# β Subunits Promote K+ Channel Surface Expression through Effects Early in Biosynthesis

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## Summary

**Voltage-gated K+ channels are protein complexes composed of ion-conducting integral membrane α subunits and cytoplasmic β subunits. Here, we show that, in transfected mammalian cells, the predominant β subunit isoform in brain, Kvβ2, associates with the Kv1.2 α subunit early in channel biosynthesis and that Kvβ2 exerts multiple chaperone-like effects on associated Kv1.2 including promotion of cotranslational N-linked glycosylation of the nascent Kv1.2 polypeptide, increased stability of Kvβ2/Kv1.2 complexes, and increased efficiency of cell surface expression of Kv1.2. Taken together, these results indicate that while some cytoplasmic K+ channel β subunits affect the inactivation kinetics of α subunits, a more general, and perhaps more fundamental, role is to mediate the biosynthetic maturation and surface expression of voltage-gated K+ channel complexes. These findings provide a molecular basis for recent genetic studies indicating that β subunits are key determinants of neuronal excitability.**

## Introduction

Voltage-gated K+ channels are a diverse group of plasma membrane proteins that are fundamental in determining the electric properties of excitable cells (Hille, 1992). Over a dozen genes encoding the pore-forming α subunits have been isolated from mammalian sources (Chandy and Gutman, 1995); these polypeptides associate in homo- and heterotetrameric complexes that display distinct properties in both heterologous expression systems (Ruppersberg et al., 1990; Christie et al., 1990) and in mammalian central neurons in situ (Sheng et al., 1993; Wang et al., 1993; Scott et al., 1994a). Although differential assembly of α subunits provides a molecular basis for generating functional diversity among K+ channels, there are clearly contributions from other mechanisms (Jan and Jan, 1990).

Auxiliary subunits in tight association with the α subunits of Na+ and Ca2+ channels have been commonly found in neurons and other excitable tissues (Isom et al., 1994). Coexpression of these auxiliary subunits can have profound effects on α subunits, including effects on expression level, gating, and conductance (Isom et al., 1994). Recently, cDNAs encoding β subunits of K+ channels were cloned using sequence obtained from a polypeptide that copurified with α-dendrotoxin (DTX)

acceptor complexes from bovine brain (Scott et al., 1994b). Subsequently, a total of five β subunit cDNAs arising from three highly related genes, termed Kvβ1, Kvβ2, and Kvβ3, have been isolated from mammalian brain or heart (Rettig et al., 1994; Majumder et al., 1995; Morales et al., 1995; England et al., 1995a; McCormack et al., 1995; Heinemann et al., 1995; England et al., 1995b). Among them, three β subunits arise from the Kvβ1 gene by tissue-specific alternative splicing (England et al., 1995a). The encoded β subunit polypeptides are proposed to be cytoplasmic peripheral membrane proteins, interacting with the channel complex via the intracellular domains of the integral membrane α subunit polypeptides (Scott et al., 1994b).

Coexpression of any of the Kvβ1 splice variants or Kvβ3 with certain α subunits in Xenopus oocytes results in a dramatic acceleration of the macroscopic inactivation kinetics of the expressed channels (Rettig et al., 1994; Majumder et al., 1995; Morales et al., 1995; England et al., 1995a; McCormack et al., 1995; Heinemann et al., 1995; England et al., 1995b). However, Kvβ2, in spite of an ∼90% amino acid identity with the Kvβ1 family members, does not exert such effects on α subunit inactivation (Rettig et al., 1994). Mixed α and β subunit complexes have been characterized in rat brain and in transfected cells, showing Kvβ1 and Kvβ2 selectively interact with *Shaker* (Kv1) α subunit family members (Rhodes et al., 1995; Nakahira et al., submitted). These studies also showed that the "nonfunctional" Kvβ2 β subunit is by far the most abundant of the β subunit isoforms in brain, suggesting an important role for this β subunit in mammalian brain (Rhodes et al., 1995; K. J. R. et al., unpublished data).

Here, we report that Kvβ2 plays a fundamental role during K+ channel biosynthesis by promoting the cell surface expression of coexpressed α subunits. These effects presumably involve assisting the proper folding of nascent α subunit polypeptides mediated through the cotranslational interaction of Kvβ2 with the α subunit N-terminal cytoplasmic domain. These effects underlie the functional importance of K+ channel β subunits in vivo and allow increased understanding of the molecular mechanisms regulating K+ channels expression.

## Results

### In Vivo Association of Kv1.2 and Kvβ2 in Rat Brain

K+ channel β subunits were first purified from bovine brain as components of DTX-binding complexes, which have also been shown to contain the Kv1.2 α subunit (Scott et al., 1990, 1994b). To confirm that Kvβ2 and Kv1.2 associate in rat brain K+ channel complexes, we used Kvβ2- and Kv1.2-specific antibodies in immunoprecipitation reactions and for immunohistochemistry. Reciprocal immunoprecipitation showed that Kv1.2 can be detected in K+ channel complexes isolated by immunoprecipitation with the Kvβ2-specific antibodies, and vice versa, indicating that K+ channel complexes containing both Kv1.2 and Kvβ2 exist in rat brain (Figure

1A). In contrast, antibodies raised against the Kv2.1 α subunit (Trimmer, 1991) could not precipitate either Kv1.2 or Kvβ2. Likewise, Kv2.1 could be found neither in anti-Kv1.2 nor anti-Kvβ2 immunoprecipitation products (Figure 1A). These observations were further substantiated by the extensive colocalization of Kv1.2 with Kvβ2 in situ. As shown in Figures 1B and 1C, double-label immunofluorescence staining of sections taken from adult rat cerebellum shows that Kvβ2 and Kv1.2 are colocalized in basket cell terminal plexuses and in the plasma membrane at juxtaparanodal regions of axons. However, immunohistochemical staining of Kv2.1 from adult rat brain slices showed a very different pattern compared with that of Kvβ2 and Kv1.2 (Trimmer, 1991; Rhodes et al., 1995). We have also obtained evidence for similar association and colocalization of Kvβ2 with the Kv1.1 and Kv1.6 K⁺ channel α subunits (K. J. R. et al., unpublished data), which have also been identified as components in DTX-sensitive complexes in bovine brain (Scott et al., 1994a). To investigate the role of Kvβ2 in such complexes, we coexpressed the Kvβ2 cDNA in transfected COS1 cells with the Kv1.2 α subunit.

**Early Biosynthetic Association of Kv1.2 and Kvβ2**

Kv1.2 and Kvβ2 transiently coexpressed in COS1 cells interact specifically (Nakahira et al., submitted) with the

predicted stoichiometry of 1:1 (G. S. and J. S. T., unpublished data), in agreement with previous estimates obtained from analysis of K⁺ channel complexes purified from bovine brain (Parcej et al., 1992). [$^{35}$S]Methionine pulse-chase labeling of cells coexpressing Kv1.2 and Kvβ2, followed by immunoprecipitation with the subtype-specific antibodies, was used to determine the dynamic aspects of Kv1.2 and Kvβ2 association. Coprecipitation of both Kv1.2 and Kvβ2 with either anti-Kv1.2 or anti-β antibodies is detected at the earliest timepoint (5 min pulse, 0 min chase; Figure 2A) when Kv1.2 is in the endoplasmic reticulum (ER), showing that α/β subunit interaction occurs early in biosynthesis and suggesting that the subcellular site of subunit interaction is at the cytoplasmic face of the ER. The ER is the initial point of the secretory pathway in which membrane proteins
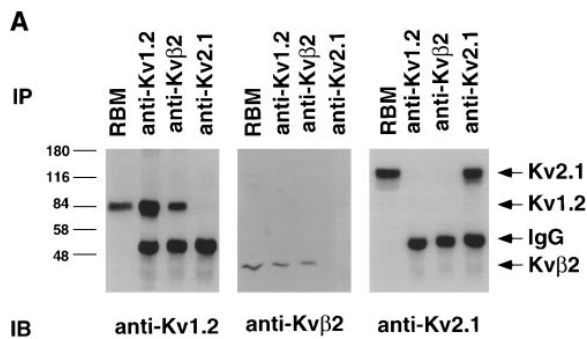


Figure 1. Association of Kv1.2 and Kvβ2 in Rat Brain

(A) Crude rat brain membranes (RBM, 25 μg) and immunoprecipitation products from reactions performed with anti-Kvβ2-, anti-Kv1.2-, and anti-Kv2.1-specific antibodies from detergent extracts of RBM were size fractionated by 9% SDS–PAGE. Samples were transferred to nitrocellulose, and the same blot was probed with anti-Kv1.2C antibody (left), then stripped and reprobed with mouse monoclonal anti-Kvβ2 antibody (K17/70, middle), or anti-Kv2.1 antibody (right), followed by incubation in the appropriate HRP-conjugated secondary antibody and ECL/autoradiography. Arrows denote mobility of α or β subunits and rabbit IgG heavy chain; note "anti-Kvβ2" panel was probed with a mouse monoclonal antibody and thus no rabbit IgG bands are apparent. Numbers to left denote mobility of prestained molecular weight standards.

(B and C) Double-label immunofluorescence labeling of Kv1.2 (green) and Kvβ2 (red) in rat cerebellar cortex. Neuronal processes containing Kv1.2 appear green, those containing Kvβ2 appear red. Processes containing both subunits appear yellow. (B) Kv1.2 and Kvβ2 are colocalized in cerebellar basket cell terminals (arrowheads) that envelop the initial segment of the Purkinje cell axon. Purkinje cells are immunoreactive for Kvβ2, but not Kv1.2, and thus appear red. (C) Kvβ2 and Kv1.2 are also colocalized at Nodes of Ranvier, where both subunits are concentrated in the axolemma (arrowheads) immediately adjacent to the node.
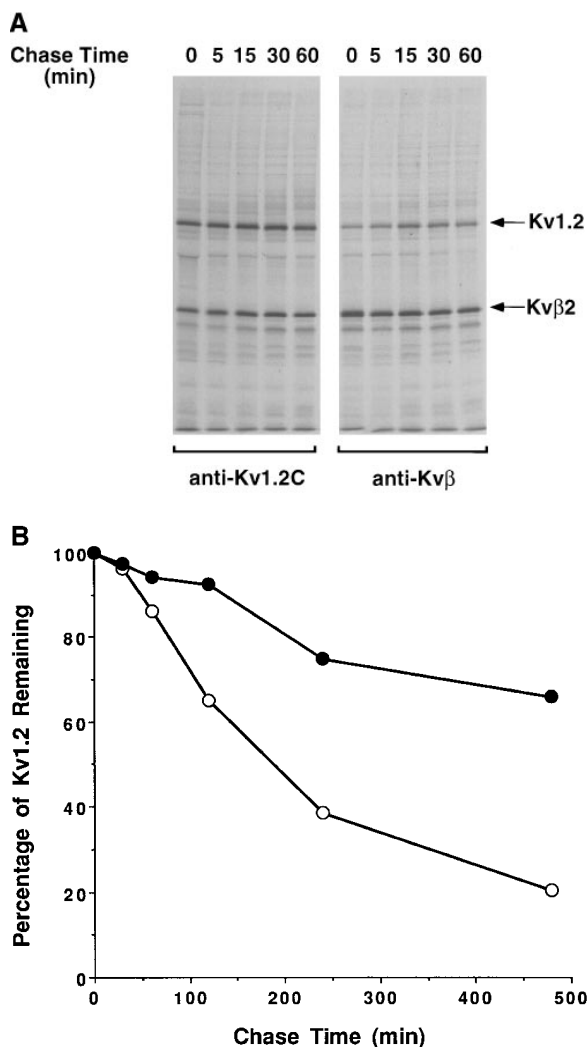
## A



## B
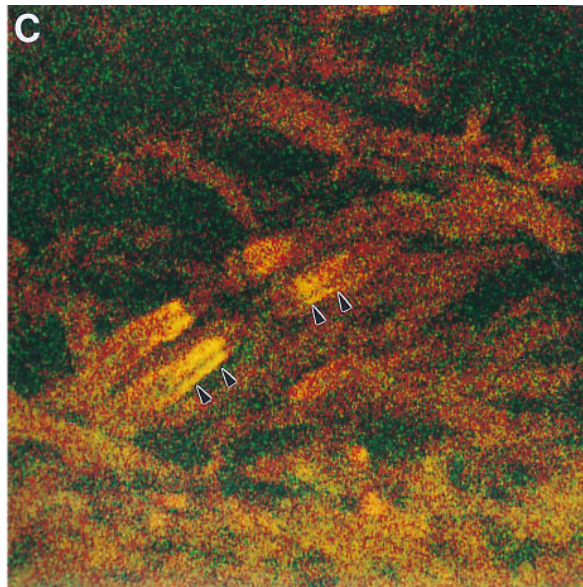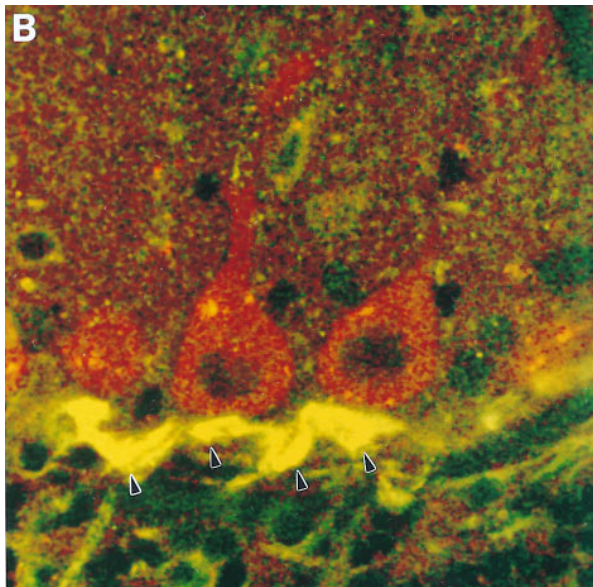


Figure 2. Association and Stabilization of Kv1.2 with Kvβ2

(A) Rapid association of the Kv1.2 α subunit and Kvβ2 β subunit. Kv1.2 and Kvβ2 in cotransfected COS1 cells were labeled with a pulse of [$^{35}$S]methionine for 5 min followed by chase media for the indicated times, followed by immunoprecipitation with anti-Kv1.2C antibody or anti-Kvβ antibody and analysis by SDS–PAGE and fluorography.

(B) Stabilization of Kv1.2 by association with Kvβ2. COS1 cells coexpressing Kv1.2 and Kvβ2 were subject to immunoprecipitation with anti-Kvβ antibody (to obtain the Kvβ2-associated Kv1.2 pool), and the remaining lysates were followed by immunoprecipitation with anti-Kv1.2C antibody (to obtain the free Kv1.2 pool). The immunoprecipitated products were fractionated by SDS–PAGE and quantified by phosphorimaging. The percentage of the initial levels of the free Kv1.2 polypeptide pool (open circles) and Kvβ2-associated Kv1.2 polypeptide pool (closed circles) remaining at the times indicated are shown.

undergo various co- or posttranslational modifications and acquire their secondary and tertiary structures (Hurtley and Helenius, 1989). This early association of α and β subunits suggests that β subunits may play a modulatory role in some of these processes.

### Stabilization of Kv1.2 by Association with Kvβ2

The total cellular Kv1.2 pool in cells coexpressing Kv1.2 and Kvβ2 is composed of two fractions: Kv1.2 not associated with Kvβ2, termed here "free Kv1.2," and Kv1.2

present in complexes with Kvβ2. These two populations can be selectively isolated by sequential immunoprecipitation with subtype-specific antibodies. Cell lysates immunoprecipitated with anti-β antibody allowed us to isolate selectively the fraction of Kv1.2 associated with Kvβ2. Free Kv1.2 in the lysates from which the Kvβ2-associated fraction had been depleted can be subsequently isolated by immunoprecipitation reactions with the anti-Kv1.2 antibody. Sequential immunoprecipitation products of [$^{35}$S]methionine pulse-chased (chase periods up to 8 hr) COS1 cell lysates were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE)/ phosphorimaging. This analysis revealed a dramatic difference in the degradation rates of the free Kv1.2 pool and the Kv1.2 associated with Kvβ2. The free Kv1.2 pool exhibits a relatively high turnover rate $t_{1/2}$ of $3 \pm 1$ hr (mean $\pm$ SD, n = 2), while the Kvβ2-associated fraction, which comprises ~50% of the total Kv1.2 pool, is quite stable, with a calculated turnover rate $t_{1/2}$ of $15 \pm 3$ hr (mean $\pm$ SD, n = 2; Figure 2B).

It is also clear from these data that although α/β subunit interaction is rapid, it is also virtually permanent. No apparent exchange of radiolabeled subunits for non-radiolabeled subunits is apparent in this experiment using a 60 min chase (Figure 2A); this holds for chase periods up to 24 hr (data not shown). This implies that α/β subunit interaction is quite stable, as the amount of radiolabeled subunits synthesized during the brief pulse is small compared with the amount of nonradiolabeled subunits synthesized both in the 24 hr before the pulse and the chase period after the pulse. Thus, as brief labeling with [$^{35}$S]methionine in the pulse-chase experiments allows only a small fraction of total Kv1.2 and Kvβ2 pool to be labeled, the fact that the relative stoichiometry of [$^{35}$S]methionine-labeled Kv1.2 and Kvβ2 present in coimmunoprecipitation products does not change demonstrates that virtually no posttranslational subunit exchange occurs. Otherwise, [$^{35}$S]methionine-labeled Kv1.2 or Kvβ2 would likely be replaced by subunits from the numerically predominant nonradiolabeled pool. The stable association of Kv1.2 and Kvβ2 thus likely persists for the lifetime of the K⁺ channel complex.

### Kvβ2 Increases the N-Linked Glycosylation Efficiency of Kv1.2

[$^{35}$S]Methionine pulse-chase experiments show that when Kv1.2 polypeptides are expressed alone in transfected COS1 cells, two populations of Kv1.2 are observed at $M_r = 63$ kDa and $M_r = 66$ kDa. These forms persist for the entire chase period (up to 4 hr), indicating that they do not represent biosynthetic intermediates. When Kv1.2 is coexpressed with Kvβ2, the lower molecular mass form (63 kDa) is not present (Figure 3A). As a single asparagine (N)-linked glycosylation site (Hubbard and Ivatt, 1981) is present in the primary sequence of Kv1.2 polypeptide at Asn-207, it was possible that the difference between the 63 kDa and 66 kDa Kv1.2 species could represent differential glycosylation of the Kv1.2 polypeptide. To test this hypothesis, we applied PNGase F to the Kv1.2 immunoprecipitated from either COS1 cells expressing Kv1.2 alone or coexpressing Kv1.2 and Kvβ2. As expected, after PNGase F digestion, the 66 kDa form shifted to 63 kDa, presumably due to the removal of the single N-linked oligosaccharide chain yielding the
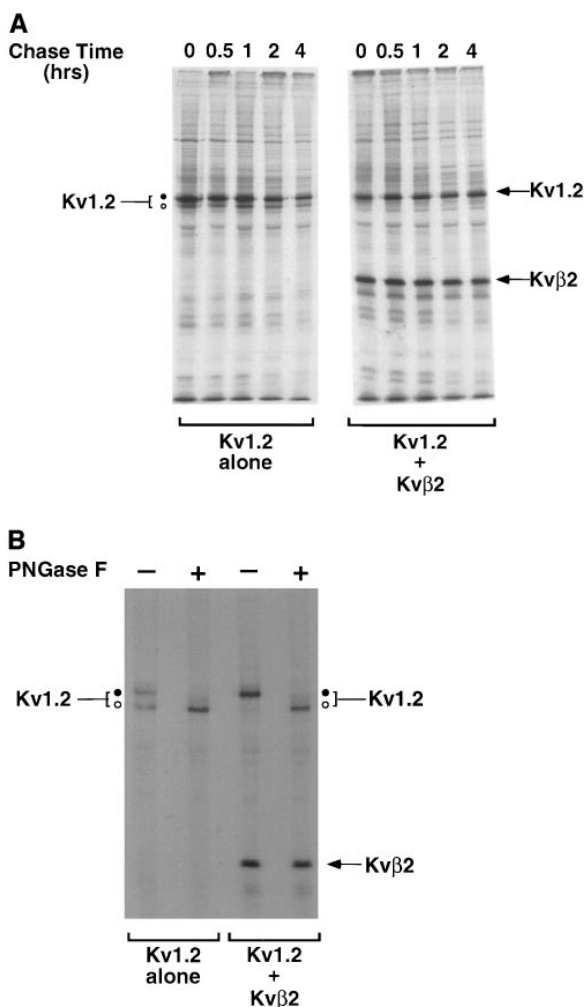
## A



## B



Figure 3. Coexpression of Kvβ2 Promotes Efficient Glycosylation of the Kv1.2 in Transfected COS1 Cells

(A) COS1 cells transfected with Kv1.2 alone or cotransfected with Kv1.2 and Kvβ2 were pulsed with [$^{35}$S]methionine for 30 min followed by chase for the indicated times. Cell lysates were immunoprecipitated with anti-Kv1.2C antibody and analyzed by SDS–PAGE and fluorography. Kv1.2 polypeptide pools migrating at 66 kDa (closed circle) and 63 kDa (open circle) are indicated.
(B) Steady-state [$^{35}$S]methionine-labeled COS1 cell lysates were prepared from cells expressing Kv1.2 alone or coexpressing Kv1.2 and Kvβ2, and Kv1.2 isolated by immunoprecipitation with the anti-Kv1.2C antibody. Aliquots were incubated in the presence (plus) or absence (minus) of PNGase F, followed by fractionation by SDS–PAGE and fluorography. Kv1.2 polypeptide pools migrating at 66 kDa (closed circle) and 63 kDa (open circle) are indicated.

unglycosylated form, and indicating that the 66 kDa form of Kv1.2 is N-glycosylated (Figure 3B). The 63 kDa form present in cells expressing Kv1.2 alone is resistant to PNGase, indicating that this form lacks the N-glycosylation present on the 66 kDa species. The 63 kDa, presumably unglycosylated, Kv1.2 fraction comprises approximately 50% of the total Kv1.2 pool in cells expressing Kv1.2 alone, while the N-glycosylated 66 kDa form is the predominant species when Kv1.2 is coexpressed with Kvβ2 (Figure 3B). It should be noted that Kv1.2 expressed in COS1 cells remains sensitive to endoglycosidase H (endo H) throughout its biosynthesis, even

at long chase periods and independent of Kvβ2 coexpression. Many other glycoproteins also retain their high mannose (endo H–sensitive) chains in their mature forms; however, the lack of acquisition of endo H resistance does not allow us to study the effect of Kvβ2 on later (post-ER) biosynthetic events. The observed increase in the efficiency of N-linked glycosylation predicts a cotranslational interaction between Kv1.2 and Kvβ2 in a region N-terminal to the glycosylation site at Asn-207, in agreement with the fast kinetics of α/β subunit interaction observed in the pulse-chase experiment shown in Figure 2A. Note that in agreement with the data presented above, the Kv1.2 polypeptide pool in cells coexpressing Kvβ2 is notably more stable than in cells expressing Kv1.2 alone (Figure 3A).

## Kvβ2 Coexpression Increases the Cell Surface Expression of Kv1.2

COS1 cells expressing Kv1.2 alone exhibit strong perinuclear immunofluorescence staining while very little, if any, staining is associated with the plasma membrane (Figure 4A). Kv1.2 staining colocalizes with staining for the resident ER protein grp78/BiP (Pelham, 1989), indicating that expressed Kv1.2 is contained within the ER (data not shown). Most cells (94.9% ± 3.7%; mean ± SD, n = 3 experiments of 100 Kv1.2-positive cells counted per experiment) that express Kv1.2 alone are thus negative when stained without prior permeabilization with an antibody ("Kv1.2E") raised to an external domain of Kv1.2 (Figure 4C). In contrast, when cotransfected with Kvβ2 (at a Kv1.2:Kvβ2 ratio of 1:4), the majority of Kv1.2-expressing cells (58.1% ± 2.0%, mean ± SD, n = 3 experiments of 100 Kv1.2-positive cells counted per experiment; Figure 4C) now exhibit cell surface Kv1.2E staining, with the remainder of the cells expressing Kv1.2 trapped in the ER (Figure 4B). Kv1.2 surface expression increases in a dose-dependent manner with the amount of cotransfected Kvβ2 cDNA (Figure 4C). It should be noted that Kvβ2 coexpression, while required for efficient surface expression, in itself does not guarantee Kv1.2 surface expression. Many cells are observed that express high levels of Kvβ2, yet the expressed Kv1.2 remains trapped in the ER. While virtually all of the cells (>95%) in cotransfected dishes express both Kvβ2 and Kv1.2 (data not shown), the percentage of Kv1.2-expressing cells exhibiting surface expression peaks at only 60%. Interestingly, the distribution of Kvβ2 in cotransfected cells not exhibiting surface expression is altered from the diffuse cytoplasmic stain typical of Kvβ2 expressed alone (Nakahira et al., submitted) to an ER-localized pattern identical to that of Kv1.2. Presumably this difference in immunoreactivity results from the association of Kvβ2 with the ER-localized Kv1.2 α subunit pool. This indicates that the lack of Kv1.2 surface expression in some cotransfected cells is not due simply to a lack of α/β subunit interaction, but perhaps reflects a requirement for the full 1:1 α/β subunit stoichiometry, or the absence of some other factors necessary for efficient surface expression that are limiting in some cells.

## Increased [$^{125}$I]DTX Binding of Cells Cotransfected with Kv1.2 and Kvβ2

The protein complex responsible for binding of the mamba venom peptide DTX in bovine brain has been
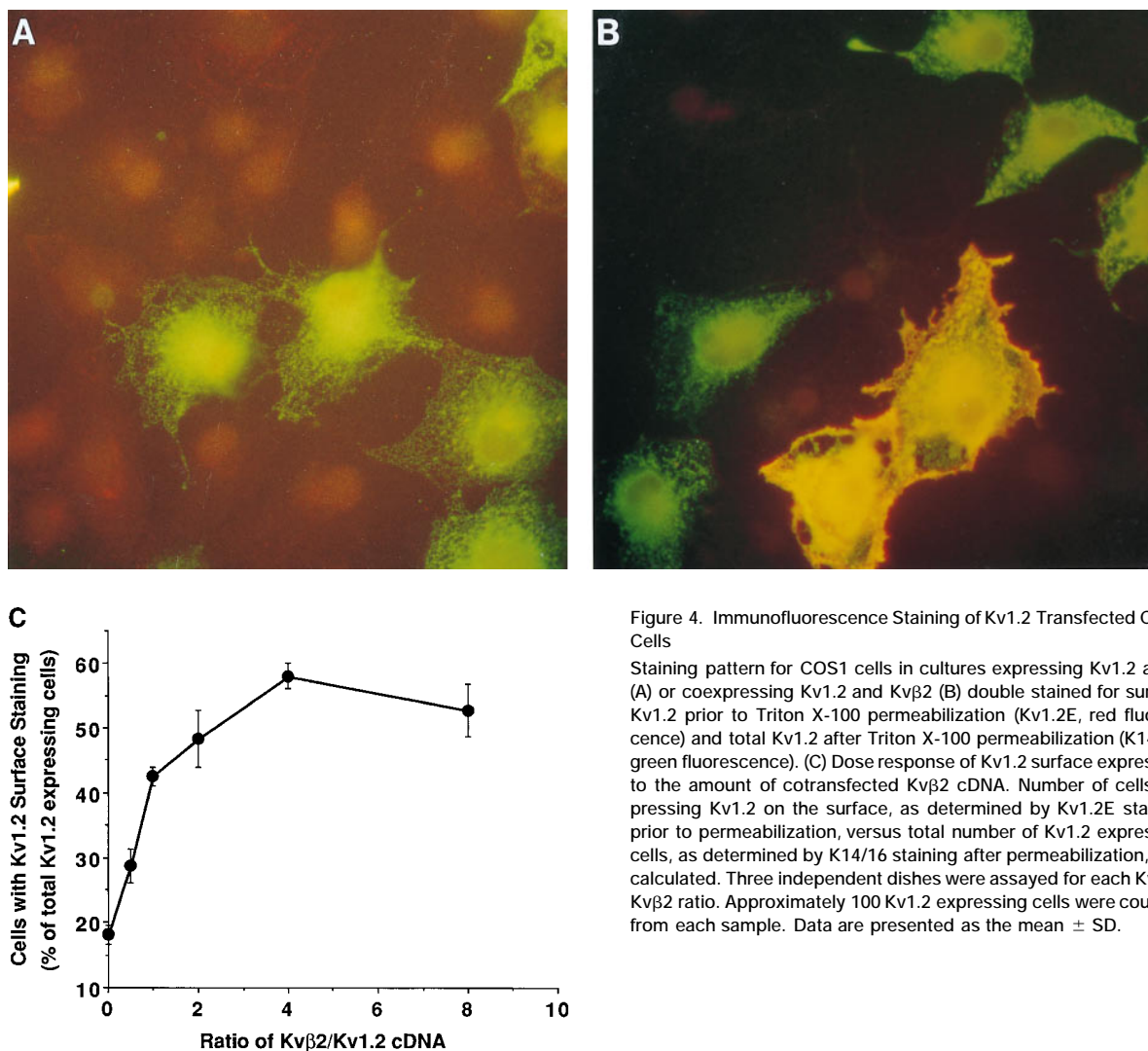
**Figure 4. Immunofluorescence Staining of Kv1.2 Transfected COS1 Cells**

Staining pattern for COS1 cells in cultures expressing Kv1.2 alone (A) or coexpressing Kv1.2 and Kvβ2 (B) double stained for surface Kv1.2 prior to Triton X-100 permeabilization (Kv1.2E, red fluorescence) and total Kv1.2 after Triton X-100 permeabilization (K14/16, green fluorescence). (C) Dose response of Kv1.2 surface expression to the amount of cotransfected Kvβ2 cDNA. Number of cells expressing Kv1.2 on the surface, as determined by Kv1.2E staining prior to permeabilization, versus total number of Kv1.2 expressing cells, as determined by K14/16 staining after permeabilization, was calculated. Three independent dishes were assayed for each Kv1.2/Kvβ2 ratio. Approximately 100 Kv1.2 expressing cells were counted from each sample. Data are presented as the mean ± SD.

shown by direct microsequencing to contain the bovine Kv1.2 α subunit, as well as the Kvβ1 and Kvβ2 β subunits (Scott et al., 1990, 1994b). Currents expressed from the Kv1.2 cRNA in Xenopus oocytes are blocked by DTX in the nanomolar range ($K_I$ = 4 nM, Stühmer et al., 1989), through binding of the peptide to specific amino acids located near the mouth of the channel pore (Hurst et al., 1991). We found that transfected COS1 cells expressing surface Kv1.2 exhibit high affinity binding of [$^{125}$I]DTX. We used the binding of this membrane-impermeant peptide to intact cells as a quantitative measurement of the steady state level of Kv1.2 cell surface expression in cells expressing Kv1.2 alone, or in cells coexpressing Kv1.2 and Kvβ2. Saturable, high affinity binding is observed to a single set of noninteracting sites on both singly transfected and cotransfected cells (Figure 5A). Specific binding is not observed in untransfected cells or cells expressing Kvβ2 alone (data not shown). Scatchard analysis (Scatchard, 1949) of the saturation binding data reveals very similar $K_d$ values of ~1 nM (Figure 5B). However, the $B_{max}$ value of cells coexpressing Kv1.2 and Kvβ2 is almost twice that of cells expressing Kv1.2 alone (Figure 5B). Thus, coexpression of Kvβ2 increases the

number of [$^{125}$I]DTX-binding sites without affecting their affinity for [$^{125}$I]DTX.

**Effects of Kvβ2 on Other Kv1 Family Members**

Two other Kv1 family K$^+$ channels, Kv1.1 and Kv1.6, are sensitive to DTX when expressed in Xenopus oocytes (Stühmer et al., 1989). We used [$^{125}$I]DTX binding to determine whether cells coexpressing Kvβ2 could affect the surface expression of these α subunits. Expression of both Kv1.1 and Kv1.6 in COS1 cells leads to low levels of specific [$^{125}$I]DTX binding (Figure 5C, top). Coexpression with Kvβ2 leads to an increase in specific [$^{125}$I]DTX binding of both Kv1.1 (4.3 ± 1.4 fold, n = 2) and Kv1.6 (3.3 ± 0.3 fold, n = 2), to an extent similar to that seen for Kv1.2 (4.4 ± 1.6 fold, n = 2) in the same experiment. These data indicate that the effects of Kvβ2 in promoting surface expression of α subunits are not restricted to Kv1.2.

[$^{125}$I]DTX binding was also used to determine if Kvβ1 coexpression would affect the levels of Kv1.2 surface expression. As shown in Figure 5C (bottom), coexpression with Kvβ1 also leads to an increase in [$^{125}$I]DTX binding in Kv1.2 expressing cells (5.3 ± 0.6 fold, n =
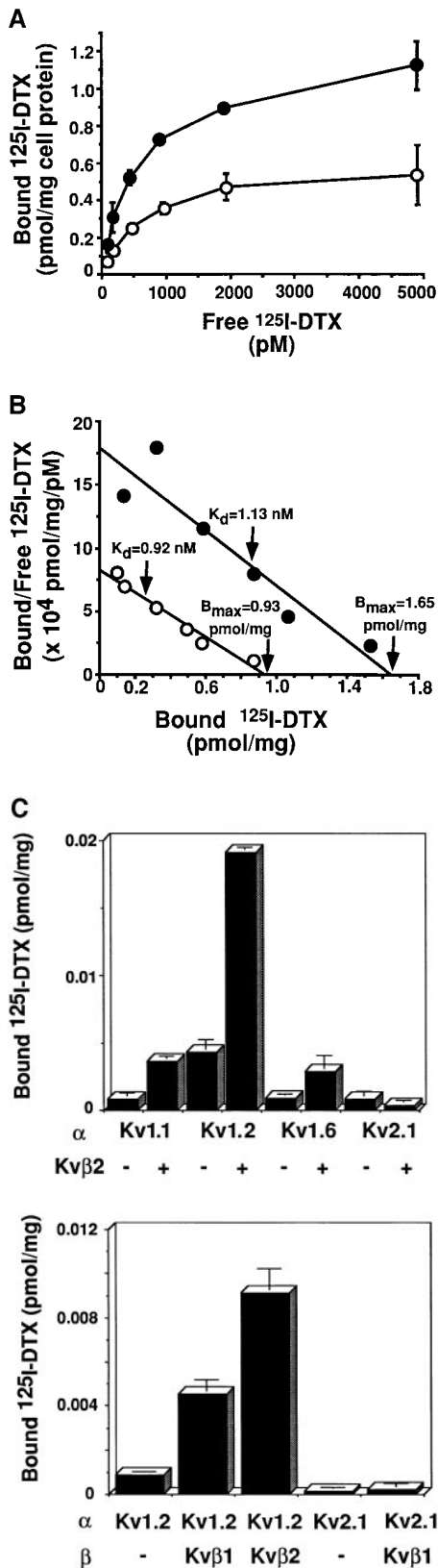
## A



## B



$K_d = 1.13$ nM

$K_d = 0.92$ nM

$B_{max} = 0.93$ pmol/mg

$B_{max} = 1.65$ pmol/mg

## C



| $\alpha$ | Kv1.1 | Kv1.2 | Kv1.6 | Kv2.1 |
|---|---|---|---|---|
| Kv$\beta$2 | - + | - + | - + | - + |



| $\alpha$ | Kv1.2 | Kv1.2 | Kv1.2 | Kv2.1 | Kv2.1 |
|---|---|---|---|---|---|
| $\beta$ | - | Kv$\beta$1 | Kv$\beta$2 | - | Kv$\beta$1 |

Figure 5. [$^{125}$I]DTX Binding to Kv1.2 in COS1 Cells

(A) Saturable binding of [$^{125}$I]DTX to Kv1.2 in transfected COS1 cells. Transfected COS1 cells were incubated with increasing amount of [$^{125}$I]DTX (0.5–5 nM). Nonspecific binding was determined as the

2). The fact that Kv$\beta$2 coexpression yielded a larger increase in the same experiment (10.7 ± 4.6 fold) may be due to factors such as transient transfection efficiency, expression levels and other technical factors, and thus may not be a true indication of quantitative differences in effects of these $\beta$ subunits on surface expression. It should be noted that while the increase in [$^{125}$I]DTX binding in cells coexpressing $\alpha$ and $\beta$ subunit polypeptides is highly reproducible, the extent of the increase in $B_{max}$ is somewhat variable (compare two panels in Figure 5C), perhaps due to differences in transient cotransfection efficiency or variable expression of other cellular factors affecting surface expression of Kv1.2. Kv$\beta$1 coexpression also leads to a change in the immunofluorescence staining pattern of Kv1.2-expressing cells similar to that seen with Kv$\beta$2 (data not shown), from that typical of an ER-localized protein (see Figure 4A) to a pattern consistent with increased surface expression (see Figure 4B). These data together indicate that the observed effects on surface expression are not limited to the Kv1.2 and Kv$\beta$2, but also occur upon other $\alpha$/$\beta$ subunit interactions.

## Discussion

Here, we show that the cytoplasmic Kv$\beta$2 $\beta$ subunit interacts with the integral membrane KvI.2 $\alpha$ subunit early (i.e., cotranslationally) in biosynthesis and that this interaction has dramatic effects on the biochemical properties of the resultant K$^+$ channel complexes. The importance of protein oligomerization during protein biosynthesis has been well established: misassembled and unassembled polypeptide are generally retained in the ER and specifically degraded (Hurtley and Helenius, 1989). Studies on a number of multisubunit ion channel proteins, such as the nicotinic acetylcholine receptor, have identified a requirement for the full complement of integral membrane subunits for efficient expression (Green and Millar, 1995). Here, we have identified a novel requirement for a cytoplasmic component, namely the newly identified $\beta$ subunits, for efficient expression of K$^+$ channel complexes. The association of Kv$\beta$2 with Kv1.2 during biosynthesis and its subsequent effects on Kv1.2 expression may reflect a fundamental role for $\beta$ subunits during biosynthesis, in addition to the previously recognized function of some $\beta$ subunits in modulating inactivation.

[$^{125}$I]DTX bound in the presence of a 0.05–5 $\mu$M concentration of unlabeled DTX. Specific binding of [$^{125}$I]DTX to COS1 cell expressing Kv1.2 alone (open circles) or Kv1.2 coexpressing with Kv$\beta$2 (closed circles) and are presented as the mean ± SD of duplicate samples. (B) Scatchard analysis of [$^{125}$I]DTX binding data. The saturation binding data presented in (A) were transformed according to Scatchard (1949). The slopes for the analysis of cells expressing Kv1.2 alone (open circles), and cell coexpressing Kv1.2 and Kv$\beta$2 (closed circles) were generated by linear regression.
(C) [$^{125}$I]DTX binding of COS1 cells transfected with different K$^+$ channel $\alpha$ subunits in the presence (plus) or absence (minus) of $\beta$ subunits. COS1 cells transfected with indicated $\alpha$ and $\beta$ subunits were incubated with 100 pM [$^{125}$I]DTX in the presence or absence of 1 $\mu$M unlabeled DTX. Specific binding was determined as the difference and plotted as the mean ± SD of duplicate samples.
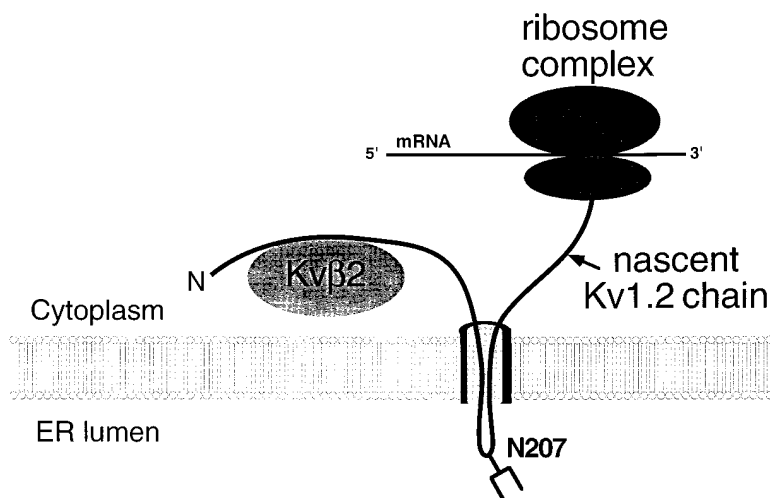
Figure 6. Schematic Model of K⁺ Channel α/β Subunit Interaction

The cytoplasmic K⁺ channel Kvβ2 β subunit associates with N-terminal domain of the partially synthesized Kv1.2 α subunit. This cotranslational association affects the efficiency of cotranslational oligosaccharide chain addition at Asn-207, presumably due to effects on Kv1.2 folding either before or during translocation of the S1 and S2 transmembrane segments.

The effect of Kvβ2 on the N-glycosylation of Kv1.2 defines a cotranslational interaction between these two channel subunits. The addition of oligosaccharide chains to Asn residues occurs while the nascent polypeptide chain is still attached to the ribosome complex, presumably as the consensus sequence is translocated across the ER membrane into the lumen (Abeijon and Hirschberg, 1992). Kv1.2 contains a single consensus site for N-linked glycosylation at Asn-207, located between the first and second transmembrane segments (Figure 6). The fact that the expression of the cytoplasmic Kvβ2 β subunit affects this event shows that Kvβ2 must already be bound as this glycosylation site is translocated into the ER lumen (Figure 6), implying that the interaction of Kvβ2 with Kv1.2 occurs via the cytoplasmic N-terminus of the α subunit (Figure 6). Consistent with these conclusions are the rapid kinetics of α/β subunit association observed in [$^{35}$S]methionine pulse-chase labeling experiments (Figure 2), and recent data from Li and his colleagues demonstrating that α/β subunit interaction is mediated by the "T" or "NAB" region located at the N-terminus of all Kv1 α subunit polypeptides (Yu et al., 1996). Similarly, rapid kinetics of α/α subunit association during K⁺ channel biosynthesis has also been reported (Deal et al., 1994), presumably mediated by this same cytoplasmic N-terminal domain (Li et al., 1992; Shen et al., 1993; Xu et al., 1995). However, the extent to which the association domains of the integral membrane α subunits and the cytoplasmic β subunits overlap within this relatively large (100 amino acid) region remains to be determined. The spatial and temporal relationships between α/α and α/β subunit interaction may be critical in regulating the rapid assembly of K⁺ channel complexes.

The rapid kinetics of K⁺ channel α/β subunit interaction is distinct from that of voltage-sensitive Na⁺ channels. Na⁺ channel α/β2 subunit association occurs much later in biosynthesis ($t_{1/2}$ = 1.2 hr) and appears to be the rate limiting step for their subsequent exit from the ER (Schmidt and Catterall, 1986). As the single N-linked oligosaccharide chain of Kv1.2 remains in its high mannose (endo H–sensitive) form throughout its biosynthesis in COS1 cells, we were unable to determine the

kinetics of ER to Golgi transit for this K⁺ channel α subunit. However, reported half-times for exit of membrane proteins from the ER vary from 15 min to 3 hr (Pelham, 1989), and the Kv1.4 α subunit expressed in COS1 cells is converted to an endo H–resistant form with a $t_{1/2}$ of ~3 hr (G. S. and J. S. T., unpublished data). Thus, it is unlikely that α/β subunit interaction ($t_{1/2}$ = 5 min) is the rate-limiting step in the exit of K⁺ channel complexes from the ER. The mechanism whereby coexpression of Kvβ2 promotes the exit of Kv1.2 from the ER is not known, but may involve effects on early folding events that are prerequisites for as yet undefined later, rate-limiting step.

The finding of the important role of the cytoplasmic β subunits in promoting the surface expression and stability of the Kv1.2 α subunit is similar in many respects to the role of ER-luminal molecular chaperones, such as calnexin, involved in the promotion of membrane protein maturation (Bergeron et al., 1994). This functional analogy presumably extends to the involvement of Kvβ2 in assisting the proper folding of partially synthesized α subunits that is necessary for efficient N-linked glycosylation. While cytosolic chaperones have been reported to be involved in the import of other cytoplasmic proteins into the mitochondria (Stuart et al., 1994), Kvβ2 is a novel example of a cytoplasmic protein that exhibits chaperone-like effects on an integral membrane protein. Like other molecular chaperones (Hartl et al., 1994), Kvβ2 may interact with exposed hydrophobic motifs of the elongating nascent α subunits, preventing premature aggregation and/or assisting the translocation of the nascent polypeptide chain. Future studies may allow us to delineate the precise mechanism that underlies the observed effects of the cytoplasmic Kvβ2 on the early events of K⁺ channel α subunit biosynthesis. It should be noted that K⁺ channel β subunits, unlike true molecular chaperones, remain associated with the channel complex as permanent structural components, in some cases altering the functional properties of the channel itself (Rettig et al., 1994).

These findings suggest an important and general role for Kvβ2 in promoting efficient K⁺ channel biosynthesis, explaining the previously observed abundance of this

β subunit polypeptide in brain relative to the channel-modulating Kvβ1 subtype (Scott et al., 1994b; Rhodes et al., 1995). The ability of Kvβ2 to associate with, and thus promote, the efficient expression of K⁺ channel α subunits, without dramatically altering the inactivation gating kinetics of associated α subunit as do Kvβ1 β subunits, may be the key to the precise role of Kvβ2. Kvβ2 can be "safely" used to promote α subunit expression and stability without dramatically altering the biophysical properties of the α subunit polypeptides. Kvβ1 isoforms, on the other hand, due to their dramatic effects on channel function, are only selectively substituted for Kvβ2 in special situations when more transient currents are appropriate. The fact that both Kvβ1 and Kvβ2 promote the stability and surface expression of interacting α subunits suggests that this is one of their fundamental roles, performed with or without the additional subtype-specific effects on channel inactivation. With the fast growing number of K⁺ channel β subunits cloned to date, it remains an interesting question to determine if other β subunits also exhibit the biosynthetic effects on α subunits that we have observed here for Kvβ1 and Kvβ2.

Recently, Chouinard et al. reported that coexpression of a Drosophila K⁺ channel β subunit encoded by the *Hyperkinetic* locus can increase current amplitude in oocytes coexpressing K⁺ channel *Shaker* α subunits (Chouinard et al., 1995). This finding is consistent with genetic analysis of *Hyperkinetic* (β subunit) mutants, which exhibit seizure activity epistatic to that caused by *Shaker* (α subunit) mutations (Stern and Ganetzky, 1989), due to a decrease in neuronal K⁺ channel expression. The data presented here on the effects of β subunit coexpression on mammalian *Shaker*-related α subunits suggest that the molecular basis for the altered electrical activity in *Hyperkinetic* mutants may be due to a failure of many of the newly synthesized *Shaker* α subunits to reach the cell surface. It also leads one to speculate as to whether β subunit dysfunction may lead to seizure activity or other neuropathological states in mammals. Further analysis of β subunit expression and the interactions among K⁺ channel α and β subunits may shed light on these fundamental elements of the control of neuronal excitability.

## Experimental Procedures

### Materials
PNGase F was provided by Dr. R. Haltiwanger (SUNY at Stony Brook, NY). COS1 cells were purchased from the Microbiology Department Tissue Culture Facility (SUNY at Stony Brook, NY). The enhanced chemiluminescence (ECL) reagents and [¹²⁵I]DTX were from Amersham (Arlington Heights, IL). [³⁵S]Methionine (Expre³⁵S³⁵S) was from DuPont–New England Nuclear (Boston, MA). HRP-conjugated secondary antibodies, Texas red–conjugated secondary antibodies, and FITC-conjugated secondary antibodies were purchased from Cappel (West Chester, PA). Pansorbin was from Calbiochem (La Jolla, CA). Prestained molecular weight standards and nonenzymatic cell dissociation solution were from Sigma (St. Louis, MO). All other reagents were from Sigma or Boehringer Mannheim (Indianapolis, IN).

### Construction of α and β Subunit Mammalian Expression Vectors
cDNAs encoding α subunits of voltage-gated K⁺ channel were provided by investigators listed below: Kv1.1 (RBK1, Christie et al.,

1989), Dr. J. Adelman (Vollum Institute); Kv1.2 (rat RAK, Paulmichl et al., 1991), Dr. K. Rhodes (Wyeth–Ayerst Research); Kv1.2 (BK2, McKinnon, 1989), Dr. D. McKinnon (SUNY at Stony Brook). We obtained similar results using either the RAT heart (RAK) or brain (BK2) cDNA; the data presented here were obtained with the RAK clone. Dr. R. Swanson (Merck Research Labs) provided Kv1.6 (Kv2, Swanson et al., 1990), and Dr. R. Joho (University of Texas Southwestern Medical Center) provided Kv2.1 (drk1, Frech et al., 1989). The Kvβ1 and Kvβ2 cDNAs were isolated by library screening and PCR amplification, respectively, as described (Nakahira et al., submitted). cDNAs were cloned into the mammalian expression vector pRBG4 for expression in COS1 cells as described in detail (Nakahira et al., submitted).

### Generation of Subunit-Specific Antibodies
The rabbit polyclonal antibody Kv1.2E reacting with S1–S2 extracellular loop of the Kv1.2 α subunit was raised against a synthetic peptide corresponding to amino acids 192–212 of Kv1.2. The rabbit polyclonal antibody Kvβ2 reacting with the unique N-terminus of the Kvβ2 β subunit was raised against a synthetic peptide corresponding to amino acids 1–17 of Kvβ2. Antibodies were produced and affinity purified essentially as described previously (Rhodes et al., 1995). Standard hybridoma methodology (Trimmer et al., 1985) was used in generating mouse monoclonal antibodies: K14/16 (anti-Kv1.2) was raised against a glutathione S-transferase (GST) fusion protein containing amino acids 428–499 of Kv1.2 (Z. Bekele-Arcuri, M. Matos, and J. S. T., unpublished data). The K14/16 epitope was subsequently mapped to the Kv1.2-specific region encompassing amino acids 463–480; K17/70 (anti-Kvβ2) was raised against GST fusion protein containing the entire Kvβ2 coding sequence and subsequently mapped to amino acids 17–22 (Z. Bekele-Arcuri, M. Matos, and J. S. T., unpublished data). The rabbit polyclonal antibodies "anti-β," reacting with the C-terminal region of β subunits; Kv1.2C, reacting with amino acids 463–480 of Kv1.2 and "KC," reacting with amino acids 837–853 of Kv2.1 were produced and affinity-purified essentially as previously described (Trimmer, 1991; Rhodes et al., 1995).

### Immunohistochemistry
Sample preparation was described by Rhodes et al. (1995). Parasagittal sections (25 μm thick) from rat cerebellar cortex were incubated in antibody vehicle containing a mixture of rabbit anti-Kv1.2 polyclonal antibody at a dilution of 1:100 and mouse anti-Kvβ2 monoclonal antibody K17/70 and were subsequently incubated in a mixture of affinity-purified goat anti-rabbit and goat anti-mouse secondary antibodies coupled to fluorescein and Texas red (1:250; Jackson ImmunoResearch), respectively. Immunofluorescence was visualized using a Molecular Dynamics Sarastro 2000 laser-scanning confocal microscope.

### Expression and Analysis of K⁺ Channel α and β Subunit Polypeptides
Procedures for COS1 cell culture, DNA transfection, immunoblot analysis, and immunoprecipitation reactions were performed essentially as described by Shi et al. (1994) and Rhodes et al. (1995). Phosphorimaging (Molecular Dynamics, Sunnyvale, CA) was used to quantitate [³⁵S]methionine-labeled immunoprecipitation products.

### [³⁵S]Methionine Pulse-Chase Metabolic Labeling
Labeling was performed essentially as described (Shi et al., 1994). Cells grown on 60 mm dishes were preincubated in methionine-free DMEM ("starved") for 15 min at 37°C, followed by incubation in methionine-free DMEM containing 300 μCi/ml of [³⁵S]methionine at 37°C for desired times. Cells were washed twice in PBS and incubated in serum-containing DMEM supplemented with an additional 5 mM L-methionine for various periods of time.

### Glycosidase Digestion
Products of immunoprecipitation reactions were washed twice in incubation buffer (75 mM Tris–HCl [pH 8.0], 10 mM EDTA), resuspended in 50 μl of incubation buffer, split into two aliquots, and PNGase F (final concentration of 8 U/ml) added to one aliquot. After

overnight incubation of both aliquots at 37°C, an equal volume of 2× sample buffer was added and digestion products were analyzed by SDS gels/fluorography.

**Immunofluorescence**
Cells were washed three times in PBS (10 mM phosphate buffer [pH 7.2], 0.15 M NaCl) containing 1 mM MgCl$_2$ and 1 mM CaCl$_2$, then fixed in the same buffer containing 2% paraformaldehyde for 30 min at 4°C. After three washes with PBS, nonspecific protein-binding sites were blocked with BLOTTO (4% nonfat dry milk powder in TBS, 10 mM Tris–HCl [pH 7.5], 0.15 M NaCl) for 1 hr at room temperature, then incubated with affinity purified anti-1.2E rabbit polyclonal antibody for 1 hr at room temperature. After washing three times with BLOTTO, cells were permeabilized with BLOTTO containing 0.1% Triton X-100 for 1 hr at room temperature. This was followed by incubation with mouse monoclonal antibody K14/16 tissue culture supernatant for 1 hr at room temperature. After primary antibody incubations, cells were washed three times in BLOTTO, incubated with Texas red goat anti-rabbit and FITC-goat anti-mouse diluted in BLOTTO for 1 hr, and washed three times with PBS. Cells were viewed under indirect immunofluorescence on a Zeiss Axioskop microscope, and scored for fluorescence on narrow wavelength fluorescein and rhodamine filter sets.

**[$^{125}$I]DTX Binding to Transfected COS1 Cells**
Cells were harvested in 0.5 ml microfuge tubes in nonenzymatic cell dissociation solution and washed twice with wash buffer (50 mM Tris–HCl [pH 7.5], 140 mM NaCl, 5 mM KCl, 1.3 mM MgSO$_4$). After the final wash, cells were resuspended in 200 μl of binding buffer (wash buffer plus 0.1% BSA) containing 0.05–5 nM [$^{125}$I]DTX and incubated at 4°C for 2 hr. Cells were washed five times by microcentrifuge centrifugation and resuspension in wash buffer before counting in a gamma scintillation counter. Duplicate assays were performed in the presence of 0.05–5 μM unlabeled DTX to determine nonspecific binding.

**References**

Abeijon, C., and Hirschberg, C.B. (1992). Topography of glycosylation reactions. Trends Biochem. Sci. *17*, 32–36.

Bergeron, J.J.M., Brenner, M.B., Thomas, D.Y., and Williams, D.B. (1994). Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. Trends Biochem. Sci. *19*, 124–128.

Chandy, K.G., and Gutman, G.A. (1995). Voltage-gated potassium channel genes. In Ligand- and Voltage-Gated Ion Channels, R.A. North, ed. (Boca Raton: CRC Press, Incorporated), pp. 1–71.

Chouinard, S.W., Wilson, G.F., Schlimgen, A.K., and Ganetzky, B. (1995). A potassium channel β subunit related to the aldo-keto reductase superfamily is encoded by the *Drosophila* hyperkinetic locus. Proc. Natl. Acad. Sci. USA *92*, 6763–6767.

Christie, M.J., Adelman, J.P., Douglass, J., and North, R.A. (1989).

Expression of a cloned rat brain potassium channel in *Xenopus* oocytes. Science *244*, 221–224.

Christie, M.J., North, R.A., Osborne, P.B., Douglass, J., and Adelman, J.P. (1990). Heteropolymeric K$^+$ channels expressed in *Xenopus* oocytes from cloned subunits. Neuron *4*, 405–411.

Deal, K.K., Lovinger, D.M., and Tamkun, M.M. (1994). The brain Kv1.1 potassium channel: *in vitro* and *in vivo* studies on subunit assembly and posttranslational processing. J. Neurosci. *14*, 1666–1676.

England, S.K., Uebele, V.N., Kodali, J., Bennett, P.B., and Tamkun, M.M. (1995a). A novel K$^+$ channel β-subunit (hKvβ1.3) is produced via alternative mRNA splicing. J. Biol. Chem. *270*, 28531–28534.

England, S.K., Uebele, V.N., Shear, H., Kodali, J., Bennett, P.B., and Tamkun, M.M. (1995b). Characterization of a voltage-gated K$^+$ channel β subunit expressed in human heart. Proc. Natl. Acad. Sci. USA *92*, 6309–6313.

Frech, G.C., Van Dongen, A.M.J., Schuster, G., Brown, A.M., and Joho, R.H. (1989). A novel potassium channel with delayed rectifier properties isolated from rat brain by expression cloning. Nature *340*, 642–645.

Green, W.N., and Millar, N.S. (1995). Ion-channel assembly. Trends Neurosci. *18*, 280–287.

Hartl, F.-U., Hlodan, R., and Langer, T. (1994). Molecular chaperones in protein folding: the art of avoiding sticky situations. Trends Biochem. Sci. *19*, 20–25.

Heinemann, S.H., Rettig J., Wunder F., and Pongs O. (1995). Molecular and functional characterization of a rat brain Kvβ3 potassium channel subunit. FEBS Lett. *377*, 383–389.

Hille, B. (1992). Ionic channels of excitable membranes (Sunderland, Massachusetts: Sinauer Associates, Incorporated).

Hubbard, S.C., and Ivatt, R.J. (1981). Synthesis and processing of asparagine-linked oligosaccharides. Annu. Rev. Biochem. *50*, 555–583.

Hurst, R.S., Busch, A.E., Kavanaugh, M.P., Osborne, P.B., North, R.A., and Adelman, J.P. (1991). Identification of amino acid residues involved in dendrotoxin block of rat voltage-dependent potassium channels. Mol. Pharmacol. *40*, 572–576.

Hurtley, S.M., and Helenius, A. (1989). Protein oligomerization in the endoplasmic reticulum. Annu. Rev. Cell Biol. *5*, 277–307.

Isom, L.L., DeJongh, K.S., and Catterall, W.A. (1994). Auxiliary subunits of voltage-gated ion channels. Neuron *12*, 1183–1194.

Jan, L.Y., and Jan, Y.N. (1990). How might the diversity of potassium channels be generated?. Trends. Neurosci. *13*, 415–419.

Li, M., Jan, Y.N., and Jan, L.Y. (1992). Specification of subunit assembly by the hydrophilic amino-terminal domain of the *Shaker* potassium channel. Science *257*, 1225–1230.

Majumder, K., DeBiasi, M., Wang, Z., and Wible, B.A. (1995). Molecular cloning and functional expression of a novel potassium channel β-subunit from human atrium. FEBS Lett. *361*, 13–16.

McCormack, K., McCormack, T., Tanouye, M., Rudy, B., and Stühmer, W. (1995). Alternative splicing of the human *Shaker* K$^+$ channel β1 gene and functional expression of β2 gene product. FEBS Lett. *370*, 32–36.

McKinnon, D. (1989). Isolation of a cDNA clone coding for a putative second potassium channel indicates the existence of a gene family. J. Biol. Chem. *264*, 8230–8236.

Morales, M.J., Castellino, R.C., Crews, A.L., Rasmusson, R.L., and Strauss, H.C. (1995). A novel β subunit increases rate of inactivation of specific voltage-gated potassium channel a subunits. J. Biol. Chem. *270*, 6272–6277.

Parcej, D.N., Scott, V.E., and Dolly, J.O. (1992). Oligomeric properties of α-dendrotoxin-sensitive K$^+$ ion channels purified from bovine brain. Biochemistry *31*, 11084–11088.

Paulmichl, M., Nasmith, P., Hellmiss, R., Reed, K., Boyle, W.A., Nerbonne, J.M., Peralta, E.G., and Clapham, D.E. (1991). Cloning and expression of a rat cardiac delayed rectifier K$^+$ channel. Proc. Natl. Acad. Sci. USA *88*, 7892–7895.

Pelham, H.R.B. (1989). Control of protein exit from the endoplasmic reticulum. Annu. Rev. Cell Biol. *5*, 1–23.

Rettig, J., Heinemann, S.H., Wunder, F., Lorra, C., Parcej, D.N., Dolly, J.O., and Pongs, O. (1994). Inactivation properties of voltage-gated K$^+$ channels altered by presence of β-subunit. Nature *369*, 289–294.

Rhodes, K.J., Keilbaugh, S.A., Barrezueta, N.X., Lopez, K.L., and Trimmer, J.S. (1995). Association and co-localization of K$^+$ channel α- and β-subunit polypeptides in rat brain. J. Neurosci. *15*, 5360–5371.

Ruppersberg, J.P., Schroter, K.H., Sakmann, B., Stocker, M., Sewing, S., and Pongs, O. (1990). Heteromultimeric channels formed by rat brain potassium-channel proteins. Nature *345*, 535–537.

Scatchard, G. (1949). The attraction of proteins for small molecules and ions. Ann. NY Acad. Sci. *51*, 660–672.

Schmidt, J.W., and Catterall, W.A. (1986). Biosynthesis and processing of the α-subunit of the voltage-sensitive Na$^+$ channel in rat brain neurons. Cell *46*, 437–444.

Scott, V.E., Parcej, D.J., Keen, J.N., Findlay, J.B., and Dolly, J.O. (1990). α-Dendrotoxin acceptor from bovine brain is a K$^+$ channel protein: evidence from the N-terminal sequence of its larger subunit. J. Biol. Chem. *265*, 20094–20097.

Scott, V.E.S., Muniz, Z.M., Sewing, S., Lichtinghagen, R., Parcej, D.N., Pongs, O., and Dolly, J.O. (1994a). Antibodies specific for distinct Kv subunits unveil a heterooligomeric basis for subtypes of α-dendrotoxin-sensitive K$^+$ channels in bovine brain. Biochemistry *33*, 1617–1623.

Scott, V.E.S., Rettig, J., Parcej, D.N., Keen, J.N., Findlay, J.B.C., Pongs, O., and Dolly, J.O. (1994b). Primary structure of a β subunit of α-dendrotoxin-sensitive K$^+$ channels from bovine brain. Proc. Natl. Acad. Sci. USA *91*, 1637–1641.

Shen, N.V., Chen, X., Boyer, M.M., and Pfaffinger, P.F. (1993). Deletion analysis of K$^+$ channel assembly. Neuron *11*, 67–76.

Sheng, M., Liao, Y.J., Jan, Y.N., and Jan, L.Y. (1993). Presynaptic A-current based on heteromultimeric K$^+$ channels detected *in vivo*. Nature *365*, 72–75.

Shi, G., Kleinklaus, A.K., Marrion, N.V., and Trimmer, J.S. (1994). Properties of Kv2.1 K$^+$ channels expressed in transfected mammalian cells. J. Biol. Chem. *269*, 23204–23211.

Stern, M., and Ganetzky, B. (1989). Altered synaptic transmission in *Drosophila hyperkinetic* mutants. J. Neurogenet. *5*, 215–228.

Stuart, R.A., Cyr, D.M., Craig, E.A., and Neupert, W. (1994). Mitochondrial molecular chaperones: their role in protein translocation. Trends Biochem. Sci. *19*, 87–92.

Stühmer, W., Ruppersberg, J.P., Schroter, K.H., Sakmann, B., Stocker, M., Giese, K.P., Perschke, A., Baumann, A., and Pongs, O. (1989). Molecular basis of functional diversity of voltage-gated potassium channels in mammalian brain. EMBO J. *8*, 3235–3244.

Swanson, R., Marshall, J., Smith, J.S., Williams, J.B., Boyle, M.B., Folander, K., Luneau, C.J., Antanavage, J., Oliva, C., Buhrow, S.A., Bennett, C., Stein, R.B., and Kaczmarek, L.K. (1990). Cloning and expression of cDNA and genomic clones encoding three delayed rectifier potassium channels in rat brain. Neuron *4*, 929–939.

Trimmer, J.S. (1991). Immunological identification and characterization of a delayed rectifier K$^+$ channel in rat brain. Proc. Natl. Acad. Sci. USA *88*, 10764–10768.

Trimmer, J.S., Trowbridge, I.S., and Vacquier, V.D. (1985). Monoclonal antibody to a membrane glycoprotein inhibits the acrosome reaction and associated Ca$^{2+}$ and H$^+$ fluxes of sea urchin sperm. Cell *40*, 697–703.

Wang, H., Kunkel, D.D., Martin, T.M., Schwartzkroin, P.A., and Tempel, B.L. (1993). Heteromultimeric K$^+$ channels in terminal and juxtaparanodal regions of neurons. Nature *365*, 75–79.

Xu, J., Yu, W., Jan, Y.N., Jan, L.Y., and Li, M. (1995). Assembly of voltage-gated potassium channels. J. Biol. Chem. *270*, 24761–24768.

Yu, W., Xu, J., and Li, M. (1996). NAB domain is essential for the subunit assembly of both α–α and α–β complexes of Shaker-like potassium channels. Neuron *16*, 441–453.

**Note Added in Proof**

The reference cited as Nakahira et al., submitted, has now been published. The reference is as follows: Nakahira, K., Shi, G., Rhodes, K.J., and Trimmer, J.S. (1996). Selective interaction of voltage-gated K$^+$ channel β-subunits with α-subunits. J. Biol. Chem. *271*, 7084–7089.