Separable effects of human Kvβ1.2 N- and C-termini on inactivation and expression of human Kv1.4

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1. The Kvβ subunits of voltage-gated K^+ channels alter the functional expression and gating of non- or slowly inactivating Kvα1 subunits via two separate domains. To determine how Kvβ subunits modulate a rapidly inactivating Kvα1 subunit, we did two-microelectrode voltage clamp experiments on human Kv1.4 voltage-gated K^+ channels expressed heterologously in *Xenopus* oocytes. In addition we tested a slowly inactivating mutant of Kv1.4 lacking amino acids 2–146 of the N-terminal α-ball domain (Kv1.4ΔN2–146). Kv1.4 or Kv1.4ΔN2–146 were co-expressed with either rat Kvβ2 or human Kvβ1.2. To separate domain effects, we also used a mutant of Kvβ1.2 lacking the unique 79 amino acid N-terminal β-ball domain (Kvβ1-C).

2. For the mutant Kv1.4ΔN2–146 we found that Kvβ1-C or Kvβ2 increased current amplitude without altering activation or inactivation. By contrast Kvβ1.2 produced rapid inactivation and slowed deactivation due to block produced by the β-ball. The β-ball also increased the rate of C-type inactivation in 5 mM, but not 50 mM, external K^+ consistent with an effect of blockade on K^+ efflux.

3. For Kv1.4, Kvβ1-C produced a voltage-independent increase in the rate of inactivation and shifted the inactivation curve to more hyperpolarized potentials, but had no effect on deactivation. Kvβ1-C, Kvβ2 and Kvβ1.2 slowed recovery from inactivation similarly, thereby excluding involvement of the β-ball. Kvβ1.2 produced an additional more rapid, voltage-dependent component of inactivation, significantly reduced peak outward current and shifted steady-state inactivation towards hyperpolarized potentials.

4. Yeast two-hybrid studies showed that α–β interaction was restricted to the N-terminus of Kv1.4 and the C-terminus of Kvβ1.2 or Kvβ2. Direct interaction with the α-ball did not occur. Our interpretation is that Kvβ1-C and Kvβ2 enhanced N-type inactivation produced by the Kv1.4 α-ball allosterically.

5. We propose that Kvβ1.2 has three effects on Kv1.4, the first two of which it shares with Kvβ2. First, Kvβ1-C and Kvβ2 have a current-enhancing effect. Second, Kvβ1-C and Kvβ2 increase block by the α-ball allosterically. Third, the β-ball of Kβ1.2 directly blocks both Kv1.4 and Kv1.4ΔN2–146. When both α- and β-balls are present, competition for their respective binding sites slows the block produced by either ball.

Transient A-type K^+ currents are important for regulating the firing frequency of neurons (Connor & Stevens, 1971) and for regulating action potential propagation in axons (Debanne *et al.* 1997). Molecular cloning, co-precipitation and co-localization experiments and current measurements suggest that hetero-oligomeric assemblies of Kv1.4 and modulatory Kvβ subunits may underlie inactivating potassium channels in central neurons *in vivo* (Sheng *et al.* 1993; Rettig *et al.* 1994; Rhodes *et al.* 1995, 1997; Sewing *et al.* 1996; Yu *et al.* 1996; Shamotienko *et al.* 1997). Kvβ subunits have been shown to alter the kinetics (Heinemann *et al.* 1994; Rettig *et al.* 1994; Majumder *et al.* 1995; England *et al.* 1995; Morales *et al.* 1995; Wang *et al.* 1996; Heinemann *et al.* 1996; De Biasi *et al.* 1997; Accili *et al.* 1997*a*,*b*) and expression (Shi *et al.* 1996; Accili *et al.* 1997*a*) of non-inactivating Kvα1 subunits. For Kvβ1.2, two separate domains are involved; the N-terminus ball domain (β-ball) identified by its net positive charge (Rettig *et al.* 1994) was shown to be responsible for the kinetic effects as a result of open channel blockade (Wang *et al.* 1996; DeBiasi *et al.* 1997; Accili *et al.* 1997*a*) whereas the C-terminus was shown to be responsible for changes in functional expression (Accili *et al.* 1997*a*).

For the inactivating ferret Kv1.4 channel, Kvβ1.2 accelerated inactivation and slowed recovery from inactivation. For a slow C-type inactivating mutant of Kv1.4 lacking the positively charged α-ball peptide (Castellino *et al.* 1995), Kvβ1.2 conferred rapid inactivation but did not alter recovery (Castellino *et al.* 1995), leading the authors to infer an interaction with the α-ball. Kvβ2, which shares extensive amino acid homology with Kvβ1.2 in their C-termini, has been shown to accelerate inactivation of voltage-dependent *Shaker* K^+ channels (McCormack *et al.* 1995) and rat Kv1.4 channels (McIntosh *et al.* 1997) but does not introduce rapid, N-type inactivation into non- or slowly inactivating Kvα1 channels (Rettig *et al.* 1994). Taken together these results suggest that Kvβ subunits may alter inactivation by interacting directly with the α-ball of Kv1.4.

In the present experiments we found that Kvβ1.2 C-terminus (Kvβ1-C) and Kvβ2 had enhancing effects on Kv1.4 currents. In addition, both increased inactivation of Kv1.4 but had no effects on the kinetics of Kv1.4ΔN2–146, a mutant lacking the α-ball. Both slowed recovery from inactivation of wild-type Kv1.4 in a manner identical to Kvβ1.2. Because our yeast two-hybrid studies showed that direct α–β interaction did not involve the α-ball, we propose that the effects on inactivation are allosterically mediated. The β-ball blocked Kv1.4 and the non-inactivating Kv1.4 mutant, and in the case of Kv1.4 competed with block produced by the α-ball. The channel block by the β-ball increased the rate of C-type inactivation of the Kv1.4 mutant in low (5 mM) K^+ probably by inhibiting K^+ efflux. For Kvβ2 the α–β interactions show that the C-terminus domain of Kvβs enhances both expression and α-ball blockade, suggesting a novel role in modulating transient K^+ currents and hence the firing frequency of neurons *in vivo*.

METHODS

In vitro transcription of cRNAs and oocyte injection

Oocytes were isolated as previously described (Taglialatela *et al.* 1995). Stage V–VI *Xenopus* oocytes were surgically removed from anaesthetized frogs (0·2% tricaine, 30 min) and defolliculation was achieved by exposing the oocytes to collagenase (2 mg ml^{-1}, 40 min) in solution (mM): 82·5 NaCl, 2·5 KCl, 1 $MgCl_2$, 5 Hepes and 100 μg ml^{-1} gentamicin, pH 7·6. After removal of the ovarian lobes, the anaesthetized frogs were killed. Following this, oocytes were incubated at 19 °C in (mM): 100 NaCl, 2 KCl, 1·8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes, 2·5 pyruvic acid and 100 μg ml^{-1} gentamicin, pH 7·6. One day after isolation, defolliculated oocytes were injected with cRNAs. cRNAs encoding Kvβ1.2, Kvβ1-C, Kvβ2, Kv1.4ΔN2–146 and Kv1.4 were prepared as previously described (Wang *et al.* 1996; Accili *et al.* 1997*a*). cRNA concentrations were estimated on denaturing agarose gels stained with ethidium bromide by comparison with RNA standards. cRNAs were dissolved in 0·1 M KCl, stored at −80 °C, and diluted and mixed immediately prior to injection. Oocytes were injected with 46 nl of cRNA solutions in 0·1 M KCl. Final concentrations of cRNA were: 2 ng μl^{-1} for Kv1.4ΔN2–146, 5 or 10 ng μl^{-1} for Kv1.4 and 250 ng μl^{-1} for Kvβ1.2, Kvβ1-C or Kvβ2.

Electrophysiology

Measurement of *Xenopus* oocyte whole-cell currents was performed using the standard two-microelectrode voltage clamp technique as described previously (Wang *et al.* 1996; Accili *et al.* 1997*a*). Electrodes filled with 3 M KCl had resistances of 0·2–0·5 MΩ when measured in the bath solution containing (mM): 50 KOH, 55 NaOH, 0·5 $CaCl_2$, 100 Mes, 2 $MgCl_2$ and 10 Hepes, pH 7·3. We used 50 mM K^+ in bath solution to slow C-type inactivation and reduce its contribution to the inactivation of Kv1.4 during short pulses. In some experiments measurements were performed in solution containing 5 mM K^+. In this case K^+ was replaced with an equivalent concentration of Na^+. Measurements were done 3–5 days after cRNA injection. For comparison of whole cell current amplitudes, all measurements were done 3 days after oocyte injection. All recordings were made at room temperature (20–22 °C). Chemicals were purchased from Sigma.

Data acquisition and analyses were performed with pCLAMP software (Axon Instruments). Data were low pass filtered at 2 or 5 kHz before digitalization at 10 kHz. Data are reported as means ± S.E.M. Comparisons between two groups of oocytes were performed by Student's t test and means are considered to be significantly different when $P < 0·05$.

Yeast two-hybrid interaction

Protein–protein interactions were monitored with the yeast Matchmaker Two-Hybrid System from Clontech (Palo Alto, CA, USA). Full-length human Kvβ1.2 (amino acids 1–408), Kvβ1.2-N terminus (amino acids 1–79) and Kvβ1-C (the C-terminal 329 amino acids of the Kvβ1 subfamily) were subcloned into the DNA binding domain (BD) vector, pGBT9, and Kv1.4-N (amino acids 1–305) and a truncated version, Kv1.4-N (amino acids 147–305), were subcloned into the activation domain (AD) vector, pGAD424, as previously described (Wang *et al.* 1996). To subclone the partial fragment of Kv1.4-N containing the ball peptide (amino acids 1–176) into pGAD424, in frame *EcoR*I and *Sal*I sites were incorporated into the 5′ and 3′ ends, respectively, by polymerase chain reaction (PCR). The PCR amplified construct was sequenced to confirm the correct reading frame for the yeast fusion and to check that no unwanted PCR mutations were introduced. Protein–protein interactions were tested in yeast host strain Y190 by co-transformation with pairs of BD and AD fusion constructs according to the manufacturer's protocol. Transformants were initially plated on media lacking tryptophan (−trp) and leucine (−leu) and grown for 2·5 days at 30 °C. To facilitate comparison of interactions, a representative colony from each co-transformation was spotted onto another −trp/−leu plate and grown for 24 h at 30 °C prior to assay for β-galactosidase activity using a filter lift assay as specified in Clontech protocols. Development of blue colour within 8 h indicated activation of the reporter gene, *lacZ*, and was scored as a positive interaction between two fusion proteins.

RESULTS

Kvβ subunits without ball domains increase current expression of the non-inactivating Kv1.4 ball mutant

Previously we reported that the C-terminus of Kvβ1.2 (Kvβ1-C) increased Kv1.2 currents but decreased Kv1.5 currents (Accili *et al.* 1997*a*). We co-expressed Kvβ1-C with a Kv1.4 mutant lacking the ball peptide (Kv1.4ΔN2–146) to determine the effects on current amplitude without the confounding effects of α- and/or β-ball inactivation. Significant increases in current (2·69 ± 0·29 times that of

control in 5 batches of oocytes) were observed (Fig. 1*A*) although the increases were larger for Kv1.2 (Fig. 1*B*). Kvβ2, whose C-terminus is highly homologous to Kvβ1-C, also increased Kv1.4ΔN2–146 currents (3·19 times that of control in 28 oocytes from 1 batch; Fig. 1*C*).

Neither Kvβ1-C (Fig. 1*A*) nor Kvβ2 (data not shown) produced rapid inactivation of Kv1.4ΔN2–146 currents during the 100 ms test pulses. Nor did either alter the steady-state activation: half-maximal activation ($V_{0.5}$) and voltage dependence (k) values were $-30{\cdot}9 \pm 0{\cdot}9$ and $8{\cdot}4 \pm 0{\cdot}7$ mV for Kv1.4ΔN2–146 alone ($n = 6$), $-33{\cdot}1 \pm 1{\cdot}1$ and $7{\cdot}9 \pm 0{\cdot}8$ mV for Kv1.4ΔN2–146 plus Kvβ1-C ($n = 8$) and $-30{\cdot}2 \pm 1{\cdot}0$ and $8{\cdot}4 \pm 0{\cdot}8$ mV for Kv1.4ΔN2–146 plus Kvβ2 ($n = 8$), or the rate of deactivation (data not shown). We also found that Kvβ2 did not alter slow, C-type inactivation of Kv1.4ΔN2–146. Four-second voltage pulses to +40 mV produced slow inactivation of Kv1.4ΔN2–146 having single exponential time constants of $2{\cdot}84 \pm 0{\cdot}08$ s ($n = 6$) without and $3{\cdot}50 \pm 0{\cdot}13$ s ($n = 6$) with Kvβ2. Recovery from inactivation for Kv1.4ΔN2–146 without and with Kvβ2 was also similar: recovery time constants were $3{\cdot}37 \pm 0{\cdot}19$ ($n = 6$) and $3{\cdot}44 \pm 0{\cdot}14$ s ($n = 6$), respectively. Kvβ1.2 was reported not to alter recovery from inactivation of Kv1.4ΔN2–146 currents (Castellino *et al.* 1995).

The β-ball produces rapid inactivation of Kv1.4ΔN2–146

Unlike Kvβ1-C (Fig. 1*A*), Kvβ1.2 produced rapid inactivation of Kv1.4ΔN2–146 (compare Fig. 2*A* and *B*). Deactivation was also slowed (compare tails between Fig. 2*D* and *E*) and the steady-state activation curve was steeper and shifted to more negative potentials although the activation threshold was unchanged (Fig. 2*C*). The rates of rapid inactivation produced by Kvβ1.2 are voltage dependent in the range of 50 to 80 mV (Fig. 4*C*). A tail current protocol (Fig. 2*D* and *E*) established that the rate of activation was unchanged (Fig. 2*D*–*F*). It has been suggested that Kvβ1.2 modulation of C-type inactivation in the ferret Kv1.4ΔN2–146 was sensitive to external K^+ concentration (Morales *et al.* 1996). We found that inactivation of Kv1.4ΔN2–146 in the presence of Kvβ1.2 consisted of two components (Fig. 3*A*), a rapid component attributable to block by the Kvβ1.2 N-terminal ball domain and a much slower component involving a C-type inactivation. In 50 mM external K^+ the rate of C-type inactivation was not altered by Kvβ1.2.

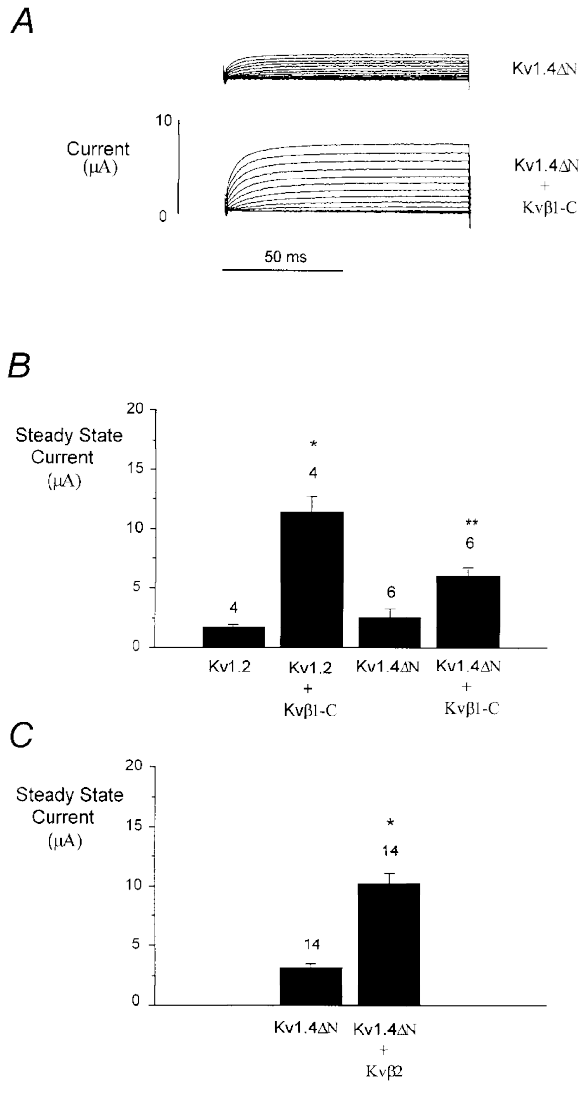

Figure 1. Kvβ subunits increase functional expression of the non-inactivating Kv1.4 mutant, Kv1.4ΔN2–146

A, whole cell currents were measured in *Xenopus* oocytes injected with cRNAs for Kv1.4ΔN2–146 alone or together with Kvβ1-C (also equivalent to the C-terminus of Kvβ1.2). Oocytes were held at −80 mV and pulsed to +80 mV in 10 mV steps of 100 ms duration. *B*, bar plot of whole-cell currents measured in Kv1.4ΔN2–146 or Kv1.2, alone or together with Kvβ1-C. Current amplitudes were measured at the end of the pulse to +70 mV. Values represent means ± S.E.M. * and ** indicate a significant difference from the value of current for Kv1.4ΔN2–146 and Kv1.2, respectively ($P < 0{\cdot}05$, t test). Numbers above bars represent the total number of oocytes in each group for a particular batch of oocytes. *C*, bar plot of whole cell currents measured in *Xenopus* oocytes injected with Kv1.4ΔN2–146 alone and with Kvβ2 (*$P < 0{\cdot}05$, t test).

However, in 5 mM K^+, C-type inactivation was faster in the presence of Kvβ1.2 (Fig. 3*B*). The fast component was insensitive to external K^+ concentration. The increased rate of C-type inactivation at 5 mM external K^+ is probably due to inhibition of K^+ efflux by β-ball blockade.

Separable effects of Kvβ1.2 N- and C-termini on inactivation of Kv1.4

To this point, we have shown that the C-terminus of Kvβ1.2 enhanced Kv1.4ΔN2–146 current as did Kvβ2 and that the N-terminus of Kvβ1.2 introduced rapid, N-type inactivation into Kv1.4ΔN2–146. Next, we compared the effects of the Kvβ1.2 C-terminus and intact Kvβ1.2 on intact Kv1.4. Normalized current traces are compared in Fig. 4*A*. In these experiments short pulses were utilized and the extracellular solution contained 50 mM K^+ to minimize the contribution of C-type inactivation. We found that both Kvβ1-C and Kvβ1.2 accelerated Kv1.4 N-type inactivation but did so in different ways. The inactivating currents produced by Kv1.4 alone or with Kvβ1-C were fitted with a single exponential function. The rate of N-type inactivation was roughly doubled in the presence of Kvβ1-C (●, Fig. 4*B*). The rates of Kv1.4 inactivation either with or without Kvβ1-C did not vary with membrane potential in the range of 50 to 80 mV.

Co-expression with intact Kvβ1.2 produced further effects on the N-type inactivation of Kv1.4. Inactivation now had

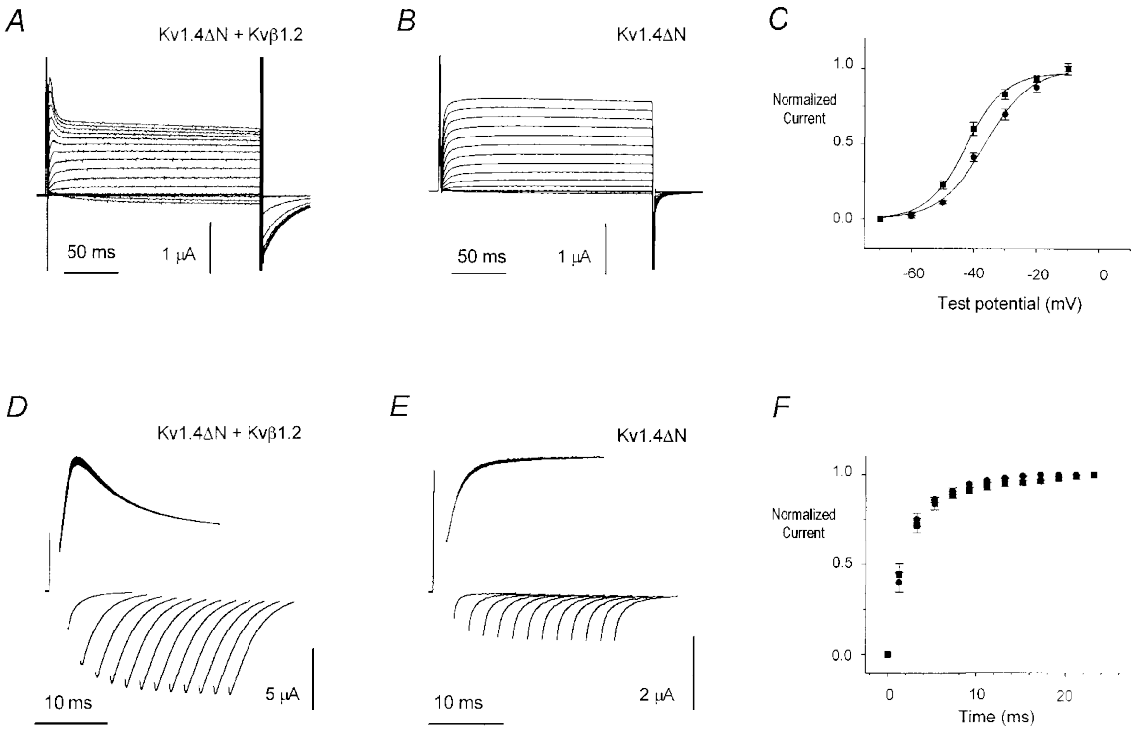

Figure 2. The Kvβ1.2 N-terminal ball domain produces N-type inactivation of the Kv1.4ΔN2–146 mutant

Whole cell currents were measured in *Xenopus* oocytes injected with cRNA for Kv1.4ΔN2–146 with (*A*) and without (*B*) Kvβ1.2. Oocytes were held at −80 mV and pulsed to +80 mV in 10 mV steps of 125 ms in duration. Kvβ1.2 produced inactivation and slowed deactivation. *C*, steady-state activation curves were generated from peak tail currents, at −80 mV normalized to the maximum and plotted against test potential. Data were fitted with the following Boltzmann function:

$$I/I_{max} = [1 + \exp((V_{0.5} - V)/k)]^{-1},$$

where I is the current, V is the test potential and k is the Boltzmann constant. ● and ■ represent normalized values determined from tail currents of oocytes injected with Kv1.4ΔN2–146 and Kv1.4ΔN2–146 plus Kvβ1.2, respectively. Kvβ1.2 shifted the curve to more negative potentials (−9·9 mV) and increased the curve steepness (k) from 10·4 ± 1·1 mV ($n = 6$) to 7·3 ± 0·5 mV ($n = 6$). Note that the threshold for activation is similar in both situations. The values for half-maximal activation ($V_{0.5}$) are −31·0 ± 1·8 mV ($n = 6$) and −40·9 ± 1·3 mV ($n = 6$). *D* and *E*, tail current protocol was used to compare activation kinetics of currents in oocytes injected with Kv1.4ΔN2–146 + Kvβ1.2 (*D*) or Kv1.4ΔN2–146 alone (*E*). Oocytes were held at −90 mV, pulsed to +120 mV for times ranging from 1 to 23 ms and repolarized to −120 mV (100 mM K^+ in external solution). *F*, normalized tail currents were plotted as a function of depolarization time. Time constants of activation were 2·8 ± 0·4 ($n = 3$) and 2·5 ± 0·3 ms ($n = 3$) for Kv1.4ΔN2–146 (●) and Kv1.4ΔN2–146 + Kvβ1.2 (■), respectively.

two rates and was fitted with a sum of two exponentials. The small (Fig. 4*C*; ■) and large time constants (Fig. 4*B*; ■) bracket the time constants associated with intact Kv1.4 with and without Kvβ1.2-C. Unlike the other time constants, the small time constant is voltage dependent. We attribute the smallest time constant to block by the β-ball of Kvβ1.2 and the larger time constant to block by the α-ball of Kv1.4 in the presence of β-ball blockade. Note that the β-ball time constant is larger in the presence of the α-ball (Fig. 4*C*) and the α-ball time constant is larger in the presence of the β-ball (Fig. 4*B*, compare ■ with ▲).

In addition to its effect on N-type inactivation, Kvβ1-C produced a significant increase in the peak current of Kv1.4 as it did for Kv1.4ΔN2–146 (Fig. 4*D*). At a test potential of +70 mV peak currents were $11{\cdot}30 \pm 2{\cdot}42$ ($n = 5$) and $21{\cdot}10 \pm 2{\cdot}08$ μA ($n = 5$) for Kv1.4 alone and Kv1.4 plus Kvβ1-C, respectively. By contrast Kvβ1.2 decreased the peak current levels: $7{\cdot}54 \pm 0{\cdot}71$ μA ($n = 5$; Fig. 4*D*).

As shown in Fig. 5*D*, Kvβ1.2-C shifts the inactivation curve of Kv1.4 to more negative potentials. However, intact Kvβ1.2 produces rapid, N-type inactivation having two time constants (Fig. 4). Using the amplitude of the slower component results in a steady-state inactivation curve shifted to more negative potentials and similar to the curve produced by Kvβ1-C (Fig. 5*B* and *D*). Using the amplitude of the fast component results in a curve shifted further to the left with a steeper slope (Fig. 5*D*).

Separable effects of Kvβ1.2 N- and C-termini on deactivation of Kv1.4

Tail currents of Kv1.4 alone or in the presence of Kvβ1-C and Kvβ1.2 are shown in Fig. 6*A*. The tails were fitted with a single exponential function and the time constants plotted against voltage (Fig. 6*B*). At potentials of −90 mV, deactivation time constants were $4{\cdot}97 \pm 0{\cdot}08$ ($n = 6$), $5{\cdot}56 \pm 0{\cdot}04$ ($n = 6$) and $16{\cdot}82 \pm 0{\cdot}79$ ms ($n = 5$) for Kv1.4 alone, Kv1.4 plus Kvβ1-C and Kv1.4 plus Kvβ1.2, respectively. The rate of Kv1.4 deactivation was not affected by Kvβ1-C whereas Kvβ1.2 produced a clear slowing of deactivation.

Effects of Kvβ1.2 N-terminal ball domain on recovery from inactivation of Kv1.4

Castellino *et al* (1995) have shown that Kvβ1.2 slows recovery from inactivation of Kv1.4. To separate C- and N-terminal effects, we compared the effects of Kvβ1.2,

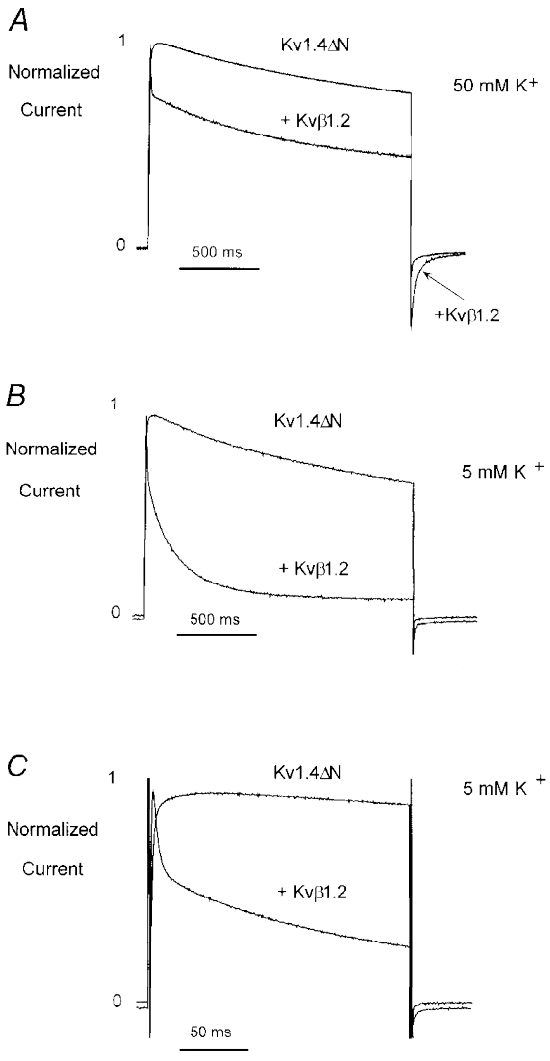

Figure 3. Kvβ1.2 increases the rate of C-type inactivation at low but not high external K^+ concentrations

A, superimposition of normalized current traces for Kv1.4ΔN2–146 alone and with Kvβ1.2 at test potential to +70 mV for 1·6 s from a holding potential of −80 mV. The C-type inactivation time constant for Kv1.4ΔN2–146 alone was 1289 ms. In 50 mM K^+, Kvβ1.2 produced a rapid inactivation but did not alter the rate of the slow C-type inactivation; time constants were 3·9 and 865 ms, respectively. *B*, the same oocytes and protocol as in *A* but in 5 mM external K^+. C-type inactivation is significantly faster in the presence of Kvβ1.2; time constants were 1012 and 227 ms without and with Kvβ1.2, respectively. Note that the fast time constant was unchanged (4·0 ms). *C*, the same oocytes as in *B* but at a faster time scale.

Kvβ1-C and Kvβ2 on Kv1.4 recovery from inactivation (Fig. 7). Recovery was slowed equally by Kvβ1.2 and Kvβ1-C, indicating that block by the N-terminus ball of Kvβ1.2 does not contribute to this effect (Fig. 7*A*). Kvβ2 also slowed recovery from inactivation of Kv1.4 (Fig. 7*B*).

Yeast two-hybrid interactions

Conserved sequences in the C-terminus of Kvβ1.2 or Kvβ2 have been shown to interact with the N-terminus of Kvα1 (amino acids (aa) 1–305 or aa 147–305) (Yu *et al.* 1996; Sewing *et al.* 1996; Nakahira *et al.* 1996; Wang *et al.* 1996). However, there is no evidence regarding the interactions of these Kvβs with the variable N-terminal region of Kv1.4 (aa 1–176) that includes the α-ball domain. Since changes in inactivation kinetics by the C-terminus of Kvβ1.2 were manifest (Fig. 4) we tested for a possible interaction between Kvβ1.2-C and the N-terminus ball domain of Kv1.4 (aa 1–176) using the yeast two-hybrid assay. We found that Kvβ1-C and the Kv1.4ΔN2–146 domain (aa 1–176) did not interact whereas interactions were present for Kvβ1-C and the N-terminus of Kv1.4 downstream from the α-ball domain (aa 147–305) (Fig. 8).

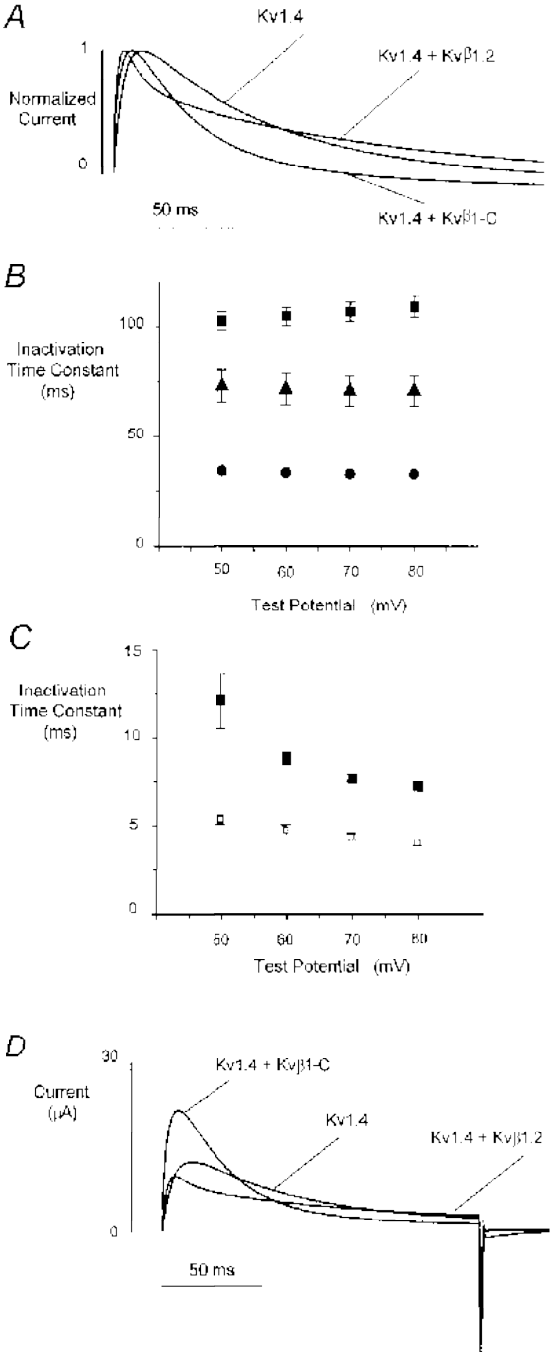

Figure 4. Kvβ1.2 C- and N-termini produce distinct changes in Kv1.4 inactivation kinetics

A, normalized whole cell currents measured in *Xenopus* oocytes injected with cRNAs for Kv1.4 alone or with Kvβ1.2 or Kvβ1-C. Oocytes were held at −80 mV, pulsed to +80 mV for 125 ms and repolarized to −80 mV. *B*, plot of inactivation time constants *versus* test potential. ■, slower second time constant of inactivating currents produced by injection of Kv1.4 with Kvβ1.2 ($n = 5$) fitted with a sum of two exponential functions. ▲ and ●, time constants of inactivating currents produced by injection of Kv1.4 alone ($n = 2$) and Kv1.4 with Kvβ1-C ($n = 5$), respectively, fitted with a single exponential function. *C*, a plot of inactivation time constants *versus* test potential. ■, faster first time constant of inactivating currents produced by injection of Kv1.4 with Kvβ1.2 (the second time constant is shown by ■ in *B*). The fast time constant is voltage dependent, decreasing by almost 50 % from +50 to +80 mV whereas the slower time constant is not. For comparison the fast time constant of inactivating currents produced by injection of Kv1.4ΔN2–146 with Kvβ1.2 is shown (□; $n = 5$). *D*, whole cell currents measured in *Xenopus* oocytes injected with cRNAs for Kv1.4 alone or with Kvβ1-C or Kvβ1.2. Oocytes were held at −80 mV, pulsed to +70 mV for 125 ms and repolarized to −80 mV.

DISCUSSION

Kvβ subunit C-terminal domains increase functional expression of the inactivating K^+ channel, Kv1.4

We have called the Kvβ1 C-terminus the 'α-expression domain' because this region is responsible for altering the functional expression of Kv1·2 and Kv1·5 (Accili *et al.* 1997*a*). Using the non-inactivating Kv1.4 mutant with amino acids 2–146 deleted, we showed that Kvβ1-C, which is the C-terminal 329 amino acids of the Kvβ1 family, produced a significant increase in the currents expressed by this channel. Taken together with the evidence from yeast two-hybrid assays (Yu *et al.* 1996; Wang *et al.* 1996) and other binding studies (Sewing *et al.* 1996) the functional effects of Kvβ1-C or Kvβ2 on expression probably result from a direct interaction with the N-terminus of Kv1.4.

The increase in Kv1.2 produced by Kvβ is the result of an increase in the number of functional channels in the membrane (Accili *et al.* 1997*a*). Single channel measurements of non-inactivating Kv1.4 mutants similar to the mutant used here have shown that the mean open probability is approximately 80% at the test potentials we have used (Tseng-Crank *et al.* 1993). Assuming no change in single channel conductance, the increase in Kv1.4 current produced by Kvβ-C in this study was also probably due to an increase

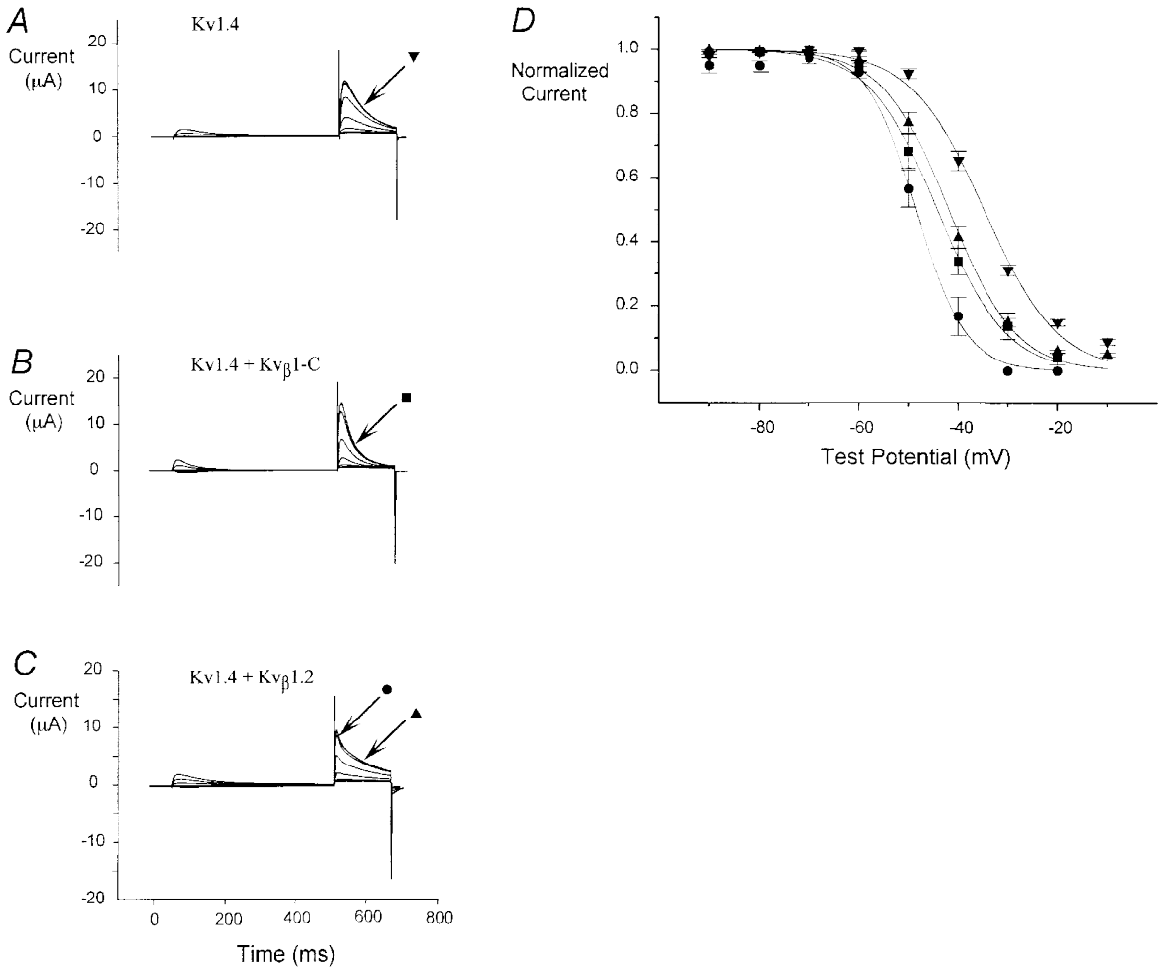

Figure 5. Kvβ C- and N-termini produce distinct changes in Kv1.4 steady-state inactivation

A, *B* and *C* show whole cell currents in oocytes held at −90 mV, pulsed to −10 mV in 10 mV steps of 500 ms duration, subsequently pulsed to +70 mV for 200 ms and repolarized to −90 mV for 15 s. *D*, steady-state inactivation–voltage curves. For Kv1.4 and Kv1.4 + Kvβ-C, curves were generated from currents at +70 mV, normalized to the maximum and plotted against test potential. For Kv1.4 + Kvβ1.2, currents were divided into fast and slow components by fitting as a sum of two exponential functions. Values of peak currents were determined for both components at +70 mV, normalized to the maximum values and both sets of data plotted against test potential. Data were fitted to a Boltzmann equation as described in Fig. 2. The following values were obtained for half-maximal activation ($V_{0.5}$) and k, respectively: −35·05 ± 0·76, −7·35 ± 0·23 mV (Kv1.4, ▼; $n = 8$), −42·06 ± 0·97, −6·59 ± 0·08 mV (Kv1.4 + Kvβ1-C, ■; $n = 6$), −44·31 ± 1·49, −6·79 ± 0·53 mV (Kv1.4 + Kvβ1.2 slow component, ▲; $n = 6$) and −47·72 ± 1·42, −4·60 ± 0·38 mV (Kv1.4 + Kvβ1.2 fast component, ●; $n = 6$).

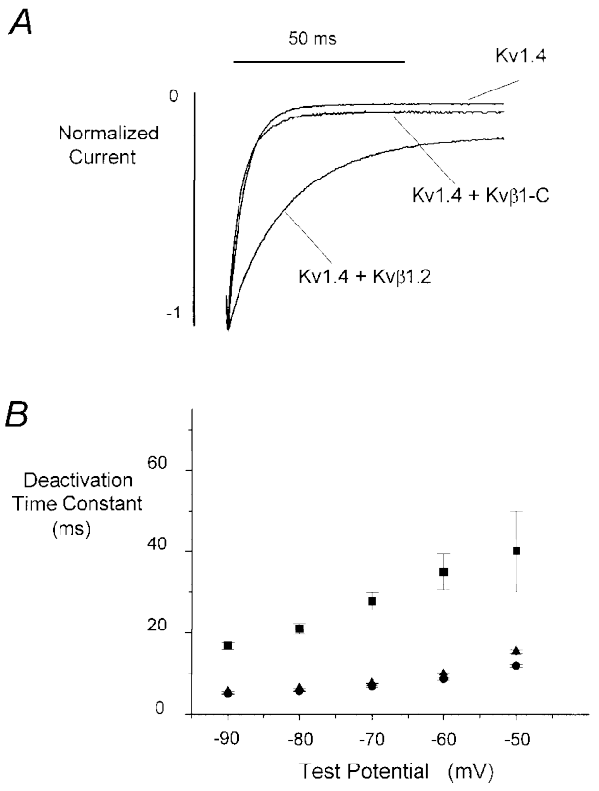

Figure 6. Kvβ1.2 slows deactivation of Kv1.4

A, normalized whole cell currents measured in *Xenopus* oocytes injected with cRNAs for Kv1.4 alone or with Kvβ1.2 or Kvβ1-C. Oocytes were held at −80 mV, pulsed to +80 mV for 125 ms and repolarized to −80 mV. *B*, plot of deactivation time constants *versus* test potential. Deactivation time constants were calculated by fitting the tail currents with a single exponential function. ■, ▲ and ● represent time constants of currents produced by injection of Kv1.4 with Kvβ1.2 ($n = 5$), Kv1.4 with Kvβ1-C ($n = 6$) and Kv1.4 alone ($n = 6$), respectively.

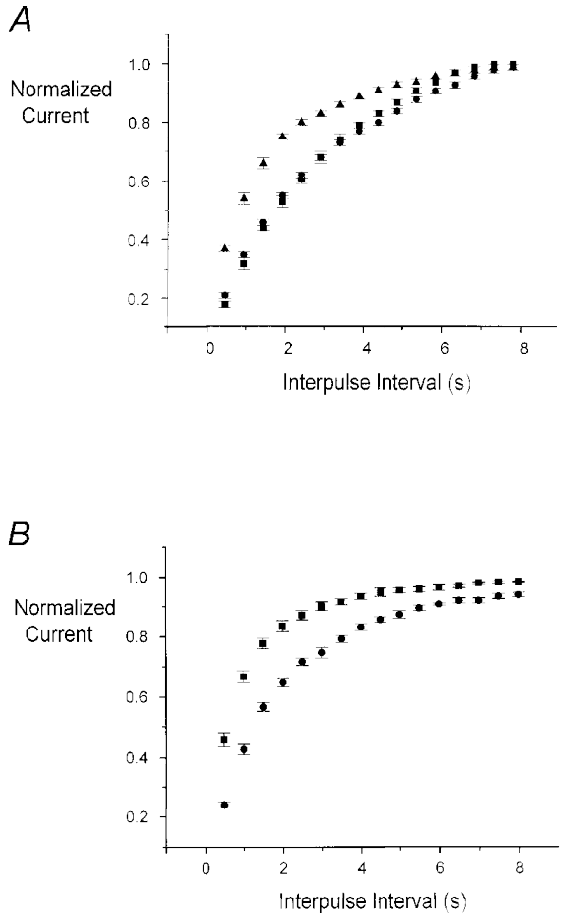

Figure 7. Kvβ1.2 slows recovery from inactivation of Kv1.4 through its C-termini and not through its N-termini

Whole cell currents were measured in *Xenopus* oocytes injected with cRNA for Kv1.4 alone, Kv1.4 with Kvβ1-C, Kv1.4 with Kvβ1.2 and Kv1.4 with Kvβ2. Oocytes were held at −90 mV, pulsed to +40 mV for 1 s, repolarized to −90 mV for times ranging from 1 to 8 s (interpulse duration) and depolarized again to +40 mV. *A*, plot of the amount of inactivated current during the second pulse normalized to the amount of inactivated current during the first pulse *versus* the interpulse duration. Recovery time constants were 1·47 ± 0·07, 3·31 ± 0·25 and 3·32 ± 0·07 s for Kv1.4 alone (▲; $n = 6$), Kv1.4 with Kvβ1.2 (■; $n = 5$) and Kv1.4 with Kvβ1-C (●; $n = 5$), respectively. *B*, recovery from inactivation for Kv1.4 alone (■; $n = 4$) and for Kv1.4 with Kvβ2 (●; $n = 4$). Recovery time constants were 1·18 ± 0·08 and 1·89 ± 0·09 s, respectively.

in the number of functional channels. That Kv1.4 single channel conductance was unchanged is reasonable since no changes in Kv1.2 single channel conductance were observed upon co-expression with Kvβ1-C (Accili *et al.* 1997*a*). The increase in Kv1.4 may be due to a more efficient maturation of the subunits leading to an increase in the surface expression as suggested for non-inactivating Kvα1 subunits (Shi *et al.* 1996).

Separable effects of Kvβ1.2 N- and C-termini on inactivation of Kv1.4

Our results show that co-expression of Kvβ1-C with Kv1.4 increased the rate of inactivation and slowed the rate of recovery from inactivation. The latter effect differs from that observed for Kvβ2 and rat Kv1.4 in the presence of Rb^+ (McIntosh *et al.* 1997). Our results agree with previous studies which have shown that recovery from inactivation was slowed by Kvβ1.2 only for Kv1.4 and not for Kv1.4ΔN2–146 (Castellino *et al.* 1995). In the absence of Kvβ1.2, the rate of recovery from inactivation was not different between Kv1.4 and Kv1.4ΔN2–146 (Rasmusson *et al.* 1995). These authors concluded that the slow recovery was dominated by C-type inactivation and that the Kv1.4 N-terminus was required for the increased inactivation and slowed recovery.

A possible explanation for such results is that Kvβ1-C, i.e. the C-terminus of Kvβ, interacts with the N-terminal α-ball domain of Kv1.4. However, our yeast two-hybrid assay results do not support this explanation. Since the Kvβ

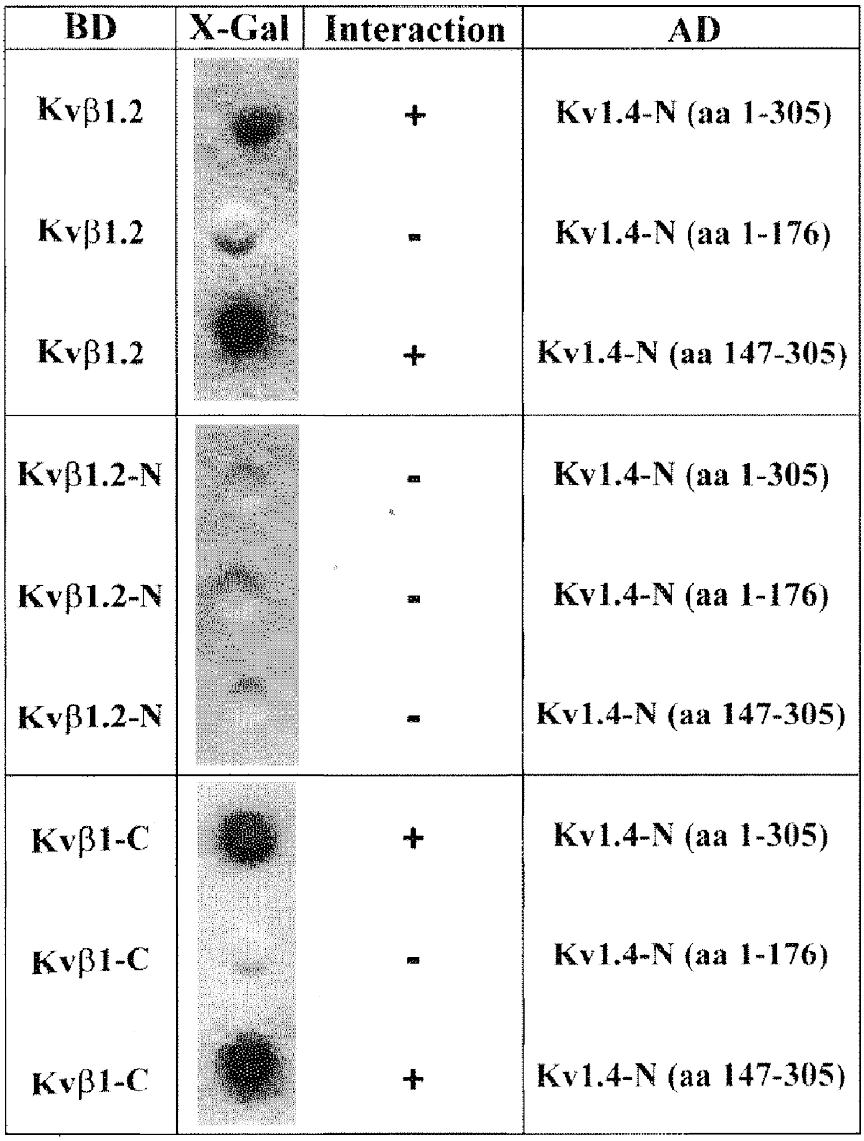

Figure 8. Yeast two-hybrid interaction of Kv1.4 N-terminal fragments with Kvβ subunits

β-Galactosidase filter lift assay (column labelled X-Gal) of Y190 yeast co-transformed with combinations of DNA binding domain (BD) and activation domain (AD) plasmids. Development of a blue colour within 8 h indicated an interaction between the two fusion proteins (scored as a + or − to the right of the X-Gal column).

C-terminus does bind to the Kv1.4 N-terminus downstream from the α-ball we propose that Kvβ1-C or Kvβ2 produces an allosteric enhancement of α-ball blockade.

The inactivation introduced by the β-ball on non-inactivating Kvα1 channels has been attributed to block of open channels (DeBiasi *et al.* 1997; Accili *et al.* 1997*a*). The results we obtained using the non-inactivating mutant of Kv1.4 probably have the same basis. As for the naturally non-inactivating Kvα1 channels, Kvβ1-C and Kvβ2 also had no effects on voltage-dependent activation or deactivation.

Block by the β-ball was observed for intact Kv1.4 where an additional voltage-dependent component of inactivation significantly reduced peak outward current. A hyperpolarizing shift and increase in slope of the steady-state inactivation curve were also observed. These effects may also be explained by open channel block. The effects of Kvβ1.2 on deactivation may be due to unblock of the N-terminus β-ball. Previous experiments using Kv1.4 and an N-terminal deletion mutant of Kv1.4 have shown that the presence of the α-ball has little effect on deactivation (Bertoli, Moran & Conti, 1994).

A slowing of block produced by the α-ball was observed in the presence of the β-ball and vice versa. An apparent slowing would be expected if block by each ball was mutually exclusive. In fully activated channels the rate of block by the β-ball was voltage dependent whereas that of the α-ball was not, suggesting differences in binding site or access between the two balls.

An increase in the rate of C-type inactivation by Kvβ1.2 was observed at 5 mM external K^+ concentration, as described previously (Morales *et al.* 1996). Kvβ1-C and

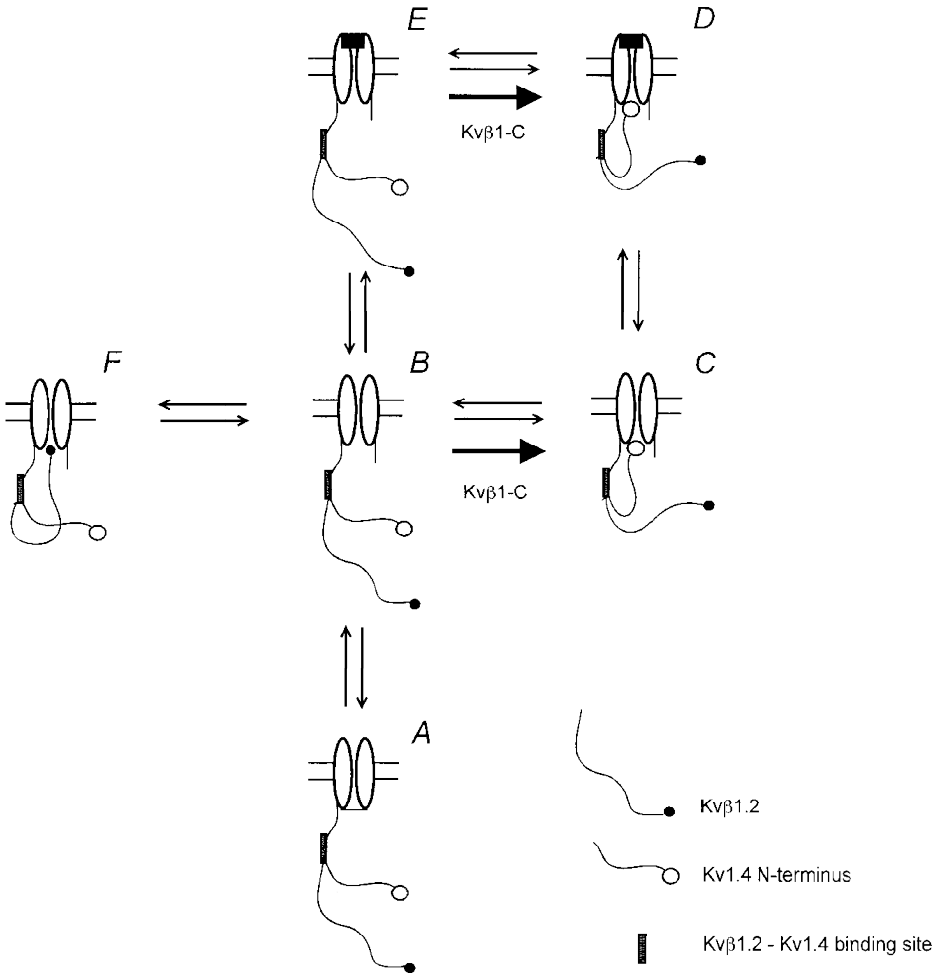

Figure 9. Proposed scheme for Kvβ1.2 effect on the inactivation of Kv1.4

The scheme shows a cutaway of an hetero-oligomeric Kv1.4–Kvβ1.2 channel in the following states: closed (*A*), open (*B*), blocked by the Kvβ1.2 N-terminus β-ball domain (*F*), blocked by the Kv1.4 N-terminus α-ball domain (*C*), undergoing C-type inactivation (*E*) and undergoing C-type inactivation during block by the Kv1.4 N-terminus α-ball (*D*). Kvβ1.2 is bound to the region of the Kv1.4 N-terminus defined by yeast two-hybrid and co-immunoprecipitation data. For simplicity, the cutaway shows two α-subunits, one N-terminus α-ball domain and one Kvβ binding site and Kvβ N-terminus β-ball.

Kvβ2 had no effect on C-type inactivation, implicating block by the β-ball in this effect. However, we found that at 50 mM K^+, C-type inactivation was unaffected by Kvβ1.2. Thus, the open channel block by the β-ball probably inhibits K^+ efflux as suggested for the α-ball effect on C-type inactivation of *Shaker* K^+ channels (Baukrowitz & Yellen, 1995).

A model of Kvβ effects on Kv1.4

Figure 9 was adapted from Hoshi *et al.* (1991) and shows how we envision inactivation in a hetero-oligomeric Kv1.4–Kvβ1.2 channel. The model can also explain the effects produced by a mixture of inactivating and non-inactivating Kvβ subunits (Accili *et al.* 1997*b*; Xu & Li, 1997). The channel goes from closed to open (state *A* to state *B*) upon depolarization and then can either be blocked by the α-ball (state *C*), or undergo C-type inactivation (state *E*). Since block by the α-ball enhances C-type inactivation (Hoshi *et al.* 1991; Baukrowitz & Yellen, 1995; Rasmussen *et al.* 1995) the channel assumes state *D*. Kvβ1.2, through its C- and N-termini, produces two separate effects on inactivation. Kvβ1-C or Kvβ2 enhances the block produced by the allosterically modified α-ball. Activation and deactivation kinetics between *A* and *B* are not affected, but the *B* to *C* transition rate for the α-ball is increased. The recovery from inactivation is thought to be rate limited by recovery from C-type inactivation (Rasmussen *et al.* 1995). In our model, increased allosterically enhanced block by the α-ball increases the transition rate constants for *E* to *D* and/or *C* to *D*. Block by the β-ball is produced by the transition from *B* to state *F*. The channel may be blocked by either ball but not by both. It is this interference that slows the blocking rate of either ball. State *F* is not coupled to *E* or *D* since no effects of β-ball blockade on C-type inactivation were observed in our experiments.

Functional roles of Kvβ subunits in modifying inactivating K^+ channels in neurons

Transient K^+ currents contribute to the regulation of firing frequency of neurons (Connor & Stevens, 1971) and more recently have been implicated in regulating action potential propagation in axons (Debanne *et al.* 1997). Heterologous expression of Kv1.4 in *Xenopus* oocytes alone, or with Kvβ subunits, produces inactivating currents similar to those found in central neurons, suggesting that this channel makes an important contribution to neuronal A-currents (Pardo *et al.* 1992; Rettig *et al.* 1994). Evidence exists for extensive co-localization of Kv1.4 and Kvβ subunits in cell bodies and axons of central neurons (Rhodes *et al.* 1995, 1997; Nakahira *et al.* 1996). Homomultimers of Kv1.4, and heteromultimers of Kv1.4 with Kvβ subunits and/or other Kvα1 subunits have been characterized from central neurons (Shamotienko *et al.* 1997). Our previous (Accili *et al.* 1997*b*) and present results, and the results of others (Castellino *et al.* 1995), offer several potential roles for Kvβ subunits in modifying the electrical properties of central neurons by acting on inactivating K^+ channels. Kvβ subunits can increase the functional expression of inactivating K^+ channels. Non-inactivating Kvβ subunits can increase inactivation rates and slow recovery from inactivation of inactivating K^+ channels. Inactivating Kvβ subunits also slow recovery, through their C-termini, as well as producing additional open channel block reducing peak current and slow deactivation. These mechanisms greatly expand the mode and range over which inactivating K^+ channels, and hence neuronal firing frequency and action potential propagation, can be regulated and underline the importance of analysing the functional relationship between inactivating K^+ channels and Kvβ subunits *in vivo*.

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