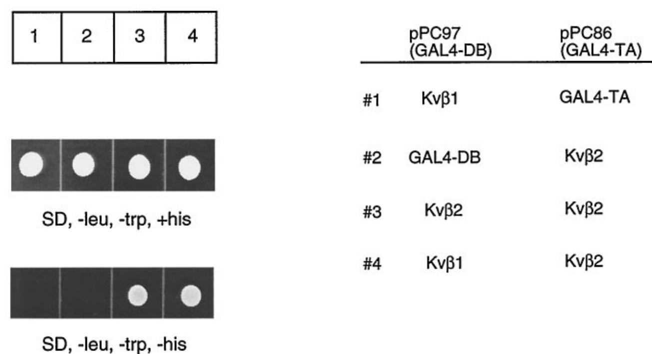

FIG. 3. **The β-β interaction tested by the yeast two-hybrid system.** Experiments similar to that described in Fig. 2 were carried out to test the homomeric and heteromeric interaction between Kvβ2 (aa 1–367) and Kvβ1 (aa 1–401). Four plasmid combinations are listed on the *right* (see "Materials and Methods" for vector construction). Their growth on the SD, −leu, −trp, +his medium is shown on the *left* (*middle panel*). Their growth on the SD, −leu, −trp, −his medium is shown in the *lower panel* on the *left*.

This indicates that Kvβ2 itself does not exert any endogenous activity that permits the yeast transformants to grow on the selection medium. By contrast, the coexpression of Kvβ2 and the NH_2-terminal domain (Fig. 2*A, number 3*), not the COOH-terminal domain (Fig. 2*A, number 4*) of Kv1.4, resulted in growth on the selection medium lacking histidine. Consistent results have been obtained using a β-galactosidase assay (data not shown). Thus, similar to Kvβ1, Kvβ2 interacts with the NH_2-terminal domain of the Kv1.4 α-subunit.

The ability of Kvβ2 to interact with the NH_2-terminal domain of Kv1.4 suggests that the resultant association may be essential for Kvβ2 to interact with α-subunits, as seen in biochemical copurification. In the case of Kvβ1, its subfamily-specific association with the NH_2-terminal domains of Kv1 α-subunits has been shown to be essential for the Kvβ1-mediated inactivation (28, 36). Coimmunoprecipitation of K^+ channel polypeptides in rat brain has indicated that Kvβ2 interacts with Kv1.2 and Kv1.4, but not Kv2.1 (24, 39). To further test the specificity of the Kvβ2, pairwise combinations of Kvβ2 and the NH_2-terminal domains of eight different α-subunits were analyzed with the yeast two-hybrid system. The eight α-subunits included were: Shaker B (40–42), Shabll, Shaw2, and Shal2 from *Drosophila* (9); Kv1.4 (or RCK4) (43), Kv2.1 (or DRK1) (44), Kv3.1 (or NGK2b) (45), and Kv4.2 (or rShal1) (46, 47) from rat. These genes belong to the four major subfamilies, one fly gene and one rat gene for each subfamily. Among the selected NH_2-terminal domains, Kvβ2 interacts only with the NH_2-terminal domains of ShB and Kv1.4 (Fig. 3*B, numbers 1* and *5*), both of which belong to the Kv1 subfamily. Furthermore, the Kvβ2 interacting site was mapped to aa 174–306 within the NH_2-terminal domain of Kv1.4 (data not shown), which coincides precisely with the domain that interacts with Kvβ1 (28, 36). Thus, both Kvβ1 and Kvβ2 interact subfamily-specifically with the Kv1 α-subunits and share the same binding site on the α-subunits.

Formation of Homo- and Heteromultimeric β-β Complexes—Based on the hydrodynamic estimates, the α-dendrotoxin acceptor (or Kv1.2) complex contains more than one Kvβ2 subunit per complex (21). It is not clear, however, whether Kvβ2 can form an oligomeric complex in the absence of α-subunits. Additionally, since expression patterns of Kvβ1 and Kvβ2 overlap *in vivo* (8, 22, 39, 52), it would be interesting to examine whether different β-subunits can interact with each other to form heteromultimers. Fig. 3 shows that Kvβ2 can indeed associate to form homomultimers as the yeast transformants grow in the selection medium lacking histidine (Fig. 3, *number 3*). The known potassium channels in yeast have distinctive topology and belong to a subclass different from the Shaker-like potassium channels (48). Therefore, the above result supports that Kvβ2 is capable of interacting with itself in the absence of α-subunits.

Both Kvβ1 and Kvβ2 are expressed in rat brain with overlapping expression patterns (8, 22, 39). Because Kvβ2 forms multimers in the absence of α-subunits (Fig. 3, *number 3*) and has considerable overall sequence homology (73%) to Kvβ1, we tested the possible interaction between Kvβ1 and Kvβ2 and found that Kvβ1 and Kvβ2 can also interact (Fig. 3, *number 4*). This implies that Kvβ1 and Kvβ2 can form heteromultimers in the absence of the pore-forming α-subunits.

Inhibition of the Kvβ1-mediated Inactivation by Kvβ2—Both Kvβ2 and Kvβ1 interact with the Kv1 α-subunits by recognizing the same region in the Kv1 α-subunits (Fig. 2 and Ref. 28). Additionally, Kvβ2 interacts with itself and/or Kvβ1 to form homo- and/or heteromultimers (Fig. 3). Because Kvβ1, not Kvβ2, induces the fast inactivation of the Kv1 α-subunits that lack fast inactivation (Fig. 1), these data suggest that one potential function of Kvβ2 would be to alter the efficacy of the Kvβ1-mediated inactivation. One predicted outcome would be that Kvβ2 weakens the ability of Kvβ1 to inactivate, as Kvβ2 may compete with Kvβ1 for the binding site on α-subunits and/or associate with Kvβ1 to form Kvβ1-Kvβ2 heteromultimers containing fewer inactivation particles.

One experiment to test this hypothesis would be to coexpress Kvβ1 and a compatible α-subunit in the presence or absence of Kvβ2 and ask whether Kvβ2 alters the ability of Kvβ1 to inactivate. We cotransfected COS cells with noninactivating ShBΔ(6–46) and Kvβ1 in a 1:6 plasmid ratio of α/Kvβ1. Fig. 4*A* shows three representative traces, one from each group, that were superimposed and normalized. These traces were recorded by stepping up the holding potential from −87 mV to a test potential of +13 mV for a duration of 300 ms. ShBΔ(6–46) alone produced a trace with fast activating kinetics lacking N-type fast inactivation. When Kvβ1 was included in the transfection, we observed a majority of transfected cells that show the Kvβ1-mediated fast inactivation (Fig. 4*A*). If, however, both Kvβ1 and Kvβ2 were included in a plasmid ratio of α/Kvβ1/Kvβ2 of 1:6:5, much fewer transfected cells showed fast interaction induced by Kvβ1 (see below and Fig. 4*B*).

The lack of fast inactivation by Kvβ1 in the presence of Kvβ2 in the example shown in Fig. 4*A* could have resulted from variable transfection rates of the three plasmids. To address this, we recorded 17 Shaker-positive cells for the ShBΔ(6–46) transfection, 40 cells for the ShBΔ(6–46) + Kvβ1 transfection, 44 cells for the ShBΔ(6–46) + Kvβ1 + Kvβ2 transfection. Since recorded cells were selected solely based on the presence of Shaker current and the expression of CD_4 antigen that was cotransfected in all experiments, the percentage of recorded cells in a given transfection shown, fast and/or slow inactivation can then be determined. Among the recorded traces, some exhibited both fast and slow inactivation, the others showed only slow inactivation. The traces were fit by a double exponential function, and the resultant inactivation constants were plotted against the cell number in percentage (Fig. 4*B*). Fig. 4*B* shows plots using one inactivation constant per recorded cell, *i.e.* if the inactivation consists of two (fast and slow) components, only the fast inactivation constant was plotted. For the ShBΔ(6–46) transfection, we observed that all recorded cells lacked the fast inactivation and gave a slow inactivation constant (τ_2) larger than 128 ms (Fig. 4*B, top panel*). In the 40 recorded cells for ShBΔ(6–46) + Kvβ1 (Fig. 4*B, middle panel*), we observed that more than 60% of recorded cells possessed a fast inactivation component (τ_1 = 1–64 ms), while about 35% of

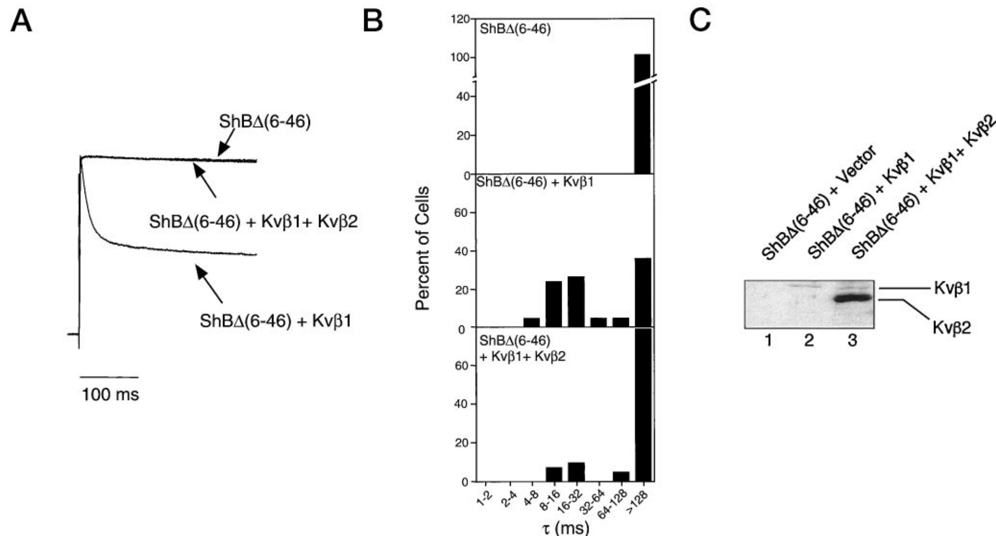

FIG. 4. **Inhibition of the Kvβ1-mediated inactivation by Kvβ2.** *A*, normalized K^+ currents obtained by whole-cell voltage clamp recording. The current responses were recorded from COS cells transfected with ShBΔ(6–46), ShBΔ(6–46) + Kvβ1, or ShBΔ(6–46) + Kvβ1 + Kvβ2. Plasmid inputs were 3 μg for ShBΔ(6–46), 18 μg for Kvβ1, and 15 μg for Kvβ2. Typical responses to a voltage step from −87 mV to +13 mV of one cell from each group were normalized according to the peak response and superimposed. The traces for ShBΔ(6–46) + Kvβ1 and ShBΔ(6–46) + Kvβ1 + Kvβ2 have been fit by a two-exponential function to yield inactivation constants. ShBΔ(6–46) + Kvβ1: A2/(A1 + A2) = 0.19; $\tau 1$ = 11.9 ms; $\tau 2$ = 154 ms. ShBΔ(6–46) + Kvβ1 + Kvβ2: A2/(A1 + A2) = 1; $\tau 1$ (not available since A1 = 0); $\tau 2$ = 178 ms. *B*, distribution of inactivation time constants. A total of 17 cells positive in Shaker currents were recorded for ShBΔ(6–46), 40 cells recorded for the ShBΔ(6–46) + Kvβ1 transfection, and 44 cells were recorded for the ShBΔ(6–46) + Kvβ1 + Kvβ2 transfection. The decay phase of current responses (300 ms duration) to a voltage step from −87 mV to +13 mV was fit by a double exponential function to obtain the onset parameters of inactivation. The cell number (in percentage normalized to the total cell number recorded for that group) was plotted against the inactivation constants. For a given recorded trace, if the response shows both fast and slow inactivation, only $\tau 1$ is used in this plot. If a trace shows no fast inactivation (*i.e.* A1 = 0), the $\tau 2$ is used in this plot. *Top panel*, ShBΔ(6–46); *middle panel*, ShBΔ(6–46) + Kvβ1; *bottom panel*, ShBΔ(6–46) + Kvβ1 + Kvβ2. The statistical significance was calculated according to a standard formula for comparison of two proportions (see "Materials and Methods"). Comparison of ShBΔ(6–46) + Kvβ1 with ShBΔ(6–46) + Kvβ1 + Kvβ2: $z = 4.111$ and $p < 0.001$. *C*, expression of Kvβ1 and Kvβ2 polypeptides in COS cells detected by immunoblot analysis. Total protein lysates from the three groups of transfected COS cells (see legend of *A*) were prepared. The Kvβ1 and Kvβ2 polypeptides were detected by the 12CA5 monoclonal antibody according to the protocol described in the legend to Fig. 1. *Lane 1*, COS transfected with ShBΔ(6–46) alone; *lane 2*, ShBΔ(6–46) + Kvβ1; *lane 3*, ShBΔ(6–46) + Kvβ1 + Kvβ2. The Kvβ1 and Kvβ2 signals are marked and labeled on the side.

cells showed no fast inactivation, which presumably is due to low or no expression of Kvβ1. By contrast, we observed that only 17% of cells showed fast inactivation when Kvβ2 was coexpressed (Fig. 4*B, bottom panel*). As the amount of plasmid input for both ShBΔ(6–46) and Kvβ1 was identical in both transfections, it is unlikely that the population of the recorded cells, which showed no fast inactivation, is due to the lack of Kvβ1 plasmids. Furthermore, statistical analysis shows that the difference was statistically significant (see legend to Fig. 4*B* and "Materials and Methods").

Despite the constant plasmid input of Kvβ1 in both experiments, the inhibition of the Kvβ1-mediated inactivation by Kvβ2 can be subjected to several interpretations. For example, it is not known whether the presence of Kvβ2 decreases the expression of other subunits at the protein level and/or at the level of channel surface expression, thereby resulting in the inhibition of the Kvβ1-mediated inactivation. To examine whether coexpression of β-subunits differentially altered the channel surface expression, we plotted the current amplitude of ShBΔ(6–46) in the presence of Kvβ1 and Kvβ1 + Kvβ2. The result indicates that the averaged current amplitudes were similar: 6.5 nA for the ShBΔ(6–46) + Kvβ1 transfection and 6.7 nA for the ShBΔ(6–46) + Kvβ1 + Kvβ2 transfection ($p >$ 0.2, Student's t test). Furthermore, there was no obvious correlation between current amplitude and inactivation properties (data not shown). To examine the expression level of Kvβ1 in the presence or absence of Kvβ2, aliquots of the transfected cells in the experiment (Fig. 4*A*) were collected, and the total cell lysates were prepared and separated by SDS-polyacrylamide gel electrophoresis. Indeed, the Kvβ1 expression was found to be comparable, regardless of the presence of Kvβ2 (Fig. 4*C, lanes 2* and *3*). Together, these results suggest that when both Kvβ1 and Kvβ2 are expressed in the cells, Kvβ2 is capable of inhibiting the Kvβ1-mediated inactivation. Because the Kvβ1 and ShBΔ(6–46) expression remained relatively constant, both in the presence and absence of Kvβ2, the inhibition by Kvβ2 is likely to be caused by differential subunit assembly, *i.e.* Kvβ2 competes with Kvβ1 for the binding site on α-subunits and/or Kvβ1 and Kvβ2 form heteromultimeric complex(es).

Regions Involved in β-β and α-β Interactions—If the differential subunit assembly indeed plays a role in inhibiting the Kvβ1-mediated inactivation, then the binding of Kvβ2 to α-subunits and/or Kvβ1 would be essential for the Kvβ2-mediated inhibition.

Deletion analysis for Kvβ1 has shown that the conserved core region of Kvβ1, *i.e.* amino acids 73–401, is sufficient for the Kvβ1-α interaction (28). Among the 47 positions that harbor different residues between Kvβ1 and Kvβ2, 27 are conservative changes. Additionally, both Kvβ1 and Kvβ2 recognize the same subset of α-subunits, *i.e.* the Kv1 α-subunits. These suggest that the corresponding region in Kvβ2 may serve a similar function in binding to the α-subunits. Indeed, when a truncated fragment representing the conserved core region of Kvβ2 was subjected to the yeast two-hybrid test, the core region is sufficient to interact with the NH_2-terminal domain of ShB (*NShB*) (Fig. 5).

One possibility for inhibiting the Kvβ1-mediated inactivation is that Kvβ2 can somehow interact with the inactivation particle of Kvβ1, which is located at the NH_2 terminus (8). This interaction may lead to the defective activity of Kvβ1 to inactivate the Kv1 α-subunits. Taking this into consideration, we chose to first carry out deletion analysis using Kvβ1 to map the

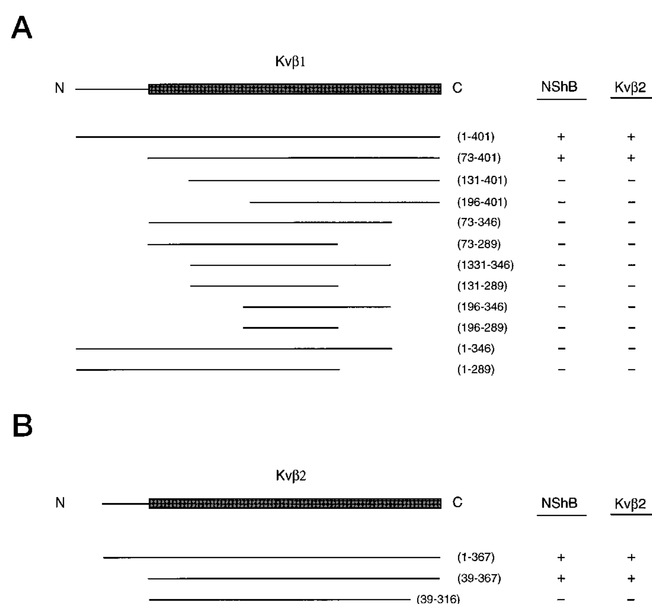

FIG. 5. **Regions in β-subunits that are involved in α-β and β-β interactions tested by the yeast two-hybrid system.** *A*, deletion mapping of the region(s) in Kvβ1 for interaction with the NH_2-terminal domain of ShB (*NShB*) and Kvβ2. A diagram representing the coding sequence of Kvβ1 is shown. The *filled box* indicates the COOH-terminal "core region" of Kvβ1. Different coding sequences, as indicated in *parentheses*, were cloned into the pPC97 vector for the yeast two-hybrid test together with either pPC86-NShB or pPC86-Kvβ2 as described in the legend to Fig. 2 and under "Materials and Methods." The + indicates growth, the – indicates no growth. *B*, interaction of Kvβ2 and ΔKvβ2(39–367) with NShB and Kvβ2, as tested by the yeast two-hybrid system. Data representation is identical to that described in *A*.

region involved in the Kvβ1-Kvβ2 interaction to test whether the inactivation particle is necessary for the heteromultimeric β-β interaction. A total of 11 Kvβ1 deletion mutants were constructed (Fig. 5*A*). Their ability to interact with Kvβ2 was tested by the yeast two-hybrid system using growth selection. The minimal region in Kvβ1 required for interacting with Kvβ2 was mapped to the conserved core region, which indicates that the NH_2-terminal 72 residues of Kvβ1 are not required for the Kvβ1-Kvβ2 interaction in the yeast two-hybrid test. Similar to Kvβ1, the corresponding core region of Kvβ2 is sufficient for interacting with Kvβ2 (Fig. 5*B*). Thus, the conserved core region of β-subunits is involved in both α-β and β-β interactions.

Inhibition of the Kvβ1-mediated Inactivation by Truncated Kvβ2 Subunits—The results in Fig. 5 suggest that the formation of heteromultimeric α-Kvβ2 and/or Kvβ1-Kvβ2 complexes is responsible for Kvβ2 to inhibit the Kvβ1-mediated inactivation. If this is true, one prediction is that the truncated Kvβ2 polypeptide containing the mapped interacting region, *i.e.* the conserved core region, should be capable of inhibiting the Kvβ1-mediated inactivation. To test this, we constructed two Kvβ2 deletion mutants: ΔKvβ2(39–367) and ΔKvβ2(39–316) (Fig. 6*A*). The ΔKvβ2(39–367) mutant contains the intact interacting region mapped by the yeast two-hybrid analysis (Fig. 5), while 51 residues COOH-terminal to the interacting region of Kvβ2 were truncated in ΔKvβ2(39–316). The resultant mutant can no longer interact with either Kvβ1 or NH_2-terminal domains of the Kv1 α-subunits (Fig. 5). Because the ability to interact should correlate with the activity in inhibiting the Kvβ1-mediated inactivation, we carried out experiments similar to those in Fig. 4, which involved cotransfecting ShBΔ(6–46) and Kvβ1 in the presence of either ΔKvβ2(39–367) or ΔKvβ2(39–316), and examined the subsequent inactivation properties. Fig. 6*B* shows that the protein levels of Kvβ2 and the two deletion mutants are comparable. Among the 39 recorded Shaker-positive cells transfected in the presence of ΔKvβ2(39–367), we found only 22.5% of cells that showed fast inactivation (Fig. 6, *C* and *D, panel b*). Indeed, ΔKvβ2(39–367) acts similarly to Kvβ2 by decreasing the number of cells that exhibit the fast inactivation (Fig. 6, *C* and *D, panels a* and *b*). The difference between Kvβ1 alone and Kvβ1 + ΔKvβ2(39–367) was statistically significant ($p < 0.001$ and see legend to Fig. 6*D*). In contrast, among the 49 recorded cells that were transfected in the presence of ΔKvβ2(39–316), 61% of cells showed fast inactivation. The distribution of inactivation constant from this group of cells is similar to that obtained from cells transfected by ShBΔ(6–46) + Kvβ1 (Fig. 6*D, panels c* and *d*). Thus, the ability of the mutated Kvβ2 to interact with ShBΔ(6–46) and/or Kvβ1 directly correlates with their ability to inhibit the Kvβ1-mediated inactivation.

DISCUSSION

Using transient expression of different combinations of α-subunits and β-subunits, we have observed a functional role of Kvβ2 in modulating channel inactivation. Since the activity of Kvβ2 to inhibit the Kvβ1-mediated inactivation directly correlates with the ability of Kvβ2 to associate with α-subunits and Kvβ1, differential subunit assembly is a likely mechanism responsible for the Kvβ2 activity. This subunit interaction may be important for tuning the K^+ channel diversity *in vivo*.

Consistent with the biochemical data from copurification of Kv1.2 and Kvβ2 (7, 22), our results (Fig. 2) show that Kvβ2 interacts with the NH_2-terminal domains of α-subunits in a subfamily-specific manner. Curiously, a recent report suggested that both Kvβ1 and Kvβ2 interact with Kv1 and Kv4 α-subunits (23). This is inconsistent with results from biochemical binding and electrophysiological analysis (28, 36). Because Kvβ2 plays a role in both channel expression (24) and channel properties (this report), important future experiments would be to investigate the *in vivo* specificity of α-β interaction.

The formation of heteromultimeric complexes as a mechanism to increase K^+ current diversity has been studied in several channel systems. This includes the formation of functional channels by different pore-forming subunits (*e.g.* nicotinic acetylcholine receptor, voltage-gated K^+ channels, etc.) and by assembly of pore-forming subunits with various auxiliary subunits (*e.g.* voltage-gated sodium or calcium channels) (see review by Catterall (49)). The heteromultimeric oligomerization by auxiliary subunits in the absence of pore-forming subunits has not been reported. The biochemical evidence of two homologous auxiliary subunits "competing" with same set of α-subunits and the formation of heteromultimeric auxiliary subunits in the absence of pore-forming subunits suggests yet another potential mechanism to create and tune their electrical diversity. The ability to form homo- or heteromultimers of β-subunits in the absence of α-subunits does not necessarily imply the α-β and β-β subunit assembly that happens in separate steps *in vivo*. Future experiments are needed to determine the physiological existence of heteromeric complexes of β-subunits and molecular cascade for assembling α-β heteromultimers.

The results reported here demonstrate that Kvβ2 is an active player in determining the fast inactivation mediated by other β-subunits. How does Kvβ2 inhibit the Kvβ1-mediated inactivation? Our data can be best explained by the following mechanism (Fig. 7*A*). In the absence of Kvβ2, the expression of Kvβ1 will permit it to coassemble with compatible α-subunits. Depending upon whether the interacting α-subunits contain an inactivation gate, Kvβ1 either accelerates or induces fast inactivation (Fig. 7*A, I*). In the presence of the high concentration of Kvβ2, Kvβ2 occupies most of the sites on α-subunits as homomultimeric Kvβ2 complexes. As a result, it prevents (or re-

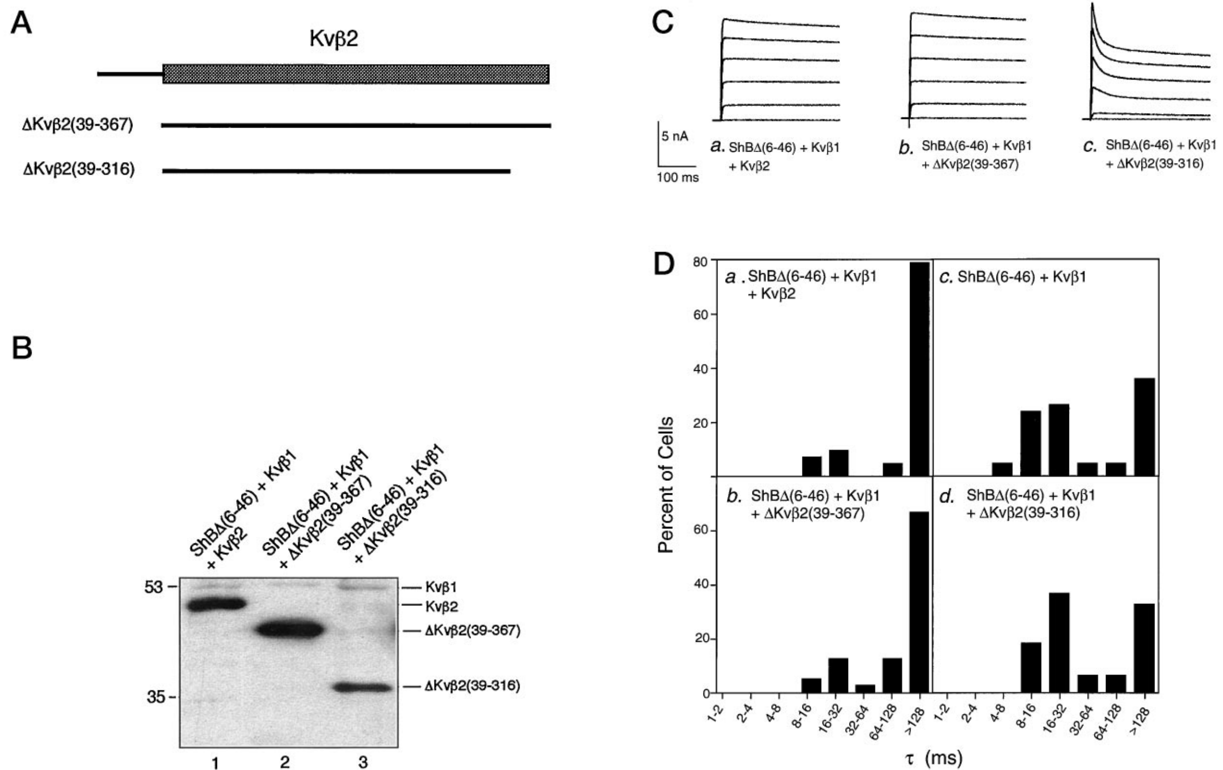

FIG. 6. **Inhibition of the Kvβ1-mediated inactivation by truncated Kvβ2 subunits.** *A*, schematic diagram of Kvβ2 and its truncations which were subcloned and expressed in COS cells. The *filled gray box* indicates the COOH-terminal conserved core region of Kvβ2. The ΔKvβ2(39–367) mutant is a deletion of Kvβ2 that contains only the COOH-terminal core region. The ΔKvβ2(39–316) mutation was constructed by further deleting 51 amino acids from its COOH terminus of the core region. *B*, immunoblot detection of Kvβ1 and Kvβ2 mutants expressed in COS cells. COS cells were transfected with ShBΔ(6–46) and Kvβ1 in the presence of either ΔKvβ2(39–367) or ΔKvβ2(39–316). Aliquots of the transfected cells were collected and subjected to immunoblot analysis using the mAb12CA5 (see the legend to Fig. 1*C* for the experimental protocol). The plasmid inputs were 3 μg for ShBΔ(6–46), 18 μg for Kvβ1, and 15 μg for either ΔKvβ2(39–367) or ΔKvβ2(39–316). The identity of the mAb12CA5 signals is marked and indicated on the *side*. *C*, whole-cell voltage clamp recordings were obtained from transfected COS cells described in *B*. A total of 39 cells positive for Shaker current were recorded for the ShBΔ(6–46) + Kvβ1 + ΔKvβ2(39–367) transfection, 49 cells for the ShBΔ(6–46) + Kvβ1 + ΔKvβ2(39–316) transfection. Typical current responses of one cell from each transfection are shown. Voltage steps are from −87 mV to +33 mV in 20-mV increments. *D*, distribution of inactivation time constants. The recorded traces from the two groups of cells were analyzed according to the procedures described and as in the legend to Fig. 4. Inactivation time constant distribution was plotted against percent of recorded cells. *Panel a*, ShBΔ(6–46) + Kvβ1 + Kvβ2; *panel b*, ShBΔ(6–46) + Kvβ1 + ΔKvβ2(39–367); *panel c*, ShBΔ(6–46) + Kvβ1; *panel d*, ShBΔ(6–46) + Kvβ1 + ΔKvβ2(39–316). Statistical analyses of the two-group comparison (see legend to Fig. 4*B* and "Materials and Methods") were: $z_{a,b} = 0.555$, $p = 0.291$; $z_{a,c} = 4.111$, $p < 0.001$; $z_{a,d} = 4.387$, $p < 0.001$; $z_{c,d} = 0.165$, $p = 0.433$.

moves) Kvβ1 from interacting with α-subunits (Fig. 7*A, II*), thereby inhibiting the Kvβ1-mediated inactivation. The coexpression experiments in the present study show that Kvβ2 inhibited the Kvβ1-mediated inactivation, consistent with the results of immunoblot analyses in which we found that the expression of Kvβ2, despite its comparable plasmid input, was considerably higher than that of Kvβ1 (Figs. 1*C*, 4*C*, and 6*B*). Thus, under the conditions of our experiments, most α-subunits presumably were occupied by homomultimeric Kvβ2 complexes, which prevents the limited numbers of Kvβ1 subunits to bind, thereby eliminating the Kvβ1-mediated inactivation without altering the protein expression of Kvβ1 (Fig. 4*D*).

Conceivably, one should observe an intermediate situation where Kvβ1 and Kvβ2 are present in a comparable concentration (or different concentrations with an appropriate ratio for heteromultimeric interaction, since the Kvβ1 and Kvβ2 may have considerable difference in affinity for subunit interaction). Under such conditions, most compatible α-subunits should be interacting with heteromultimeric β-complexes to produce intermediate effects, *i.e.* Kvβ2 weakens but does not remove the Kvβ1-mediated inactivation. In an attempt to test this, we performed experiments with lower inputs of Kvβ2 plasmid. Thus far, we have not identified an input plasmid ratio that gives rise to currents with a prominent intermediate inactivation constant. A different approach to alter protein expression level for β-subunits might be necessary to test this hypothesis. The failure to identify intermediate inactivation could also be interpreted by other mechanism(s). For example, compared with Kvβ1, Kvβ2 has considerably higher affinity for α-subunits and/or itself. In this case, the higher affinity and/or avidity for α-Kvβ2 and/or Kvβ2-Kvβ2 interactions would yield predominantly α-Kvβ2 complex(es).

In mammalian systems, two genes encoding three forms of mammalian β-subunits, *i.e.* Kvβ1, Kvβ3 (a putative splice variant of Kvβ1), and Kvβ2, have been found expressed in brain with an overlapping expression pattern (7, 8, 39). Although Kvβ3 has not been demonstrated directly to bind either α-subunits or β-subunits, the fact that the Kvβ3 amino acid sequence within the interacting region is identical to that of Kvβ1 suggests that Kvβ3 is likely to share features for subunit interaction found in Kvβ1 and Kvβ2 (17, 18, 20). Based on the results of studying subunit interaction between α-subunits and β-subunits, an interesting new regulatory pathway is emerging. All cloned β-subunits consist of a conserved core region critical for α-β and β-β interaction and variable NH_2-terminal domains that determine the modulatory activity (Fig. 7*B*). Their distinct modulatory effects on α-subunits and ability to form heteromultimers have revealed yet another potential *in vivo* mechanism for creating and tuning the diversity of potassium currents. Although our present study probes the formation of

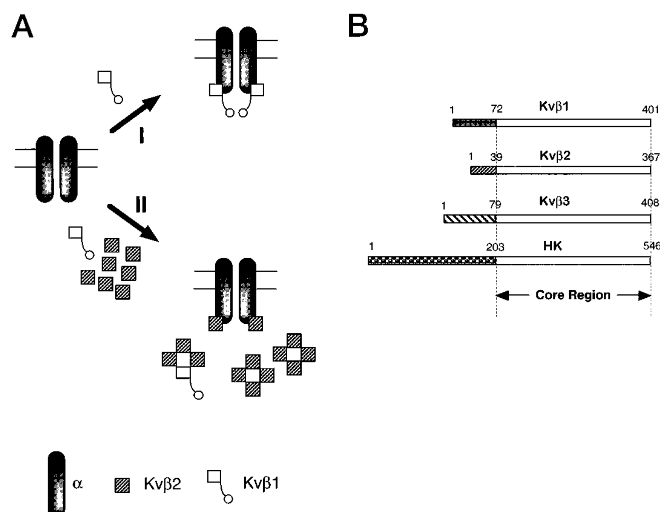

FIG. 7. **A model for the Kvβ2-mediated inhibition.** *A*, a schematic diagram illustrates the postulated Kvβ2-mediated inhibition. The model may not represent the actual stoichiometry of β-β complex(es). *B*, comparison of the four well characterized β-subunits.

heteromultimers by examining the protein-protein interaction and the resultant alterations of inactivation properties, there is increasing evidence suggesting that the functional roles of β-subunits may not be restricted to modulation of inactivation (16, 24, 50). Thus, future experiments should be focused on addressing whether the heteromultimeric assembly of β-subunits is indeed present *in vivo* and whether these heteromultimeric α-β combinations could specify a wide spectrum of modulatory activities, which may include, but are not limited to, tuning inactivation properties and surface expression of α-subunits *in vivo*.

Acknowledgments—We thank Magdalena Bezanilla for the ΔKvβ2(39–316) construct; Dr. Weifeng Yu for help on patch clamp techniques; Dr. Lily Jan for ShB and rshal1 (Kv4.2); Dr. R. Joho and Dr. A Brown for DRK1; Dr. D. Mckinnon for RCK1; Dr. L. Salkoff for fshal2, fshaw2, and fshabl1; and Dr. R. Aldrich for the ShBΔ(6–46) mutant. We also thank Magdalena Bezanilla, Gerda Breitwieser, Xiaodong Li, Rajini Rao, and Weifeng Yu for critical reading of this manuscript.

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