

# NAB Domain Is Essential for the Subunit Assembly of both $\alpha$ - $\alpha$ and $\alpha$ - $\beta$ Complexes of Shaker-like Potassium Channels

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## Summary

There are at least five subfamilies of Shaker-like K<sup>+</sup> channels. The diverse functions of K<sup>+</sup> channels are thought to be further modulated by hydrophilic  $\beta$  subunits. Here we report that Kv $\beta$ 1 inactivates RCK4 and Shaker B K<sup>+</sup> channels of the Kv1 subfamily, but not Shal2 of the Kv4 subfamily. This correlates the subfamily-specific binding of Kv $\beta$ 1 to the cytoplasmic N-terminal domains of Kv1  $\alpha$  subunits. We map the Kv $\beta$ 1-binding site to a region overlapping NAB<sub>Kv1</sub>, a domain that specifies different Kv1  $\alpha$  subunits to form heterotetramers. Using chimeric  $\alpha$  subunits, we demonstrate that NAB<sub>Kv1</sub> is essential for the Kv $\beta$ 1-mediated inactivation. These results suggest that Kv $\beta$ 1 modulates a subset of K<sup>+</sup> channels through the specific assembly of  $\alpha$ - $\beta$  complexes and reveal the dual function of the NAB domain in mediating the assembly of both  $\alpha$ - $\alpha$  and  $\alpha$ - $\beta$  complexes.

## Introduction

K<sup>+</sup> channels comprise a diverse family of membrane proteins that regulate action potentials, cardiac pacemaking, and neurotransmitter release in excitable tissues (Connor and Stevens, 1971; Byrne, 1980; Rogawski, 1985; Hille, 1991). In nonexcitable cells, they play important roles in hormone secretion, cell proliferation, cell volume regulation, and lymphocyte differentiation (Dubois and Rouzair-Dubois, 1993). A functional K<sup>+</sup> channel has four identical and/or homologous  $\alpha$  subunits that together form a central conduction pore for K<sup>+</sup> ions (MacKinnon, 1991; Li et al., 1994). The functional diversity of the K<sup>+</sup> channels arises from several mechanisms. These include a large number of K<sup>+</sup> channel genes (Jan and Jan, 1989, 1990), the ability of different  $\alpha$  subunits to form heteromultimeric channels (Christie et al., 1990; Isacoff et al., 1990; Ruppertsberg et al., 1990; Covarrubias et al., 1991; Li et al., 1992), and regulatory interactions with distinct hydrophilic  $\beta$  subunits (Rettig et al., 1994; Scott et al., 1994; Majumder et al., 1995; Morales et al., 1995). Thus, a combination of differential expression and selective formation of heteromultimeric channels by different membrane-bound  $\alpha$  subunits and hydrophilic  $\beta$  subunits may allow individual cells to acquire their own characteristic K<sup>+</sup> current properties.

There are at least five subfamilies of  $\alpha$  subunit genes that encode the Shaker-like K<sup>+</sup> channels: Kv1 (Shaker), Kv2 (Shab), Kv3 (Shaw), Kv4 (Shal), and Kv5 (Butler et

al., 1990; Wei et al., 1990; Salkoff et al., 1992; Chandy and Gutman, 1993; Zhao et al., 1994). They share a common design: each subunit has a hydrophobic core region flanked by cytoplasmic N- and C-terminal domains (see Miller, 1991; Jan and Jan, 1992, for review). Each  $\alpha$  subunit can form oligomers with members of the same subfamily, as shown by results from studying K<sup>+</sup> channels expressed in heterologous expression systems and in rat brain (Covarrubias et al., 1991; Li et al., 1992; Sheng et al., 1993; Wang et al., 1993; Deal et al., 1994). The regions responsible for subfamily-specific assembly of heteromultimeric channels were localized to the cytoplasmic N-terminal domain. This was determined by coexpression of mutated Kv1 and Kv2 channels (Li et al., 1992; Lee et al., 1994) and by testing the specificity of physical association between truncated polypeptides of Kv1 and Kv3 channels (Li et al., 1992; Shen and Pfaffinger, 1995). Based on systematic binding studies of the N-terminal domains of  $\alpha$  subunits of the Kv1, Kv2, Kv3, and Kv4 subfamilies, we have identified a highly conserved region within the N-terminal domain that mediates subfamily-specific association (Xu et al., 1995). This conserved region has been named N-terminal A and B box (NAB) according to the original nomenclature by Drewe et al. (1992). Based on the ability of NABs to associate with each other, they were divided into four classes: NAB<sub>Kv1</sub>, NAB<sub>Kv2</sub>, NAB<sub>Kv3</sub>, and NAB<sub>Kv4</sub> (Xu et al., 1995). Consistent with their binding specificity, NAB sequences are conserved in a subfamily-specific manner (Xu et al., 1995). It is possible that the NAB domain also determines other subfamily-specific functions.

The regulation of K<sup>+</sup> channel function by the hydrophilic  $\beta$  subunits is less well understood. This group of proteins was originally identified in membrane preparations from bovine brain as soluble proteins that copurified with Kv1.2 (RCK5) K<sup>+</sup> channel protein (Scott et al., 1990). This led to the isolation of genes that encode two homologous  $\beta$  subunit proteins, Kv $\beta$ 1 and Kv $\beta$ 2 (Rettig et al., 1994; Scott et al., 1994). These  $\beta$  subunits share over 85% amino acid identity in their core region, but differ significantly in the length and sequence of their N-terminal regions. The *in vivo* function(s) of  $\beta$  subunits is unknown. However, coexpression of rat Kv $\beta$ 1 with RCK1 or RCK4 in *Xenopus laevis* oocytes accelerates the N-type inactivation. The excised-patch experiments showed that a synthetic peptide of the Kv $\beta$ 1 N-terminus (amino acids [aa] 1–34) accelerates the inactivation of RCK4 and suggest that the N-terminal region of Kv $\beta$ 1 functions like the inactivation gates found in some  $\alpha$  subunits (Hoshi et al., 1990; Rettig et al., 1994). It is not known, however, whether Kv $\beta$ 1 can inactivate all  $\alpha$  subunits or just a subset of the  $\alpha$  subunits. Experiments with a third  $\beta$  subunit, Kv $\beta$ 3 (cloned by homology to Kv $\beta$ 2), suggest that  $\beta$  subunits may act selectively. When coexpressed in *Xenopus* oocytes, Kv $\beta$ 3 accelerates the N-type inactivation of hKv1.4 but not that of hKv1.1, RCK1, hKv1.2, or hKv2.1 (Majumder et al., 1995; Morales et al., 1995). If Kv $\beta$ 1 selectively inactivates a subset of  $\alpha$  subunits, it is important to understand how Kv $\beta$ 1 interacts specifically.

† These authors contributed equally to this work.

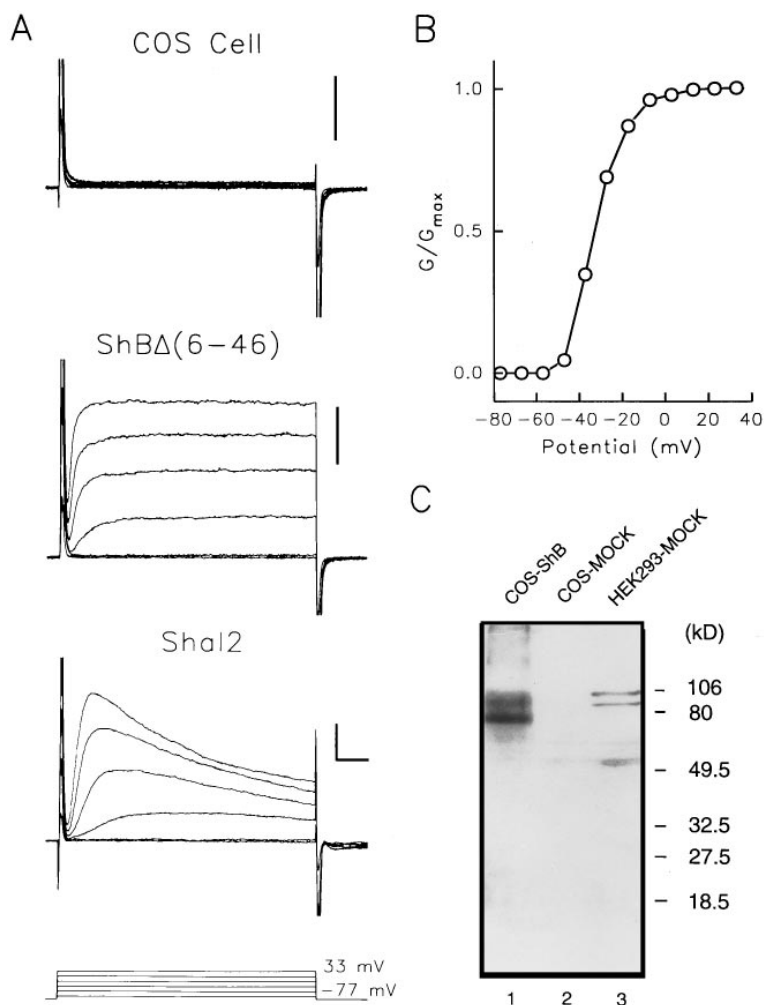


Figure 1. Expression of K<sup>+</sup> Channels in COS Cells

(A) K<sup>+</sup> currents recorded from transfected COS cells. Current responses to different holding potentials were recorded from a mock-transfected COS cell (n = 8; top), a cell transfected with ShBΔ(6-46) (n = 6; middle), and a cell transfected with Shal2 (n = 6; bottom). The bottom trace indicates the voltage steps from -77 to +33 mV at 20 mV increments. Bars, 5 ms and 500 pA.

(B) Voltage-dependent activation of ShBΔ(6-46) expressed in COS cells. Conductance (G) was calculated and normalized (see Experimental Procedures) according to the maximum conductance (G/G<sub>max</sub>; y-axis) and plotted against potentials (x-axis). Data were taken from the same cell illustrated in (A).

(C) Immunoblot analysis of the ShB expression in transiently transfected COS cells. Cell lysates containing approximately equal amounts of protein were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The ShB and possibly related endogenous polypeptides were detected by an affinity-purified anti-NShB antibody. COS-ShB, COS cells transfected with ShB; COS-MOCK, mock-transfected COS cells; HEK293-MOCK, mock-transfected HEK293 cells. The protein molecular weight markers (in kilodaltons) are indicated on the right.

We have now studied in detail the interactions between Kvβ1 and different α subunits. The specificity of the Kvβ1-mediated inactivation was tested by patch-clamp recording on transfected cells that coexpress Kvβ1 with different α subunits. The regions involved in the specific interaction between the N-terminal domain of α subunits and Kvβ1 were determined by deletion analysis using the yeast two-hybrid system. The functional consequences of this interaction were analyzed by testing the ability of Kvβ1 to inactivate channels formed by chimeric α subunits, which contain either compatible or incompatible Kvβ1-binding sites.

## Results

### Functional Expression of K<sup>+</sup> Channels in COS Cells

We used a tissue culture system to express cloned genes encoding different subunits of K<sup>+</sup> channels. Cultured mammalian cells have potentially large and inconsistent endogenous voltage-sensitive K<sup>+</sup> currents. To identify a suitable host for K<sup>+</sup> channel expression, we compared cell lines using patch-clamp recording and immunoblot analysis. Figure 1A (top) shows the recorded traces of a typical mock-transfected COS cell

in a 50 ms pulse by stepping the holding potential from -77 mV to a series of command potentials. Under these conditions, the mock-transfected COS cells showed low endogenous currents and were therefore suitable for further studies. Transient expression of different K<sup>+</sup> channel subunits was achieved using a mammalian expression vector containing the SV40 replication origin. Transcription was driven by the human cytomegalovirus (CMV) immediate-early promoter. A plasmid expressing the CD4 surface antigen was included as 10% of the total input DNA, which allowed us to identify the transfected cells using beads coated with the anti-CD4 antibody (Jurman et al., 1994). The Shaker B (ShB) and other K<sup>+</sup> channel α subunits driven by the CMV promoter have currents up to 10 nA, depending upon the channel and the amount of transfected DNA (Figure 1A). Figure 1B shows the relative peak conductance as a function of voltage for ShBΔ(6-46), a mutated ShB K<sup>+</sup> channel that lacks the inactivation gate (Hoshi et al., 1990). The voltage dependence of the peak current is qualitatively similar to that seen for Shaker currents recorded in *Drosophila* muscle and *Xenopus* oocytes (Iverson et al., 1988; Timpe et al., 1988; Zagotta et al., 1989). To compare the possible endogenous Kv1 homologous polypeptides in different cells, total protein lysates from

transfected cells were prepared and analyzed by immunoblot using an affinity-purified rabbit antibody, anti-NShB (Li et al., 1992) (see Experimental Procedures). Because the affinity purification of antibody was done using a fusion protein containing mainly the NAB region of ShB (NAB<sub>ShB</sub>), which is highly conserved within the Kv1 subfamily, the resultant immunoglobulin cross-reacts with other Kv1  $\alpha$  subunits (data not shown). Under these conditions, little endogenous  $\alpha$  subunit protein was detectable in the mock-transfected COS cells (Figure 1C, COS-MOCK). In contrast, high expression of the ShB polypeptide was detected in COS cells transfected with ShB cDNA (Figure 1C, COS-ShB; the heterogeneity in mobility may be due to differential posttranslational modification[s]). As a control, two presumed endogenous  $\alpha$  subunits were detected in human embryonic kidney cells (HEK293; Figure 1C, HEK293-MOCK), which also have higher endogenous voltage-sensitive K<sup>+</sup> currents (data not shown). These experiments showed that the COS cell line is a reasonable system for transient expression of K<sup>+</sup> channels, particularly those with fast-activating kinetics, such as ShB.

#### Kv $\beta$ 1 Selectively Inactivates the $\alpha$ Subunits of the Kv1 Subfamily

Coexpression of rat Kv $\beta$ 1 with the rat Kv1  $\alpha$  subunits, such as RCK1 and RCK4, accelerates their N-type inactivation (Rettig et al., 1994). To test whether the Kv $\beta$ 1-mediated inactivation has any dependence on the subfamily to which the  $\alpha$  subunit belongs and/or the species from which the genes encoding  $\alpha$  subunits were cloned, we coexpressed Kv $\beta$ 1 with RCK4 from rat and with ShB (Kv1 subfamily) or Shal2 (Kv4 subfamily) from *Drosophila*. Figure 2A shows an example of the responses of RCK4 when expressed in COS cells in the presence or absence of Kv $\beta$ 1. Consistent with results observed in *Xenopus* oocytes (Rettig et al., 1994), cotransfection of RCK4 with Kv $\beta$ 1 accelerated the intrinsic N-type inactivation of RCK4. Similarly, Kv $\beta$ 1 accelerated the N-type inactivation when coexpressed with ShB (Figure 2B). This indicates that the action of Kv $\beta$ 1 is not species dependent. To test whether Kv $\beta$ 1 displays any subfamily specificity, we coexpressed Kv $\beta$ 1 with an  $\alpha$  subunit from a different subfamily. Sequence alignment indicated that the Kv1 and Kv4 subfamilies are more closely related to each other than to other subfamilies (Milkman, 1994). Shal2 is a *Drosophila*  $\alpha$  subunit of the Kv4 subfamily with sequence and kinetic similarity to the Shaker (Kv1) channels (Butler et al., 1990) and therefore provided a test of the ability of Kv $\beta$ 1 to inactivate with a different subfamily. In contrast to ShB, the inactivation properties of Shal2 did not change in the presence of Kv $\beta$ 1, as determined by comparing their time constants of inactivation (see Figure 2C and legend). These results supported the idea that Kv $\beta$ 1 interacts selectively with  $\alpha$  subunits of the Kv1 subfamily.

#### Subfamily-Specific Association of Kv $\beta$ 1 with the N-Terminal Cytoplasmic Domains of $\alpha$ Subunits

There is biochemical evidence for a physical association between the RCK5 (or Kv1.2) protein and Kv $\beta$ 2 (Scott

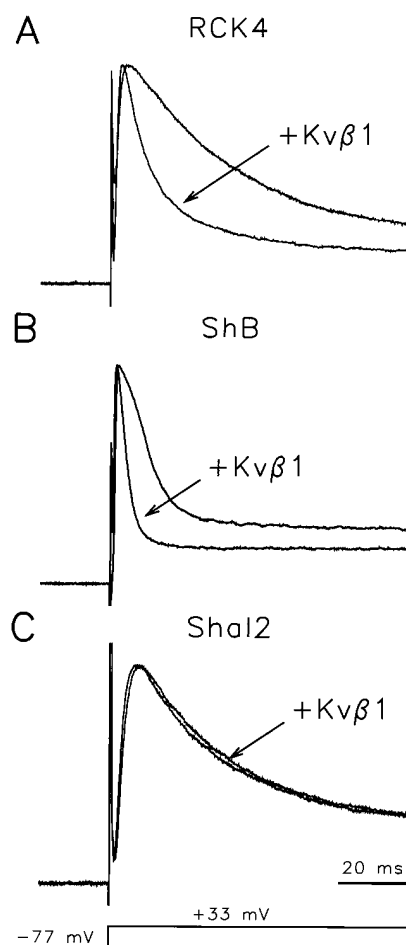


Figure 2. Selective Inactivation of K<sup>+</sup> Channels by Kv $\beta$ 1

(A) Whole-cell voltage-clamp recordings were carried out on COS cells transfected with RCK4 ( $n = 6$ ) or RCK4 plus Kv $\beta$ 1 ( $n = 6$ ) (indicated by the arrow). Current responses to a voltage step from  $-77$  to  $+33$  mV (indicated in the bottom trace) were normalized according to the peak current and superimposed. The accelerated N-type inactivation of RCK4 is indicated by the arrow.

(B) Whole-cell recordings were obtained from COS cells transfected with ShB ( $n = 5$ ) or ShB plus Kv $\beta$ 1 ( $n = 5$ ). Current responses to a voltage step from  $-77$  to  $+33$  mV (indicated in the bottom trace) were normalized according to the peak current and superimposed. The accelerated N-type inactivation of ShB is indicated by the arrow.

(C) Whole-cell recordings were obtained from COS cells transfected with Shal2 with or without Kv $\beta$ 1. Current responses to a voltage step from  $-77$  to  $+33$  mV (indicated in the bottom trace) were normalized according to the peak current and averaged within the group. The control trace shows the averaged current response recorded from COS cells transfected with Shal2 ( $\tau = 24.3 \pm 1.5$  ms, mean  $\pm$  SEM;  $n = 6$ ). The second trace (indicated by the arrow) illustrates the averaged current response obtained from COS cells cotransfected with Shal2 and Kv $\beta$ 1 ( $\tau = 26.4 \pm 2.9$  ms, mean  $\pm$  SEM;  $n = 8$ ). There is no significant difference between the time constants of these two groups ( $p < .05$ ,  $t$  test).

et al., 1990). To identify a region(s) within the  $\alpha$  subunit that binds to Kv $\beta$ 1, we performed binding studies using biochemical methods and the yeast two-hybrid system (Fields and Song, 1989; Chevray and Nathans, 1992). Since the cytoplasmic Kv $\beta$ 1 acts on the Kv1  $\alpha$  subunits (Figure 2), we reasoned that the Kv $\beta$ 1 subunit should

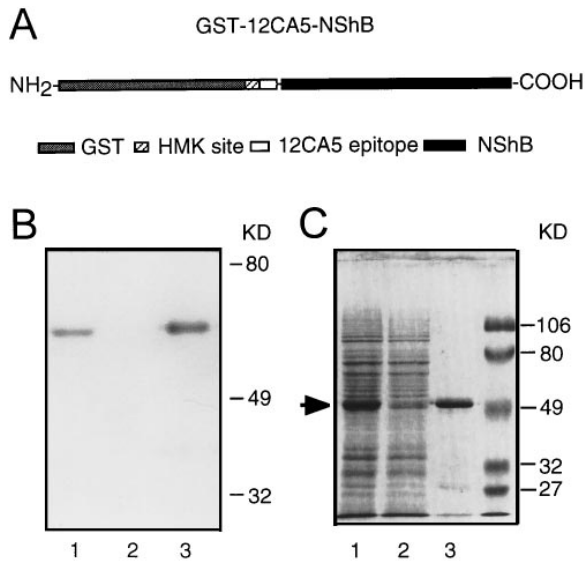


Figure 3. Affinity Binding of Kv $\beta$ 1 to NShB

(A) The NShB fusion protein was prepared in Sf9 cells using a recombinant baculovirus (see Experimental Procedures). As indicated in the diagram, the NShB fusion protein contains GST, heart muscle kinase (HMK) recognition sequence (Li et al., 1992), and a 12CA5 epitope.

(B) Soluble Sf9 lysate containing GST-NShB was incubated with glutathione-Sepharose to generate the NShB affinity column. The GST-NShB polypeptide in different fractions was detected by SDS-PAGE and immunoblot using rabbit anti-NShB (Li et al., 1992). Lane 1, soluble Sf9 cell lysate; lane 2, wash; lane 3, elution with 20 mM glutathione.

(C) Kv $\beta$ 1 binds to the NShB affinity column. The soluble bacterial lysate (see Experimental Procedures for a detailed protocol) was incubated with NShB affinity matrix. The Kv $\beta$ 1 polypeptide with an apparent molecular weight of 51 kDa (indicated by an arrow) in each fraction was analyzed by SDS-PAGE and detected by Coomassie staining. Lane 1, crude bacterial lysate; lane 2, unbound material; lane 3, eluted material by the binding buffer supplemented with 0.5 M NaCl.

interact with cytoplasmically exposed region(s) of the  $\alpha$  subunits, perhaps with N-terminal domains since they are conserved in a subfamily-specific manner (Xu et al., 1995). We expressed the N-terminal domain of ShB (NShB; aa 1–227) as a glutathione S-transferase (GST) fusion protein (GST-NShB) in Sf9 cells using a recombinant baculovirus (Figure 3A). Using GST-NShB and glutathione-Sepharose, an affinity column was generated (Figure 3B). If Kv $\beta$ 1 bound to NShB, one would expect that the Kv $\beta$ 1 protein would be retained on the column. To prepare Kv $\beta$ 1 protein, we expressed Kv $\beta$ 1 in *Escherichia coli* cells as a histidine-tagged protein. The Kv $\beta$ 1 expression was induced by isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG); soluble lysate that contains the Kv $\beta$ 1 fusion protein was prepared. The Kv $\beta$ 1 protein is the major band, with a molecular weight of 51 kDa (Figure 3C, lane 1). Indeed, when this lysate was allowed to pass through the affinity column, Kv $\beta$ 1 was specifically retained on the column, whereas the bacterial proteins were largely in the flowthrough fraction (Figure 3C, lane 2). The bound Kv $\beta$ 1 can be eluted by 0.5 M NaCl (Figure 3C, lane 3). This result suggests a physical association between Kv $\beta$ 1 and NShB.

To test the interaction between Kv $\beta$ 1 and various portions of  $\alpha$  subunits, the yeast two-hybrid system was used (Chevray and Nathans, 1992). Cytoplasmic N- and C-terminal domains of different  $\alpha$  subunits were subcloned and expressed as fusion proteins of the GAL4 DNA-binding (DB) domain (see Experimental Procedures). Kv $\beta$ 1 was expressed as a fusion protein of either the GAL4 DB domain or the transcription activation (TA) domain. The Kv $\beta$ 1 fusion proteins did not activate transcription by themselves, since yeast transformants containing either GAL4-DB-Kv $\beta$ 1/GAL4-TA or GAL4-TA-Kv $\beta$ 1/GAL4-DB did not grow in synthetic medium lacking histidine (selection medium) (Figure 4, #3 and #4). When Kv $\beta$ 1 was coexpressed with the N-terminal domain of RCK4 (NRCK4; aa 1–306), an  $\alpha$  subunit sensitive to the Kv $\beta$ 1-mediated inactivation, the yeast transformants grew on selection medium (Figure 4, #1). Given that NRCK4 does not have endogenous transcription activation activity (Xu et al., 1995), this suggests that NRCK4 also has a binding site for Kv $\beta$ 1. In contrast, coexpression of Kv $\beta$ 1 and the C-terminal domain of RCK4 (CRCK4; aa 566–651) did not permit growth on the selection medium (Figure 4, #2). These results suggest that Kv $\beta$ 1 binds to the N-terminal domain of RCK4.

Combinatorial pairwise expression of the N-terminal domains of different  $\alpha$  subunits in the yeast two-hybrid system has shown that the N-terminal domains of Kv1, Kv2, Kv3, and Kv4 associate with each other strictly within each subfamily (Xu et al., 1995). To test the possible specificity of the  $\alpha$ - $\beta$  interaction, Kv $\beta$ 1 was coexpressed with the N-terminal domains of  $\alpha$  subunits, including ShB (Kv1), DRK1 (Kv2.1), NGK2b (Kv3.1), and rshal1 (Kv4.2) (Xu et al., 1995). Yeast transformants containing Kv $\beta$ 1 and the N-terminal domain of ShB, but not that of DRK1 (aa 1–182), NGK2b (aa 1–180), or rshal1 (aa 1–228), grew on the selection medium (Figure 4, #5–#8). In addition, coexpression of Kv $\beta$ 1 with the N-terminal domain of *Drosophila* Shab11 of the Kv2 subfamily, Shaw2 of the Kv3 subfamily, or Shal2 of the Kv4 subfamily did not permit the transformants to grow on the selection medium (data not shown). Thus, the binding specificity of Kv $\beta$ 1 to the N-terminal domain of the  $\alpha$  subunits coincides precisely with the Kv1 subfamily.

#### Regions Involved in $\alpha$ - $\beta$ Interactions

Because Kv $\beta$ 1 binds to both NShB and NRCK4 (Figures 3 and 4), one would predict that the NAB region, a highly conserved region within the N-terminal domains of all Kv1  $\alpha$  subunits, may be responsible for the interaction. Kv $\beta$ 1 is a rather basic (or positively charged) protein with a calculated isoelectric focusing constant (pI) of 9.43. Interestingly, unlike NShB, NRCK4 has 30 residues with positively charged side chains and 61 negatively charged residues, which are densely located N-terminal to NAB<sub>NRCK4</sub>. To map the region(s) in the  $\alpha$  subunits to which Kv $\beta$ 1 binds and to test whether regions rich in negative charges play a role in the  $\alpha$ - $\beta$  interaction, NRCK4 was chosen for deletion analysis. Deletion mutants corresponding to different segments of NRCK4 were constructed. Their ability to interact with NRCK4 was tested by the yeast two-hybrid test. The minimal region in NRCK4 capable of associating with Kv $\beta$ 1 in

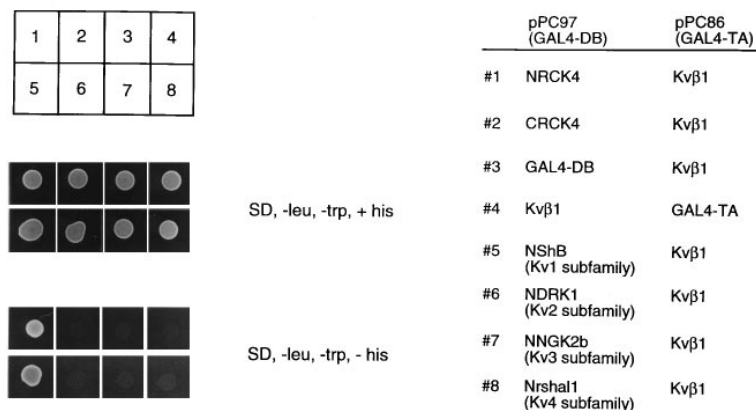


Figure 4. Binding of Kvβ1 to the N-Terminal Domains of the Kv1 α Subunits Tested by the Yeast Two-Hybrid System

YGH1 yeast cells were transformed by different pairwise combinations of the two-hybrid constructs that express either fusion proteins of the DNA-binding domain of GAL4 (DB and pPC86) or the transcription activation domain of GAL4 (TA and pPC97) (see Xu et al., 1995, and Experimental Procedures for vector construction). The transformants carrying the two different fusion proteins were first selected by dextrose synthetic dropout 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). The transformants were allowed to grow at 30°C for 65 hr. Eight different plasmid combinations are listed on the right. Their growth in the SD, -leu, -trp, +his medium is shown in the middle panel on the left. Their growth in the SD, -leu, -trp, -his medium is shown in the bottom panel on the left.

the two-hybrid test was a peptide fragment of amino acids 174–306, which overlaps with NAB<sub>RCK4</sub> (aa 174–279; Figure 5A). It also indicates that the clusters of negative charges present upstream of NAB<sub>RCK4</sub> are not required for the Kvβ1–NRCK4 interaction. Sequence alignment between the mapped region of RCK4 and that of ShB reveals more than 80% amino acid identity (Figure 5B). In contrast, the same comparison between RCK4 and Shal2 shows only 24% amino acid identity. These results suggest that the NAB<sub>Kv1</sub> region of the α subunits is a critical determinant of subfamily-specific α–Kvβ1 binding (Figure 5B).

Inactivation accelerated by Kvβ1 is mechanistically similar to the N-type inactivation present in some K<sup>+</sup> channels (Rettig et al., 1994). However, it was possible that the interaction between Kvβ1 and the N-terminal domain of the Kv1 α subunits (see Figure 4) was mediated by the inactivation particle of Kvβ1. To test this possibility, we constructed deletion mutants of Kvβ1 and tested their association with NRCK4 using the two-hybrid system. No detectable difference in association with RCK4 was found between wild-type Kvβ1 and Kvβ1Δ(1–72), which lacks the Kvβ1 inactivation gate (Figure 5C). Thus, the Kvβ1 conserved region (aa 73–401) is sufficient to bind NRCK4. We conclude that the interaction between Kvβ1 and the N-terminal domains of α subunits seen in our two-hybrid test is different from the interaction between the Kvβ1 inactivation gate and its receptor. Further deletions of Kvβ1 significantly reduced its ability to associate with NRCK4 (Figure 5C), suggesting that amino acids 73–401 are directly and indirectly involved in the α–Kvβ1 association. The mapped region (aa 73–401), i.e., the core region, for Kvβ1 binding is highly conserved among Kvβ1, Kvβ2, and Kvβ3 (Figure 5D). This may imply that the corresponding regions of other β subunits are involved in association with the N-terminal domains of the α subunits.

#### The Core Region of Kvβ1 Is Necessary for the Kvβ1-Mediated Inactivation

The interaction between the core region of Kvβ1 and NAB<sub>Kv1</sub> suggests an α–β association that is independent

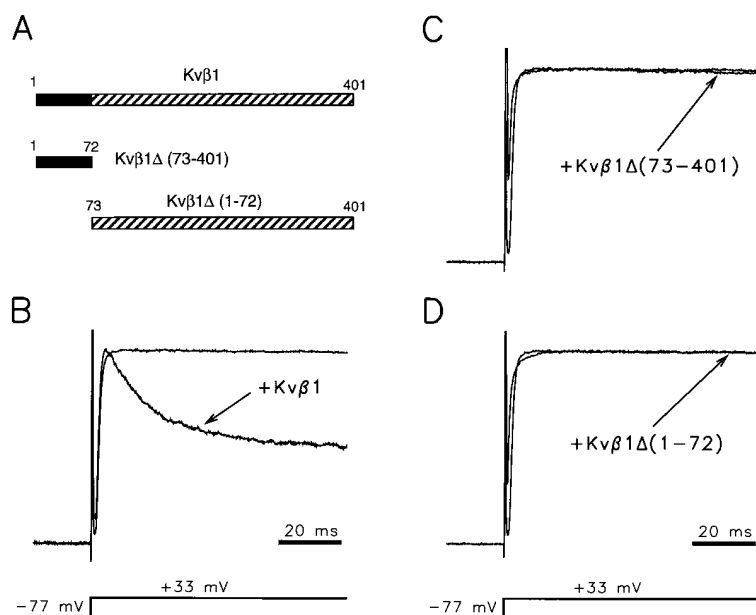
from that of the Kvβ1 inactivation particle with its putative receptor. This is supported by three lines of evidence. First, deletion of the Kvβ1 inactivation particle had no effect on Kvβ1–NRCK4 association (Figure 5C). Second, N-type inactivation does not require the NAB domain, since the deletion of amino acids 28–283 of hKv1.4, which includes the NAB domain, decreases its expression without altering the N-type inactivation (Lee et al., 1994). Third, at high concentrations, soluble peptides representing the inactivation particles of α subunits or Kvβ1 can inactivate channels, as tested by excised-patch recording (Zagotta et al., 1990; Rettig et al., 1994). Thus, one possible function for the NAB<sub>Kv1</sub>–Kvβ1 interaction is to recruit Kvβ1 to the acting site (see Discussion for a proposed model).

To test whether this interaction is required for the Kvβ1-mediated inactivation, we constructed two Kvβ1 deletion mutants, Kvβ1Δ(1–72) and Kvβ1Δ(73–401), in which either the region including the inactivation particle or the core region is removed (Figure 6A). To increase sensitivity for testing the ability of the truncated Kvβ1 to inactivate α subunits, ShBΔ(6–46) was used since its intrinsic N-type inactivation was completely removed (Hoshi et al., 1990). Figure 6B shows that Kvβ1 induces the inactivation of ShBΔ(6–46), in which the inactivation has an average time constant of 18.7 ± 2.8 ms (mean ± SEM; n = 9) at a holding potential of +33 mV. In contrast, when cotransfected with either Kvβ1Δ(73–401) (Figure 6C; n = 11) or Kvβ1Δ(1–72) (Figure 6D; n = 8), ShBΔ(6–46) showed no fast inactivation. This result suggests that, within an intact cell, neither the core region nor the inactivation particle of Kvβ1 is sufficient to induce the Kvβ1-mediated inactivation. It also supports the notion that the interaction of the Kvβ1 core region with NAB<sub>Kv1</sub> of α subunits serves to secure the α–β complex within a cell. This increases the local concentration of Kvβ1 inactivation particles, which thereby accelerates inactivation (see Discussion and Figure 8).

#### The NAB<sub>Kv1</sub> Region of α Subunits Is Necessary for Inactivation by Kvβ1

The failure of Kvβ1Δ(73–401) to inactivate ShBΔ(6–46) (Figure 6C) implies that the interaction between the Kvβ1





**Figure 6. The Core Region of Kvβ1 Is Necessary for the Kvβ1-Mediated Inactivation**

A schematic diagram (A) shows the wild-type Kvβ1 and two Kvβ1 deletion constructs. The voltage-clamp recordings were obtained from COS cells. The current responses to a voltage step from  $-77$  to  $+33$  mV (indicated at the bottom of [B] and [D]) were normalized according to the peak response. The control responses were obtained from COS cells transfected with ShBΔ(6–46). This trace was superimposed with the response from cotransfection of ShBΔ(6–46) with Kvβ1 (B;  $\tau = 18.7 \pm 2.8$  ms; mean  $\pm$  SEM;  $n = 9$ ), Kvβ1Δ(73–401) (C;  $n = 11$ ), or Kvβ1Δ(1–72) (D;  $n = 8$ ).

with the mapped Kvβ1-binding site of RCK4 (aa 174–306; see Figures 5A and 7A), it restored the ability of Kvβ1 to inactivate this new chimera (Figure 7B, NRCK4/ShB).

The failure to detect inactivation of NShal/ShB by Kvβ1 is unlikely to be a result of lower expression of Kvβ1, for the following reasons. The input DNA and the ratio of the plasmids encoding the  $\alpha$  subunits (6  $\mu$ g) and Kvβ1 (12  $\mu$ g) were identical in these experiments. In addition, it is known that the  $\alpha$ : $\beta$  plasmid ratio is at a level at which Kvβ1 is saturated, since the Kvβ1-mediated inactivation was observed when ShBΔ(6–46):Kvβ1 was 2:1 (data not shown). Although the transfection efficiency may vary from experiment to experiment, the whole-cell voltage-clamp recordings were carried out only on the CD4-positive cells, i.e., the transfected cells. To test expression of the Kvβ1 polypeptide directly, immunoblot analysis was performed using monoclonal antibody (MAb) 12CA5 to detect the tagged Kvβ1 protein in various transfected cells (see Experimental Procedures). Figure 7C shows that MAb 12CA5 detected three polypeptides with sizes ranging from 37 to 48 kDa that are present only in transfections containing Kvβ1 plasmid (see Figure 7 legend). The heterogeneity in molecular weight is presumably due to posttranslational modification(s). Judging by the intensity of the bands and their relative ratio, levels and heterogeneity of the Kvβ1 polypeptides in both NShal/ShB–Kvβ1 and ShB–Kvβ1 cotransfections were similar (Figure 7C). Therefore, the ability of Kvβ1 to inactivate NRCK4/ShB but not NShal/ShB further supports the idea that a compatible interaction (i.e., subfamily-specific association) between Kvβ1 and the N-terminal domain of a Kv1  $\alpha$  subunit is necessary for the Kvβ1-mediated inactivation.

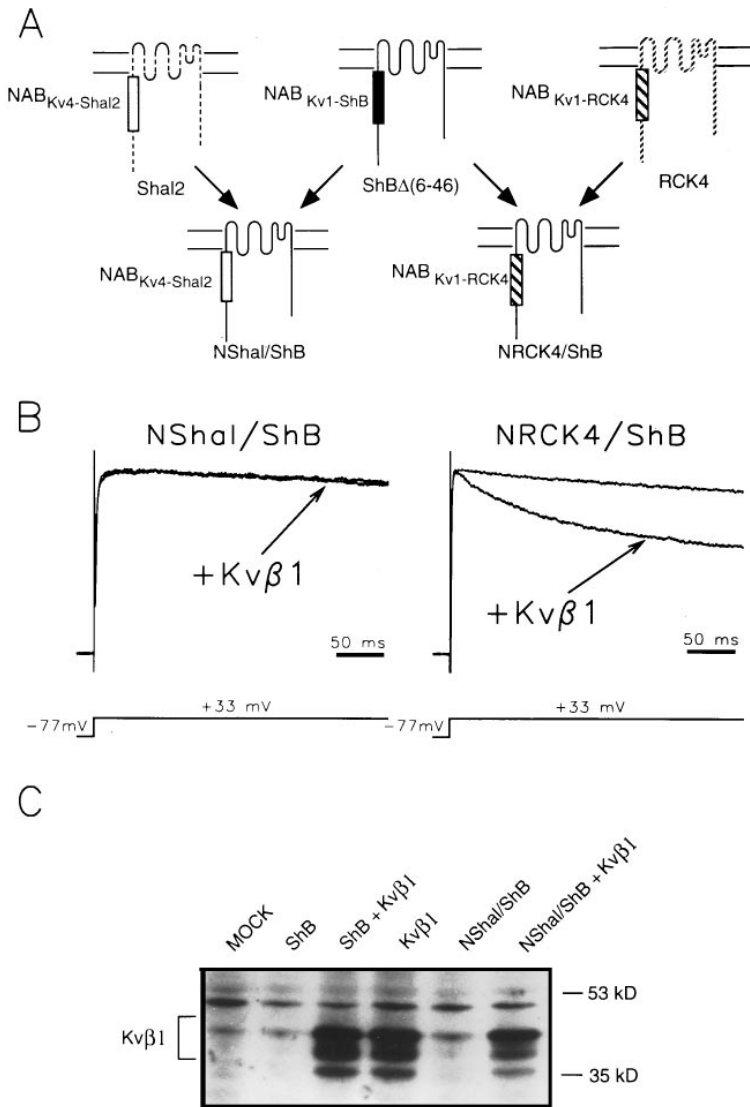
## Discussion

We have shown that Kvβ1 inactivates RCK4 and ShB of the Kv1 subfamily but not Shal2, a member of the

Kv4 subfamily. Using biochemical binding and the yeast two-hybrid system, we found that Kvβ1 binds to the N-terminal domain of the Kv1  $\alpha$  subunits but not the C-terminal domain of RCK4. Coexpression of Kvβ1 with the N-terminal domains of Kv1, Kv2, Kv3, and Kv4 in the yeast two-hybrid system revealed that Kvβ1 binds only to the N-terminal domains of the Kv1  $\alpha$  subunits and not to those of the Kv2–Kv4 subfamilies. Furthermore, the binding site on the Kv1  $\alpha$  subunits for Kvβ1 was mapped to a region overlapping NAB<sub>Kv1</sub>, a structural motif known to mediate subfamily-specific associations of  $\alpha$  subunits (Li et al., 1992). Replacing the binding site with the corresponding region of Shal2, a region that does not bind to Kvβ1, resulted in a chimeric ShB channel that was no longer sensitive to Kvβ1. The sensitivity to Kvβ1 can be restored when the Shal2 sequence is replaced by the corresponding region of RCK4. Together, these data suggest that the assembly of  $\alpha$ – $\beta$  complex(es) through the NAB<sub>Kv1</sub>–Kvβ1 association is necessary for the Kvβ1-mediated inactivation.

## The Binding Sites for $\alpha$ –Kvβ1 Assembly

Our results suggest a molecular basis for the subfamily-specific action of Kvβ1. Namely, the NAB regions of the  $\alpha$  subunits (Kv1–Kv4 subfamilies) interact to form subfamily-specific heteromultimers (Xu et al., 1995). Comparison of available NAB sequences in the cloned  $\alpha$  subunits has revealed that all NAB regions share about 40% amino acid identity (Drewe et al., 1992; Xu et al., 1995). A common structural scaffold in this region is also predicted. Nevertheless, different subfamilies can be identified, since within a given subfamily NAB regions share more than 70% amino acid identity. Residues that are conserved only in a subfamily-specific manner have been identified (Xu et al., 1995), and these may play a role in determining subfamily-specific functions. The present studies demonstrated that the binding site of  $\beta$  subunits overlaps NAB<sub>Kv1</sub>, revealing the dual function of



**Figure 7. Compatible Binding Site in the N-Terminal Domains of  $\alpha$  Subunits Is Necessary for the Kv $\beta$ 1-Mediated Inactivation**

(A) A diagram illustrates the construction of the two chimeric ShB  $\alpha$  subunits. The coding segment representing amino acids 95–227 of ShB $\Delta$ (6–46), including the Kv $\beta$ 1-binding site, was replaced by the corresponding region of Shal2 (aa 40–185; NShal/ShB; left) or by the corresponding region of RCK4 (aa 174–306; NRCK4/ShB; right).

(B) Whole-cell recordings were carried out on COS cells transfected with either NShal/ShB (left) or NRCK4/ShB (right) in the presence or absence of Kv $\beta$ 1. Current responses to a voltage step from  $-77$  to  $+33$  mV were normalized according to the peak current. The left panel shows the recordings from NShal/ShB-transfected COS cells in the presence ( $n = 6$ ) or absence ( $n = 3$ ) of Kv $\beta$ 1. The right panel shows the recordings from NRCK4/ShB-transfected COS cells in the presence ( $n = 7$ ) or absence ( $n = 3$ ) of Kv $\beta$ 1. Arrows indicate the recording from a cell co-transfected with chimera and Kv $\beta$ 1.

(C) Immunoblot analysis of Kv $\beta$ 1 expression. Total protein lysates were prepared from the cells transfected with the indicated combinations of plasmid(s). The expression of Kv $\beta$ 1 was detected by the affinity-purified MAB against the 12CA5 tag fused to the C-terminus of Kv $\beta$ 1 (see Experimental Procedures). MOCK, no plasmid DNA; ShB, 18  $\mu$ g; ShB+Kv $\beta$ 1, 6  $\mu$ g + 12  $\mu$ g; Kv $\beta$ 1, 18  $\mu$ g; NShal/ShB, 18  $\mu$ g; NShal/ShB+Kv $\beta$ 1: 12  $\mu$ g + 6  $\mu$ g. The molecular weight standards are indicated on the right.

the NAB<sub>Kv1</sub> domain in assembling both  $\alpha$ - $\alpha$  and  $\alpha$ -Kv $\beta$ 1 complexes.

The conserved core region (aa 73–401) of Kv $\beta$ 1 was sufficient for Kv $\beta$ 1 to associate with the N-terminal domain of the  $\alpha$  subunits, indicating that the inactivation gate of Kv $\beta$ 1 is not required for this interaction. The core regions of the three cloned  $\beta$  subunits are over 85% identical in amino acid sequence. However, further studies are needed to determine whether this means that Kv $\beta$ 2 and Kv $\beta$ 3 also interact exclusively with members of the Kv1 subfamily.

Figure 5 shows the identification of two interacting regions, amino acids 174–306 in the RCK4  $\alpha$  subunit and amino acids 73–401 in Kv $\beta$ 1, that are important for the  $\alpha$ - $\beta$  interaction. These results should help our future experiments to pinpoint the amino acid residues that mediate the interaction. For example, the inability of Kv $\beta$ 1(131–401) and Kv $\beta$ 1(73–346) (Figure 5C) to bind NRCK4 raises the possibility that the regions of amino acids 73–131 and 346–401 may contain residues that

are important for the interaction. This hypothesis can now be tested directly by several approaches, including site-directed mutagenesis.

Our results do not allow us to determine whether Kv $\beta$ 1 binds to a single NAB domain or to an area formed by more than one NAB. We hope that this question can be answered using point mutations defective in NAB–NAB interactions combined with binding studies using genetic or biochemical approaches.

#### Inability of Kv $\beta$ 1 $\Delta$ (73–401) to Inactivate ShB $\Delta$ (6–46)

A peptide representing a fragment of amino acids 1–34 of Kv $\beta$ 1, when applied to an inside-out excised patch at 100  $\mu$ M, partially restored the Kv $\beta$ 1-mediated inactivation on RCK4 (Rettig et al., 1994). In contrast, Kv $\beta$ 1 $\Delta$ (73–401), which contains the inactivation particle, failed to inactivate ShB $\Delta$ (6–46) in the transfected cells (see Figure 6). This inconsistency is likely due to the fact that the concentration of the Kv $\beta$ 1 $\Delta$ (73–401) peptide

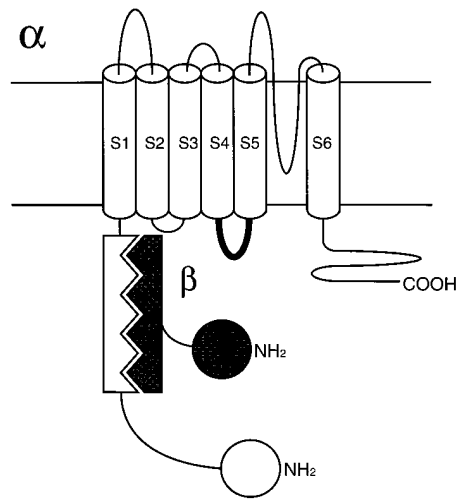


Figure 8. A Working Model for  $\alpha$ -Kv $\beta$ 1 Interaction

The diagram represents the postulated  $\alpha$ -Kv $\beta$ 1 interaction. The putative membrane-spanning segments of the  $\alpha$  subunit are designated as S1–S6, and the inactivation gate (present in some Kv1  $\alpha$  subunits) is represented as an open circle. The S4–S5 loop, the putative receptor for the inactivation gate of the  $\alpha$  subunit, is highlighted as a thicker line. The N-terminal domain of the  $\alpha$  subunit that mediates the  $\alpha$ -Kv $\beta$ 1 association is illustrated as an open box. The shaded model represents a Kv $\beta$ 1 subunit, in which the inactivation gate of Kv $\beta$ 1 is designated as a circle. The model may not represent the real stoichiometry of the  $\alpha$ -Kv $\beta$ 1 complex.

within intact cells was not high enough to induce detectable inactivation. This discrepancy can also be subjected to other interpretations. For example, the Kv $\beta$ 1 $\Delta$ (73–401) peptide expressed in the transfected cells could be incorrectly targeted.

#### Selectivity of the Kv $\beta$ 1-Mediated Inactivation

Kv $\beta$ 1 accelerates the inactivation of RCK1 and RCK4 when coexpressed in *Xenopus* oocytes (Rettig et al., 1994); it has similar effects on RCK4 and ShB expressed in COS cells (see Figure 2). Our results indicate that the compatible interactions between the Kv $\beta$ 1 core region and the N-terminal domain of the Kv1  $\alpha$  subunits are necessary for the Kv $\beta$ 1-mediated inactivation. First, deletion of the Kv $\beta$ 1 inactivation particle had no effect on the association of Kv $\beta$ 1 with NRCK4 (see Figures 4 and 5). Deletion of the Kv $\beta$ 1 core region abolished the inactivation induced by Kv $\beta$ 1 (see Figure 6). Second, when replacing NAB<sub>ShB</sub> in ShB $\Delta$ (6–46) with other NAB regions, we found that Kv $\beta$ 1 inactivates only the chimera with a compatible NAB domain (see Figure 7). However, it is not known whether this compatible interaction is sufficient for Kv $\beta$ 1 to inactivate Kv1 channels. This is an interesting question, since Kv $\beta$ 3 was reported to inactivate a subset of Kv1 channels (Majumder et al., 1995; Morales et al., 1995). In an attempt to test this possibility, we have constructed a reverse chimera by replacing the N-terminal domain of Shal2 with the corresponding region of ShB. When expressed in COS cells, the resultant chimera was not functional. In future experiments, after the identification of the receptor to which the Kv $\beta$ 1

inactivation gate binds, it would be interesting to construct a functional chimeric  $\alpha$  subunit containing a receptor from a different subfamily for the Kv $\beta$ 1 inactivation gate but a compatible N-terminal domain. Testing the ability of Kv $\beta$ 1 to inactivate this chimeric channel would help address whether the association of Kv $\beta$ 1 with the N-terminal domain of the Kv1  $\alpha$  subunits is sufficient for determining the specificity.

#### Function of the Subfamily-Specific $\alpha$ -Kv $\beta$ 1 Associations

One obvious effect of having a preassembled  $\alpha$ - $\beta$  complex is to increase the local concentration of the Kv $\beta$ 1 inactivation gate. It is not clear whether the inactivation gate of  $\alpha$  subunits and that of Kv $\beta$ 1 share the same receptor. The fact that the accelerated inactivation by Kv $\beta$ 1 on ShB is much faster than that on ShB $\Delta$ (6–46), which lacks the endogenous inactivation gate (see Figures 2 and 6), suggests that neither the  $\alpha$  nor the  $\beta$  inactivation gate has a dominant effect. Similar results were also observed when Kv $\beta$ 1 was coexpressed with RCK4 or with RCK4 $\Delta$ (1–110), in which the inactivation gate was partially removed (Rettig et al., 1994). These results support the possibility of additive effects by the inactivation gates of  $\alpha$  and  $\beta$  subunits and are consistent with studies in which increasing the number of inactivation gates in channels formed by  $\alpha$  subunits accelerated the N-type inactivation (MacKinnon et al., 1993).

The specificity of forming  $\alpha$ - $\beta$  complexes suggests another level of regulation of K<sup>+</sup> currents. Since a given cell may possess multiple classes of K<sup>+</sup> channel systems (see review by Salkoff et al., 1992) and the reported modulatory effect of Kv $\beta$ 1 is to increase inactivation (Rettig et al., 1994), perhaps it would be disadvantageous for cells to have a generic “inactivator” for all K<sup>+</sup> channels. Given the increasing evidence that indicates that some inactivation gates of  $\alpha$  subunits display broad specificity (Hoshi et al., 1990; Zagotta et al., 1990; Ruppersberg et al., 1991; Foster et al., 1992; Toro et al., 1992; Beirao et al., 1994; Stephens and Robertson, 1995), it seems logical for the cell to achieve the specificity by other type(s) of  $\alpha$ - $\beta$  interactions, such as the NAB-Kv $\beta$ 1 interactions, that appear to be independent of the interaction between the inactivation gate and its receptor.

#### A Working Model

Voltage-gated K<sup>+</sup> channels of the Kv1 subfamily are assembled by associating four identical or homologous  $\alpha$  subunits. Their functions can be modulated by interacting with hydrophilic  $\beta$  subunit(s) (Figure 8). Based on the hydrodynamic analysis of the RCK5-Kv $\beta$ 2 complex, the stoichiometry of the  $\alpha$ - $\beta$  complex was proposed to be  $\alpha_4\beta_4$  (Parcej et al., 1992). The specificity of  $\alpha$ - $\alpha$  association is mediated by the subfamily-specific interactions between NAB regions located within the cytoplasmic N-terminal domains. We propose that the acceleration of the N-type inactivation by Kv $\beta$ 1 requires two independent and sequential interactions: the conserved core region of Kv $\beta$ 1 binds to the NAB<sub>Kv1</sub> region on the  $\alpha$  subunits, and the inactivation gate of Kv $\beta$ 1 interacts

Table 1. Sequences of Oligonucleotide Primers

Oligos	Sequences	Genes	Amino Acid Positions
ML108	GGCGTCGACGGAGGTGGCAATGGTGAG	RCK4	2
ML109	GACGCGGCCGACTAGCCCCTGGCAGGGCTGG	RCK4	310
ML111	GACGCGGCCGACTATCTGGCGGCTTGGCAAC	ShB	227
ML120	GGCCCCGGGGCCGCCGTTGCCGGC	ShB	2
ML125	GACGGATCCGGTCGACGATGGCAGCCGGTGTGC	rshal1	1
ML126	GACGCGGCCGCGGATCCTCACATGGTGCTGGTGTGGG	rshal1	183
ML127	GACGGATCCGGTCGACGATGACGAAGCATGGCTCG	DRK1	1
ML128	GACGCGGCCGCGGATCCTCACACCGACGAGTTGGGC	DRK1	182
ML129	GACGGATCCGGTCGACGATGGGCCAAGGGGACG	NGK2	1
ML130	GACGCGGCCGCGGATCCTCACCGCGCATAGCGGATG	NGK2	180
ML900	CAGCCCCGGAACCATGGTCGGGCAATTGCAAG	fshabl1	1
ML901	CAGGCGGCCGCCTACCGGGCGGCGAAACTAG	fshabl1	435
ML903	CAGAGATCTTACGGACGACGAGAAGCTCC	fshal2	38
ML904	CAGGCGGCCGCCTACAGGGCGCTCGTCGACG	fshal2	185
ML905	CAGCCCCGGTACCATGGATCTGATCAACATGGACTC	fshaw2	3
ML906	CAGGCGGCCGCCTACTTGGCTGCATTGGAAGT	fshaw2	174
ML1016	CAGGTGACAGAACGCGTGGTAATAAATGTG	RCK4	174
ML1017	CAGGTGACAGACCCTGAGAAGAGGAC	RCK4	202
ML1018	CAGGCGGCCGCGCCCTCATCTCACGG	RCK4	268
ML1019	CAGGTGACCCACAGAGAGACTGAAAACG	RCK4	566
ML1020	CAGGCGGCCGACATCAGTCTCCACAGC	RCK4	651
ML1021	CAGGAATCAAGATCTCAATGCAAGTCTCCATAGC	Kvβ1	1
ML1022	CAGAGATCTCATATAGGAATCTTGGG	Kvβ1	73
ML1023	CAGAGATCTTGGTATTCTGGGGAGC	Kvβ1	131
ML1026	CAGGCGGCCGCTACAGGCACCATGCCACAGC	Kvβ1	341
ML1027	CAGGCGGCCGCTAGCCTTAGGATCTATAGTCC	Kvβ1	398
ML1028	CAGGCGGCCGCTCACTTCTCAGGGTCTCCC	RCK4	201
ML1034	CAGAAGCTTTAACGGACGACGAGAAGC	fshal2	38
ML1035	CAGAAGCTTTCGTGACGCTGTGCGG	fshal2	178

The underlined sequences represent the coding sequences or the complementary coding sequence of the indicated K<sup>+</sup> channel genes. For all antisense primers, a stop codon (UGA) was included.

with the compatible receptors on  $\alpha$  subunits. One function of assembling  $\alpha$ -Kv $\beta$ 1 complexes through binding of Kv $\beta$ 1 subunits to the NAB<sub>Kv1</sub> domain is to increase the local concentration of the inactivation gates, and thereby to accelerate the rate of N-type inactivation.

#### Experimental Procedures

##### Yeast Strains

PCY (*MATaDgal4Dgal80URA3::GAL1-lacZ lys2-801<sup>amber</sup>His3 D200t rp1D63ILEU2 ADE2-101<sup>ochre</sup>*) is a gift from Dr. D. Nathans. It contains a genomic *GAL1-lacZ* reporter gene, while the endogenous *GAL4* and *GAL80* genes were deleted (Chevray and Nathans, 1992). YGH1 (*LYS2::GAL1UAS-GAL1tat $\alpha$ -HIS3, URA3::GAL1-lacZ*) is a gift from Dr. D. Beach. In addition to the deletion of the endogenous *GAL1* and *GAL80* genes, it also contains a *GAL1-HIS3* reporter gene.

##### cDNAs and Plasmids

The full-length cDNA clones of Shab1, Shal2, Shaw2 were gifts from Dr. L. Salkoff (Butler et al., 1990). DRK1 was kindly provided by Drs. R. Joho and A. Brown (Frech et al., 1989). Two-hybrid vectors that carry the DNA-binding domain (pPC97) and the transcription activation domain (pPC86) of the GAL4 transcription factor were gifts from Dr. D. Nathans (Chevray and Nathans, 1992). The Kv $\beta$ 1 cDNA was cloned by high fidelity PCR amplification based on the sequence deposited in GenBank using oligonucleotides listed in Table 1; the nucleotide sequence was confirmed by dideoxynucleotide sequencing using the Sequenase kit (Amersham).

##### Vector Construction

Plasmid vector construction was performed according to standard recombinant DNA techniques (Sambrook et al., 1989). The vectors that express partial cDNA fragments were constructed by a high fidelity PCR cloning strategy according to the procedures described previously (Li et al., 1992). The oligos used are listed in Table 1. In

yeast, the expression of different fusion proteins of  $\alpha$  subunits and Kv $\beta$ 1 was carried out by inserting the cDNA fragments into the SmaI-NotI, Sall-NotI, or BglII-NotI sites of pPC97 and pPC86 vectors (Xu et al., 1995). The expression of the DNA-binding domain and the transcription activation domain fusion proteins was driven by a constitutive promoter of the alcohol dehydrogenase (*ADH*) gene. The expression of cDNAs encoding partial or entire  $\alpha$  subunits in COS cells was driven by the CMV promoter using the pRc/CMV vector (Invitrogen, CA). Construction of a tagged Kv $\beta$ 1 subunit was carried out by inserting an oligonucleotide (GAATCCGACAGCTTCCGTGAGTAGTTACCCATACGATGTCCCTGACTACGCATCGCTAGGAGACCTTACGG) representing a heart muscle kinase recognition sequence and the 12CA5 monoclonal epitope (PYDVPDYASL) at the end of the coding sequence of Kv $\beta$ 1 before the stop codon.

##### Preparation of Fusion Proteins

###### *Sf9 Baculovirus Expression System*

The overall procedure was similar to standard methods (Summers and Smith, 1987). A vector pV-IKS provided by Dr. Lewis William (University of California, San Francisco) was used. It carries GST and one copy of the 12CA5 epitope (SYPYDVPDYASLGGPS) to allow convenient purification and detection using Mab 12CA5 (see Figure 3A for diagram). NShB (aa 1–227) was inserted into pV-IKS C-terminal to the 12CA5 epitope. The resultant shuttle vector (5  $\mu$ g) was cotransfected with 1  $\mu$ g of linearized baculovirus DNA (Invitrogen) into 75% confluent Sf9 cells using the Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> transfection procedure recommended by the manufacturer. At 50 hr posttransfection, the supernatants were harvested. The recombinant virus and the level of expression by each viral stock were determined by immunoblot analysis using Mab 12CA5 and anti-NShB antibody (Li et al., 1992). The protocols for viral infection and protein production are identical to those described previously (Li et al., 1994).

###### *T<sub>7</sub> Bacteria Expression System*

The pRset vector (Invitrogen) carrying the Kv $\beta$ 1 cDNA fragment was transformed into BL21 cells. Isolated colonies were picked and allowed to grow in LB medium plus 50  $\mu$ g/ml ampicillin to OD<sub>600</sub> =

0.3. The expression of Kv $\beta$ 1 fusion protein was then induced by 0.2 mM IPTG for 4 hr at 32°C.

#### Affinity Binding of Kv $\beta$ 1 to NShB

##### Protein Preparation

Sf9 cells at 48 hr after infection were pelleted by  $2000 \times g$  for 10 min. The cells were lysed by freeze-thaw in a buffer containing 20 mM K<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Cell debris was then removed by spinning the lysate at  $14,000 \times g$  for 20 min at 4°C. The GST fusion proteins in the supernatants were allowed to bind glutathione-Sepharose (Pharmacia) to generate the NShB affinity column. The resultant column matrix was washed repeatedly by the above phosphate buffer supplemented with 0.5 M NaCl.

The preparation of bacterial lysate containing Kv $\beta$ 1 fusion protein was performed according to a published procedure (Li et al., 1992).

##### Affinity Binding

The crude protein lysate containing Kv $\beta$ 1 was dialyzed against a binding buffer (Tris-HCl [pH 8.5], 50 mM NaCl, 2 mM dithiothreitol, 0.1% Nonidet P-40). Before application to the column, insoluble material was removed by centrifugation ( $14,000 \times g$  for 10 min). The resultant soluble lysate (100  $\mu$ l at 0.5 mg protein/ml) was then loaded on the 1 ml bed volume NShB affinity column. The loading was repeated three times. The unbound protein was washed by the binding buffer. The bound protein was eluted using the binding buffer containing 0.5 M NaCl (pH 7.0).

#### Transient Expression of K<sup>+</sup> Channel Subunits

DH-5 $\alpha$  cells were used for the plasmid transformation, and plasmid DNA was prepared using the Maxi DNA isolation kit (Promega) according to the protocol provided by the manufacturer. COS cells were cultured in DMEM/F12 medium (Mediatech) at 37°C and 5% CO<sub>2</sub>. Transfections were carried out using the calcium phosphate precipitation method. In brief, a total of 20  $\mu$ g of plasmid DNA was added to 0.5 ml of HBS solution (21 mM HEPES [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.7 mM Na-PO<sub>4</sub>, 6.6 mM glucose) and mixed well. DNA precipitates were formed by adding 51  $\mu$ l of 2 M CaCl<sub>2</sub>. The precipitated DNA was then evenly dropped into a 10 cm dish containing about 70% confluent COS cells. The medium was replaced with fresh medium 8–16 hr after transfection. For immunoblot analysis, cells were harvested 48 hr after transfection. For patch-clamp studies, cells were recorded 36–72 hr after transfection.

#### Expression of NAB<sub>ShB</sub> and Affinity Purification of the Anti-NShB Antibody

A polypeptide representing amino acids 83–227 of the N-terminal domain of ShB was kindly provided by A. Ou and D. Leahy and purified to apparent homogeneity as judged by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The purified protein was covalently coupled to Affi-Gel 10 (Bio-Rad) to form an affinity matrix containing 5–10 mg protein/ml of gel using the protocol provided by the manufacturer. The polyserum made against the N-terminal domain of ShB (aa 1–227; Li et al., 1992) was allowed to bind to the column in the presence of 0.5 M NaCl (pH 7.0). The unbound material was removed by multiple washes, and the bound immunoglobulin (anti-NShB) was eluted with 0.1 M glycine (pH 2.2) and neutralized with Trisbase.

#### Protein Preparation and Immunodetection

Preparation of cell lysates was carried out by harvesting the transiently transfected cells at about 48 hr after transfection. After washing three times with 5 ml of phosphate-buffered saline (PBS) per dish, the cells were dislodged from dishes by incubating with PBS supplemented with 5 mM EDTA. The harvested cells were washed once with ice-cold PBS. The cell lysates were prepared by resuspending the cells in 200  $\mu$ l of lysis buffer containing 20 mM HEPES (pH 7.4), 1 mM EDTA, 120 mM KCl, 10% glycerol, and 2% Triton X-100 (w/v) for 30 min on ice. The insoluble material was removed by centrifugation at  $12,000 \times g$  for 15 min at 4°C.

Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Nonspecific binding to the membrane was blocked by PBS solution supplemented with 0.1% Tween-20, 2% dehydrated milk, and 3% normal goat serum (ICN) for 30 min at

room temperature. The primary antibodies used were the affinity-purified rabbit anti-NShB antibody (0.05–0.1 mg/ml final) or MAb 12CA5 (1:500). The unbound material was removed by three washes with PBS solution supplemented with 0.1% Tween-20. The binding of primary antibody was detected by adding corresponding horseradish peroxidase-conjugated secondary antibody (1:5000) followed by enhanced chemiluminescence (ECL, Amersham) detection.

#### The Yeast Two-Hybrid System

Vectors (pPC86, pPC97, and their derivatives) carrying coding sequences of interest were introduced into yeast cells by lithium acetate transformation according to a published procedure (Gietz et al., 1992) using  $\sim 1 \mu$ g of plasmid DNA. To test the possible protein-protein interactions, the cells containing both plasmids were first selected using supplemented dextrose synthetic dropout medium containing no leucine or tryptophan. The interaction was detected through selection of growth when a YGH1 host was used. In this strain, the GAL4 transcription factor controls the expression of the *HIS3* gene, which is required for the cells to grow in the synthetic medium lacking histidine.

#### Whole-Cell Patch-Clamp Recording

Whole-cell voltage-clamp recordings were carried out according to the published protocol (Hamill et al., 1981). The electrodes (Kimble) were pulled from a two stage vertical puller (Narishige). When filled with intracellular solution, they have a resistance of 2–8 M $\Omega$ . The intracellular solution contained 60 mM KF, 50 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EDTA, and 10 mM HEPES (pH 7.2, adjusted with KOH). During recording, the cells were constantly superfused with the extracellular solution (122 mM NaCl, 10 mM KCl, 1.8 mM CaCl<sub>2</sub>, 10 mM glucose, 10 mM HEPES; pH 7.4, adjusted with NaOH). All chemicals were obtained from Sigma. The liquid junction potential was calculated to be 7.2 mV using JPCalc software (Barry, 1994) and corrected from the holding potential.

An Axopatch 200A (Axon Instruments) amplifier was used in the experiments. Whole-cell capacitance and series resistance were compensated. Voltage protocols were generated by pCLAMP6 software (Axon Instruments). Typically, the cell was held at  $-77$  mV, and the holding voltage was then jumped from this potential to  $+53$  mV by 10 mV increments for 300 ms. Current data were filtered at 1 kHz, digitized at 100 ms intervals, and stored in a computer (Dell, 486/33) for later analysis. Data analysis was done using Clampfit (pCLAMP6, Axon Instruments). Basal leak current was subtracted. Data were transferred into Sigma plot (Jandel) for final analysis.

The conductance was calculated according to the equation  $G = I_p / (V_m - V_r)$ , where  $I_p$  is the peak current,  $V_m$  is the membrane potential during the step, and  $V_r$  is the reversal potential for the K<sup>+</sup> current. In our experiments, the  $V_r$  is  $-62$  mV, calculated according to the Nernst equation based on our intra- and extracellular solutions.

#### Acknowledgments

We thank Drs. R. Joho and A. Brown for providing us with DRK1 cDNA; Dr. D. Mckinnon for RCK1 cDNA; Dr. L. Salkoff for fshal2, fshaw2, and fshabl1 cDNAs; Dr. R. Aldrich for the ShB $\Delta$ (6–46) mutant; Dr. Q.-J. Hu for MAb 12CA5; and A. Ou and D. Leahy for purified NShB protein. We also thank Magdalena Bezanilla and Kathy Wilson for critical reading of this manuscript. This work was supported in part by a National Institutes of Health Shannon Award and a grant from the Council for Tobacco Research, Inc. (to M. L.). M. L. is a Neuroscience Fellow of the Alfred P. Sloan Foundation and the Esther A. and Joseph Klingenstein Fund.

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Received July 12, 1995; revised October 23, 1995.

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