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Episodic ataxia type 1 mutations in the *KCNA1* gene impair the fast inactivation properties of the human potassium channels Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2

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Abstract

Episodic ataxia type 1 (EA1) is an autosomal dominant neurological disorder characterized by constant muscle rippling movements (myokymia) and episodic attacks of ataxia. Several heterozygous point mutations have been found in the coding sequence of the voltage-gated potassium channel gene *KCNA1 (*hKv1.1), which alter the delayed-rectifier function of the channel. *Shaker*-like channels of different cell types may be formed by unique hetero-oligomeric complexes comprising Kv1.1, Kv1.4 and Kvβ1.x subunits. Here we show that the human Kvβ1.1 and Kvβ1.2 subunits modulated the functional properties of tandemly linked Kv1.4-1.1 wild-type channels expressed in *Xenopus laevis* oocytes by (i) increasing the rate and amount of N-type inactivation, (ii) slowing the recovery rate from inactivation, (iii) accelerating the cumulative inactivation of the channel and (iv) negatively shifting the voltage dependence of inactivation. To date, the role of the human Kv1.4-1.1, Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 channels in the aetiopathogenesis of EA1 has not been investigated. Here we also show that the EA1 mutations E325D, V404I and V408A, which line the ion-conducting pore, and I177N, which resides within the S1 segment, alter the fast inactivation and repriming properties of the channels by decreasing both the rate and degree of N-type inactivation and by accelerating the recovery from fast inactivation. Furthermore, the E325D, V404I and I177N mutations shifted the voltage dependence of the steady-state inactivation to more positive potentials. The results demonstrate that the human Kvβ1.1 and Kvβ1.2 subunits regulate the proportion of wild-type Kv1.4-1.1 channels that are available to open. Furthermore, EA1 mutations alter heteromeric channel availability which probably modifies the integration properties and firing patterns of neurones controlling cognitive processes and body movements.

Introduction

Episodic ataxia type 1 (EA1) is an autosomal dominant disorder characterized by attacks of ataxic gait that may be brought on by fever, startle, emotional stress, exercise or fatigue (Van Dyke *et al.*, 1975). This disease is distinguished from episodic ataxia type 2 by the absence of nystagmus and by the presence of continuous and spontaneous motor unit activity (myokymia) that may become more severe during an attack or after intense muscle activity. Myokymia can be detected as a fine rippling of different skeletal muscles. Genetic linkage studies of several EA1-affected families and the subsequent screen of the *KCNA1* gene for mutations resulted in the discovery of a number of point mutations in the *Shaker*-related potassium channel hKv1.1 (Browne *et al.*, 1994; Scheffer *et al.*, 1998; Eunson *et al.*, 2000). The molecular mechanisms of EA1 have been established by determining the biophysical properties of wild-type and several mutant channels in *Xenopus* oocytes (Adelman *et al.*, 1995; Zerr *et al.*, 1998; Zuberi *et al.*, 1999; Eunson *et al.*, 2000; Imbrici *et al.*, 2003; Cusimano *et al.*, 2004; D'Adamo *et al.*, 1998, 1999). These studies have shown that EA1 mutations impair hKv1.1 function, although with highly variable effects on aspects of channel assembly, trafficking and kinetics. Recently, a mouse model of EA1 has been generated by introducing the V408A mutation into the mouse Kv1.1 gene. These animals showed impaired motor performance and altered cerebellar GABAergic transmission from the basket cells to Purkinje cells (Herson *et al.*, 2003). Although several lines of evidence indicate that defective delayed rectifier channels and cerebellar dysfunction account for some of the symptoms displayed by affected individuals, the molecular and neurological mechanisms of EA1 are not completely understood (Kullmann *et al.*, 2001).

Shaker-like potassium channels undergo fast N-type inactivation. MacKinnon and co-workers provided a detailed elucidation of the fast N-type inactivation mechanism using crystallographic data (Zhou *et al.*, 2001; Long *et al.*, 2005a, b). Their model shows that the inactivation particle works its way through one of the four lateral side portals that provide access to the pore (Long *et al.*, 2005a, b). The flexible inactivation domain reaches its final binding site by moving into the central cavity located below the selectivity filter (Zhou *et al.*, 2001). The inactivation of delayed-rectifier potassium channels is a physiologically relevant process, as it controls the firing properties of neurones and their response to input stimuli (Hille, 2001). Kv1.1 channels do not show intrinsic fast inactivation. N-type inactivation can be conferred to Kv1.1 channels by ancillary subunits, such as the Kvβ1.x, that provide inactivation particles (Rettig *et al.*, 1994; Majumder *et al.*, 1995; Heinemann *et al.*, 1996). Recently, it has been shown that the EA1 mutations E325D and V408A alter the

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hKvβ1.1-induced inactivation of Kv1.1 homomeric channels (Maylie *et al.*, 2002). However, native A-type potassium channels may result from the assembly of more than one type of Kvα subunit. In fact, detailed biochemical studies indicated that the Kv1.1 may heteropolymerize with the Kv1.2, Kv1.4 and Kv1.6 subunits in specific neurones, such as the CA3 pyramidal cells and cerebellar and hippocampal granule cells (Wang *et al.*, 1993; Veh *et al.*, 1995). Moreover, in previous studies we and others have demonstrated that human Kv1.1 subunits assemble with Kv1.2, generating heteromeric channels with characteristics different from the respective homomers, and which were altered by EA1 mutations (D'Adamo *et al.*, 1999; Rea *et al.*, 2002). Kv1.1, Kv1.4 and Kvβ1.x subunits may form heteromeric channels in the substantia nigra, globus pallidus and cortical interneurones (Rhodes *et al.*, 1997). In particular, a growing body of evidence suggests that Kv1.1, Kv1.4 and Kvβ1.1 subunits form heteromeric channels at hippocampal mossy fibre boutons (Sheng *et al.*, 1992; Rhodes *et al.*, 1997; Roeper *et al.*, 1997; Monaghan *et al.*, 2001; Trimmer & Rhodes, 2004; Schulte *et al.*, 2006). Indeed, patch-clamp recordings from such presynaptic terminals suggested that this macromolecular channel complex regulates the activity-dependent spike broadening of hippocampal mossy fibre boutons and, as a consequence, the amount of neurotransmitter released during high-frequency stimuli (Geiger & Jonas, 2000). Thus, Kv1.1 subunits may form specific hetero-oligomeric complexes which, in turn, modulate the electrical properties of specific nerve structures in unique ways. The functional characterization of complexes of known molecular composition and of the effects of EA1 mutations on the biophysical properties of these channels is therefore critical for understanding the molecular mechanisms underlying EA1. To date, the effects of EA1 mutations on the human Kv1.4-1.1, Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 channel function have not been investigated. Here we show that several EA1 mutations will not only impair the function of homomeric Kv1.1 channel but will also alter the biophysical properties of channels composed of the human Kv1.1 and Kv1.4 subunits with or without the ancillary subunits Kvβ1.1 and Kvβ1.2. In particular, EA1 mutations either in the ion-conducting pore or away from the pore alter the N-type inactivation properties of the channel.

Materials and methods

Molecular biology

The human beta subunits were kindly provided by Professor Olaf Pongs (University of Hamburg), and the hKv1.4 subunit was cloned in our laboratory. To study the activity of channels composed of two hKv1.1 and two hKv1.4 subunits, the relevant cDNAs were linked as dimers (D'Adamo *et al.*, 1998). To concatenate as dimers the hKv1.4 wild-type with the episodic ataxia hKv1.1 subunits, the stop codon of the first subunit was removed and a linker encoding 10 glutamine residues was inserted between the last codon of the 5′ subunit coding sequence and the initiator codon of the following subunit. This was achieved by using a sequential polymerase chain reaction protocol. Briefly, junctions were generated by overlap extension of the polymerase chain reaction primers which also encoded the glutamine linker. The dimers were constructed in pGEMA, a modified version of pGEM9zf- (Promega) with a stretch of 40 A–T base pairs inserted just upstream of the Tth111I site. The nucleotide sequences of all linked subunits were determined throughout the joined segments by automated sequencing. Oligonucleotides were obtained from EURO-BIO (Milan, Italy). All channel subunits were subcloned into the oocyte expression vector pBF, which provides 5′- and 3′ untranslated regions from the *Xenopus* β-globin gene flanking a polylinker containing multiple restriction sites. Capped mRNAs were synthesized *in vitro* by using the SP6 mMESSAGE mMACHINE kit (Ambion).

Oocyte preparation and RNA injection

The care and handling of *Xenopus laevis* were in accordance with the highest standards of institutional guidelines in compliance with both national (D.L. n. 116, G.U., suppl. 40, 18 Feb. 1992) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication N. 85-23, 1985 and Guidelines for the Use of Animals in Biomedical Research, Thromb. Haemost. 58, 1078-1084, 1987). Frogs donated oocytes no more than twice, separated by at least 3 weeks. They were anaesthetized with an aerated solution of 3-aminobenzoic acid ethyl ester (1.3 g/L). Stage V–VI oocytes were isolated and stored at 16 °C in fresh ND96 medium (NaCl, 96 mmol/L; KCl, 2 mmol/L; $MgCl_2$, 1 mmol/L; $CaCl_2$, 1.8 mmol/L; HEPES, 5 mmol/L; gentamicin, 50 μg/mL). hKvα1.4-1.1 and hKvβ1 mRNAs (50 nL) were injected into the oocytes (Nanoject, Drummond, Broomall, PA, USA) in a ratio of 1 : 20–1 : 40. The reagents were supplied by Sigma (Italy).

Electrophysiology

Electrophysiological recordings and data analysis were performed as previously described (D'Adamo *et al.,* 1999; Cusimano *et al.,* 2004). Briefly, two-electrode voltage-clamp recordings were performed from *Xenopus* oocytes at ~22 °C at 1–8 days after injection. A GeneClamp 500 amplifier (Axon Instruments) interfaced to a PC with an ITC-16 interface (Instrutech Corp., NY, USA) was used. Microelectrodes were filled with KCl (3 M) and had resistances of 0.1–0.5 MΩ. The recording solution contained (in mM): NaCl, 96; KCl, 2; $MgCl_2$, 1; $CaCl_2$, 1.8; HEPES, 5, pH 7.4. Currents were evoked by voltage commands from a holding potential of −80 mV as described in the figure legends. The recordings were filtered at 2 kHz and acquired at 5 kHz with PULSE software (HEKA elektronik GmbH, Germany). Data analysis was performed by using IGOR (Wavemetrics), PULSEFIT (HEKA elektronik GmbH) and KALEIDAGRAPH (Synergy Software, USA). Leak and capacitative currents were subtracted using a P/4 protocol. An unpaired Student's test was used to determine the statistical significance, and P-values < 0.05 and < 0.01 were considered significant.

Results

Functional characterization of the human Kv1.4-1.1, Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 channels

We determined the biophysical properties of the human wild-type complexes formed by Kv1.1 and Kv1.4, with and without Kvβ1.1 or Kvβ1.2 subunits, as these parameters have not been thoroughly characterized. In order to study a homogeneous population of heteromeric channels composed of two α subunits of each type, we concatenated the C-terminus of Kv1.4 with the N-terminus of Kv1.1 using a flexible glutamine linker (Fig. 1A). The Kv1.4 α subunits possess an inactivation particle, located at the N-terminus, that confers so-called N-type inactivation properties to the channel (Ruppersberg *et al.*, 1991; Majumder *et al.*, 1995; McCormack *et al.*, 1995). Thus, the tandemly linked Kv1.4-1.1 channel should possess two freely moving inactivation particles (Fig. 1A). By forming a T1 domain β subunit complex, four α and four β subunits give rise to a channel with the features of A-type potassium channels (Fig. 1B) (Gulbis *et al.*, 1999, 2000). As a consequence, the coexpression of the Kv1.4-1.1

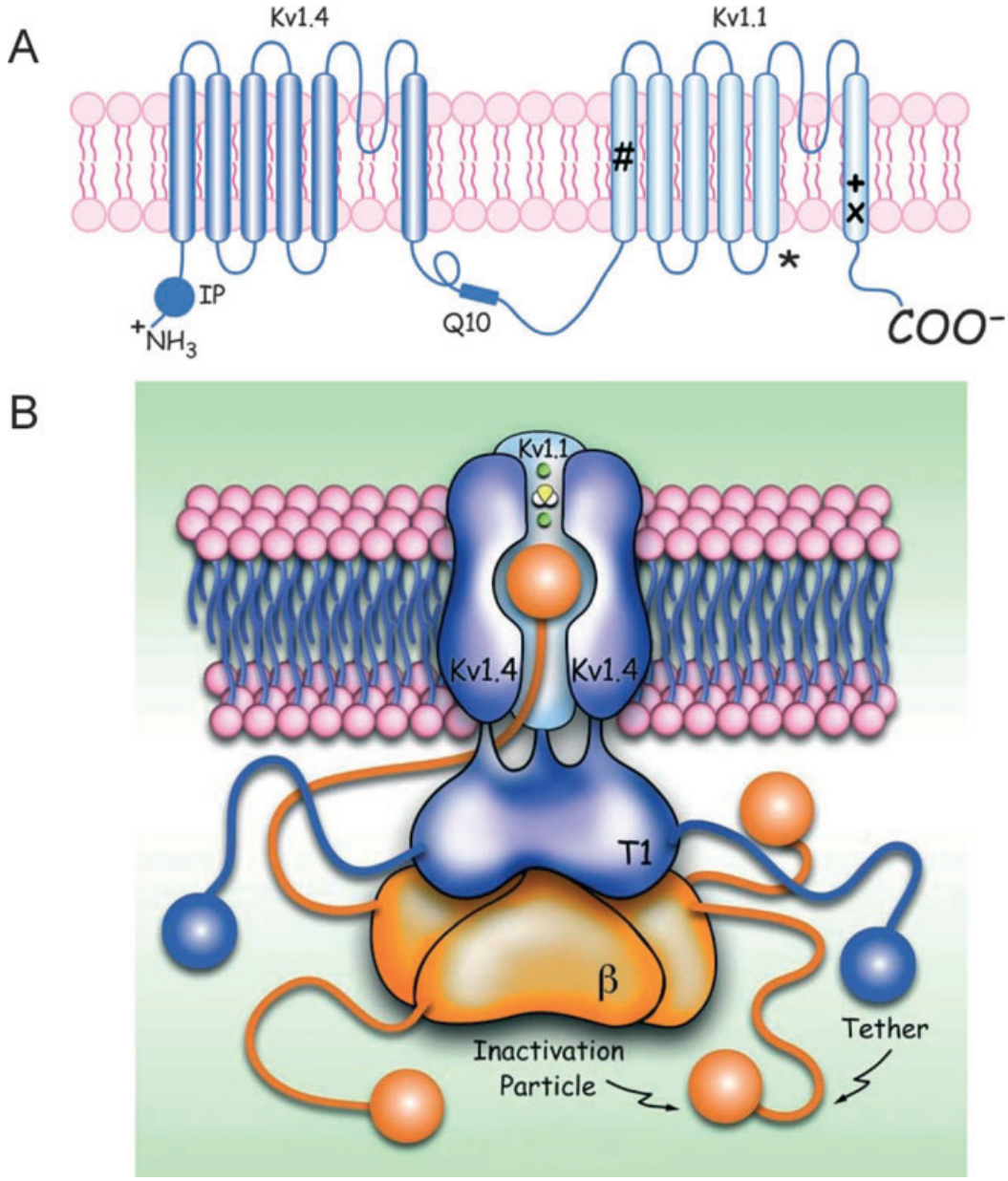

FIG. 1. Schematic representation of the Kv1.4-1.1 dimeric construct and its association with auxiliary subunits. (A) Conventional membrane topology of the human Kv1.4 subunit (blue) linked by 10 glutamines (Q10) to the Kv1.1 subunit (pale blue) as a dimer. The positions of the I177N (#), E325D (*), V404I (+) and V408A (X) mutations are marked. (B) Schematic representation illustrating the heteromeric channel Kv1.4-1.1 associated with the auxiliary subunits (β). Four inactivation particles ('balls and chain') are provided by four Kvβ1.x subunits (orange), which are anchored to the T1 domains of the α subunits. Two inactivation particles (IPs) are provided by the Kv1.4 subunits (blue). Note that the foreground Kv1.1 subunit has been removed for clarity.

construct with the Kvβ1.x subunit in *Xenopus* oocytes results in channels that should possess six freely moving inactivation particles (Fig. 1B). One particle is sufficient to occlude the channel but the availability of several particles renders the mechanism of block more effective. In fact, the inactivation time course of channels that possess only one particle is much slower (MacKinnon *et al.*, 1993).

The heterologous expression of the Kv1.4-1.1 dimer by itself gave rise to functional channels. The Kv1.4-1.1 current families recorded in two-electrode voltage-clamp mode showed a rapid inactivation process not observed when the human Kv1.1 channels were expressed alone (Fig. 2B) (cf. Figure 1 in D'Adamo *et al.*, 1998). To investigate the functional role of auxiliary subunits, the Kv1.4-1.1 and Kvβ1.x mRNAs were coinjected into *Xenopus* oocytes. The current families recorded from these cells showed that Kv1.4-1.1 inactivation was further enhanced by coexpressing the human Kvβ1.1 and Kvβ1.2 subunits (Fig. 2C and D). To quantify the rate of fast inactivation, single exponential functions were fitted to the Kv1.4-1.1 decaying current traces recorded at +60 mV; the Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 currents were best described by a sum of two exponentials. The presence of additional inactivated states of the channel, upon coexpression with beta subunits, probably could account for the additional component of current decay. The inactivation time constants and relative contributions are listed in Table 1. This analysis showed that Kvβ1.1 subunits markedly decreased the average inactivation time constant of Kv1.4-1.1 channels at +60 mV (Table 1). Thus, the greater the number of inactivating particles present in the channel the faster the time course of inactivation (MacKinnon *et al.*, 1993; Rettig *et al.*, 1994; Heinemann *et al.*, 1996). The average fast time constants

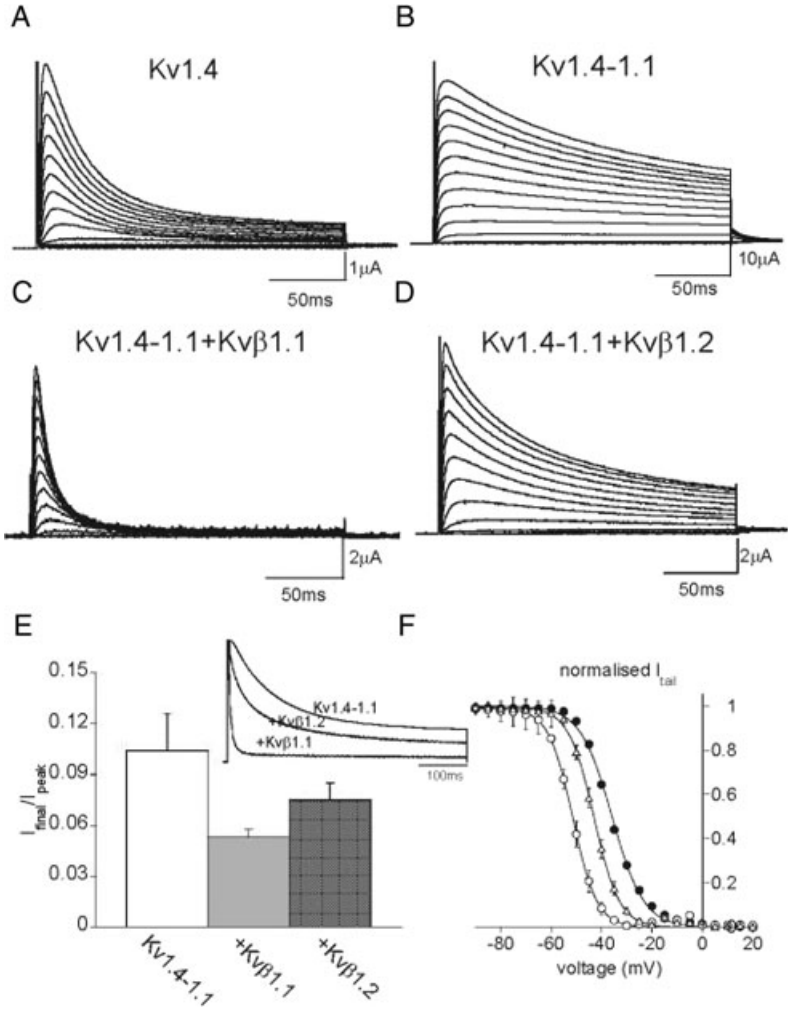

FIG. 2. Functional characterization of the hetero-dimeric channel Kv1.4-1.1 with and without auxiliary subunits. (A–D) Representative current traces recorded from *Xenopus* oocytes expressing the indicated channels. The membrane potential was held at −80 mV and the current families were evoked by 200-ms depolarizing pulses from −60 to +60 mV. (E) Bar graph of the final to peak current ratios for the Kv1.4-1.1 channel expressed without and with auxiliary subunits (+ Kvβ1.x; test potential +60 mV). The inset shows the normalized and overlaid current traces recorded at +60 mV (1000-ms pulse) from oocytes expressing the indicated channels. (F) Voltage dependence of inactivation for Kv1.4-1.1 (filled circles), Kv1.4-1.1/Kvβ1.1 (open circles) and Kv1.4-1.1/Kvβ1.2 (open triangles). The solid curves show the Boltzmann fits of the data points from which the $V_{1/2}$ and the slope factor k for the steady-state inactivation were calculated.

of Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 channel inactivation were identical (Table 1). By contrast, the slow time constant of Kv1.4-1.1/Kvβ1.1 was ∼3.8-fold smaller than Kv1.4-1.1/Kvβ1.2 channels. Moreover, the amplitude of the slow component of Kvβ1.1-induced inactivation (14%) was markedly smaller than that of Kvβ1.2-induced inactivation (72%). These results indicate that Kvβ1.1 and Kvβ1.2 subunits regulate N-type inactivation processes differently. However, the molecular mechanisms underlying these distinct effects are unclear. We next quantified the degree of inactivation as the ratio of final (at the end of the 1000-ms depolarizing pulse at +60 mV) to peak current amplitude. Figure 2E shows the amount of inactivation for Kv1.4-1.1, Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 current. This analysis indicates that Kvβ1.1 subunits inactivate the channels more efficiently than Kvβ1.2.

The voltage dependence of inactivation (steady-state inactivation) for Kv1.4-1.1 alone or when coexpressed with the Kvβ1.x subunits was then constructed (Fig. 2F). Steady-state inactivation was determined by measuring the amplitude of the outward current elicited during 100-ms depolarizing steps to +40 mV after 1-s prepulse potentials from −100 to +40 mV (5-mV increments). The steady-state inactivation curves for Kv1.4-1.1, Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 channels were steeply voltage dependent and showed 50% inactivation at −35.8, −51.4 and −42.0 mV, respectively (Fig. 2F; Table 1). These results demonstrated that the Kvβ1.1 and Kvβ1.2 subunits reduce the availability of channels by shifting the voltage dependence of Kv1.4-1.1 channel inactivation negatively. As a consequence, it is expected that each channel type confers distinct electrical properties to the nerve cells in which it is expressed by regulating the proportion of channels that are available to open.

To determine the refractory period of these channel types, the recovery from inactivation was estimated with a double-pulse protocol where the two steps (elicited from a holding potential of −80 to +40 mV for 100 ms) were separated by an interpulse interval of increasing duration (Fig. 3A–C). Figure 3D shows the amount of inactivation normalized and plotted as a function of the interpulse duration. The data points were fitted with a single exponential function and the time constants of the recovery from N-type inactivation were calculated from these fits. This analysis showed that Kvβ1.1 slowed the recovery from inactivation of Kv1.4-1.1 approximately fivefold, suggesting that the availability of this channel type is reduced by the

TABLE 1. Voltage dependence and kinetics of the Kv1.4-1.1 wild-type and mutated channels coexpressed with auxiliary subunits

	Kinetics of inactivation and recovery from inactivation						
	Voltage-dependence of inactivation		$\tau_{inactivation}$ fast component		$\tau_{inactivation}$ slow component		
Construct	*V1/2* (mV)	*K* (mV)	(ms)	(%)	(ms)	(%)	$\tau_{recovery}$ (ms)
WT	35.8 ± 0.2	6.3 ± 0.2	81 ± 13	–	–	–	1.2 ± 0.1
I177N	−13.6 ± 0.2**	7.1 ± 0.2*	152 ± 23*	–	–	–	1.1 ± 0.1
E325D	−26.1 ± 0.4*	7.3 ± 0.3	200 ± 29*	–	–	–	1.1 ± 0.2
V404I	−30.6 ± 0.3	7.7 ± 0.3*	130 ± 18*	–	–	–	2.0 ± 0.2
V408A	−34.1 ± 0:1	7.4 ± 0.3	121 ± 11*	–	–	–	0.96 ± 0.04
WT+β1.1	−51.4 ± 0.3	4.9 ± 0.3	7.6 ± 0.3	(86)	35.7 ± 2.2	(14)	4.8 ± 0.4
I177N+β1.1	−34.4 ± 0.1**	5.5 ± 0.1	10.9 ± 0.7*	(85)	61.9 ± 5.6*	(15)*	1.7 ± 0.2**
E325D+β1.1	−42.2 ± 0.1**	4.2 ± 0.1	10.6 ± 0.6**	(78)**	47.1 ± 3.3*	(22)*	1.2 ± 0.1**
V404I+β1.1	−39.5 ± 0.1*	5.8 ± 0.1	16.0 ± 0.5**	(84)**	100 ± 22*	(16)*	2.8 ± 0.2*
V408A+β1.1	−49.8 ± 0.7	6.9 ± 0.6	9.6 ± 0.5*	(83)*	43.9 ± 1.9*	(17)*	1.1 ± 0.2**
WT+β1.2	−42.0 ± 0.2	5.0 ± 0.2	7.6 ± 0.3	(28)	137 ± 5	(72)	2.5 ± 0.3
I177N+β1.2	−27.0 ± 0.1**	5.8 ± 0.1	8.0 ± 0.3	(43)	110 ± 2**	(57)**	2.8 ± 0.2
E325D+β1.2	−23.7 ± 0.3**	8.0 ± 0.3**	9.3 ± 0.4*	(14)*	185 ± 1*	(86)*	0.9 ± 0.1**
V404I+β1.2	−38.5 ± 0.2*	5.2 ± 0.1	11.8 ± 0.7**	(50)**	137 ± 9	(50)	3.6 ± 0.4*
V408A+β1.2	40.0 ± 0.2	7.2 ± 0.2*	14.5 ± 1.0**	(13)**	171 ± 6**	(87)**	1.2 ± 0.1*

The $V_{1/2}$ and k were calculated from the Boltzmann fits and represent the midpoint inactivation voltage and the slope factor. The values in parentheses are the mean amplitude of the fast and slow component expressed as percentages. The statistical significance was determined by using an unpaired Student's test. *$P < 0.05$; **$P < 0.01$. The data are means ± SEM of 6–12 cells.

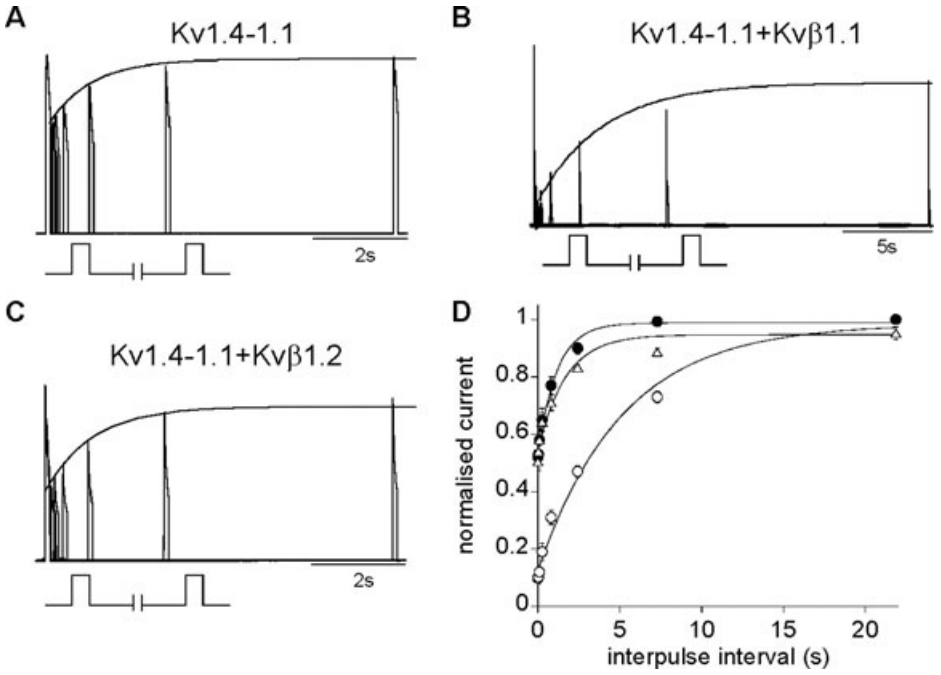

FIG. 3. The Kvβ1.1 subunits slow the recovery from inactivation. (A–C) Representative current traces showing the recovery of the indicated channels from fast inactivation which was estimated by using the two-pulse protocol (the voltage pulses are shown below each trace; note the different time scales). The solid curves indicate the fit of the peak-current amplitudes elicited by the second pulse. (D) Time course of the recovery from inactivation for Kv1.4-1.1 (filled circles), Kv1.4-1.1 + Kvβ1.1 (open circles) and Kv1.4-1.1 + Kvβ1.2 channels (open triangles). The current amplitude evoked by the second pulse (test) was divided by the first pulse (conditioning) and plotted as a function of the interpulse interval. The solid curves indicate the fit of the data points with a single exponential function from which the $\tau_{recovery}$ was calculated. Data are means ± SEM of 6–12 cells.

Kvβ1.1 subunit, whereas Kvβ1.2 did not slow the recovery as markedly and therefore it affects channel availability less (Fig. 3; Table 1). Furthermore, a slower inactivation-peptide off rate suggests that the inactivated state of Kv1.4-1.1/Kvβ1.1 is relatively more stable than that of Kv1.4-1.1/Kvβ1.2 channel.

The action potential frequency of some nerve cells can be > 100 Hz. High-frequency firing that lasts several seconds increases the amplitude and extent of the inactivation induced by Kv1.4, Kvβ1.1 and Kvβ1.2 as it enhances the recruitment of channels to the inactivated state. Therefore, we determined the inactivation properties of these channel types during a train of test pulses that mimic high-frequency firing. In detail, currents were evoked by a train of 3-ms depolarizing pulses to +40 mV with 25-ms interpulse intervals between steps. This train of impulses caused a progressive decay of

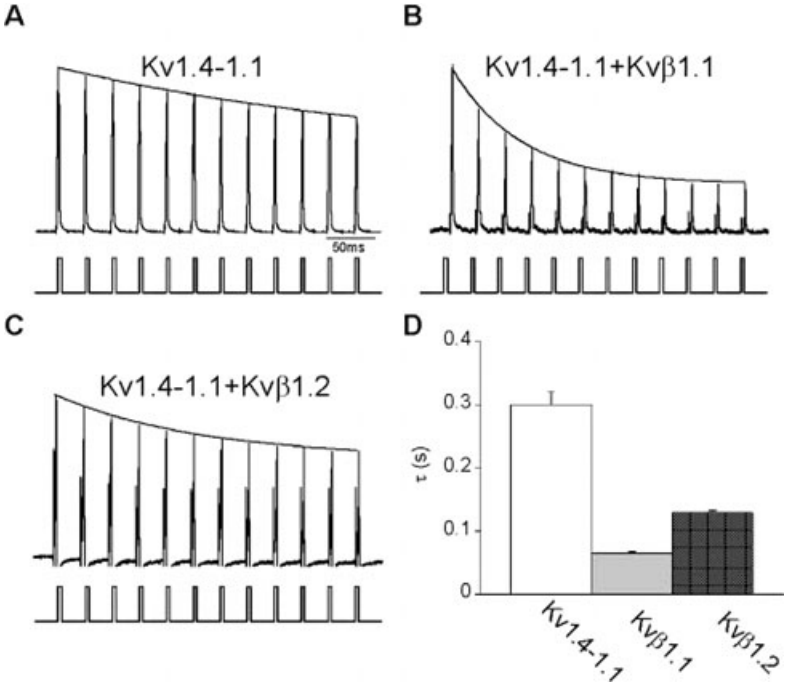

FIG. 4. Kv1.4-1.1/Kvβ1.x inactivation induced by a train of depolarizing pulses. (A–C) Representative current traces elicited by a train of pulses to +40 mV for 3 ms delivered every 25 ms (holding potential −80 mV) from oocytes expressing the indicated channels. (D) Mean decay time constants for Kv1.4-1.1, Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 channels. The decay time constant was calculated by fitting the peak current amplitudes with a single exponential function. Data are means ± SEM of 6–12 cells.

the current amplitudes that was more rapid for Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 than for Kv1.4-1.1 channels (Fig. 4). To quantify this phenomenon, single exponential functions were fitted to the decaying peak current amplitudes. Figure 4D shows that the time constants of the cumulative inactivation for Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 channels were 4.3- and 1.5-fold faster than Kv1.4-1.1, respectively.

Taken together, these results show that the Kv1.4 subunits confer fast inactivation properties to heteromeric Kv1.4-1.1 channels. Furthermore, the human Kvβ1.1 and Kvβ1.2 subunits modulated the functional properties of Kv1.4-1.1 similarly by (i) increasing the rate and amount of N-type inactivation, (ii) shifting differently the midpoint inactivation voltage to more negative potentials, further regulating the proportion of channels that are available to open and (iii) accelerating the cumulative inactivation of the channel. However, the effects exerted by the Kvβ1.1 subunits were more pronounced than those of Kvβ1.2 and showed the distinctive feature of greatly slowing the recovery rate from inactivation. Therefore, these heteromeric channels probably influence the excitability of the cell membranes in which they are expressed in distinct ways, affecting properties such as the action potential duration, firing pattern and responses of the neuron to input stimuli (Hille, 2001).

Functional effects of episodic ataxia type 1 mutations on Kv1.4-1.1, Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 channels

To investigate whether EA1 mutations alter the functional properties of Kv1.4-1.1, Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 channels, the Kv1.4 subunit was linked as a dimer with the Kv1.1 subunit carrying a distinct EA1 mutation (Fig. 1A). We first studied the effects of the E325D, V404I and V408A mutations, which are located within the ion-conducting pore (Fig. 1A) and impair several gating properties of homomeric channels (Adelman *et al.*, 1995; D'Adamo *et al.*, 1998; Zerr *et al.*, 1998). We also characterized channels carrying the I177N mutation, which resides within the S1 segment and therefore should not alter the fast inactivation kinetics (Fig. 1A). Four constructs were generated: Kv1.4-1.1E325D, Kv1.4-1.1V404I, Kv1.4-1.1V408A and Kv1.4-1.1I177N. The expression of equal amounts of concatenated wild-type and EA1 mRNAs in *Xenopus* oocytes yielded mean current amplitudes that were not statistically different (not shown). Considering our previous studies, we suspect that this effect may result from the tandem concatenation of the two genes. Figure 5 shows whole-cell currents recorded from oocytes expressing the four constructs alone or coexpressing the auxiliary subunits. These representative current traces, overlaid with the wild-type (dashed traces), clearly show that every mutation significantly affected the rate and degree of N-type inactivation induced by the Kv1.4, Kvβ1.1 and Kvβ1.2 subunits. To quantify these effects, the decaying current traces for the mutated Kv1.4-1.1 dimers were fitted with single exponential functions, whereas the currents recorded from oocytes coexpressing the mutated dimers with Kvβ1.1 or Kvβ1.2 were best described with a sum of two exponentials. These time constants were plotted as a bar chart, which showed that the time constants for the heteromeric Kv1.4-1.1 channels carrying the E325D, V404I, V408A and I177N mutations were 1.5–2.5-fold slower than the wild-type (Fig. 6A; Table 1). Again, the respective fast and slow components of the time constants for the Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 channels carrying the EA1 mutations were slower than those of wild-type channels (Fig. 6A; both components are listed in Table 1). Moreover, the amplitude of the slow component of the inactivation induced by the Kvβ1.2 subunits was larger than the fast component as was previously noticed for wild-type channels. The degree of inactivation for each channel type was quantified as the ratio of final to peak current amplitude (see above for

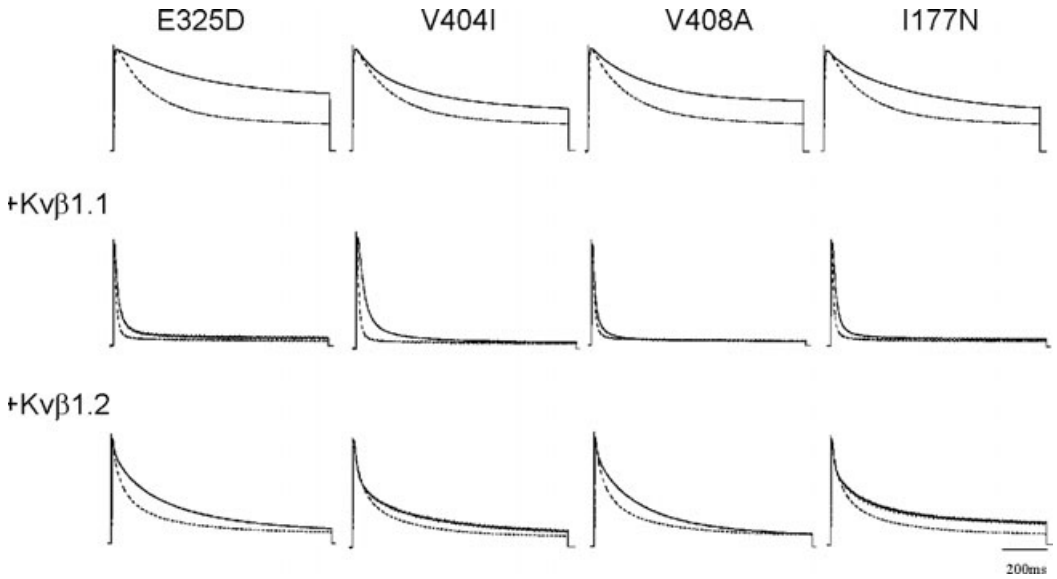

FIG. 5. N-type inactivation induced by Kv1.4, Kvβ1.1 and Kvβ1.2 subunits in channels carrying episodic ataxia type 1 mutations. Representative current traces recorded from oocytes expressing the dimeric channels Kv1.4-1.1E325D, Kv1.4-1.1V404I, Kv1.4-1.1V408A and Kv1.4-1.1I177N by itself (top traces) and coexpressed with Kvβ1.1 (central traces) or with Kvβ1.2 (bottom traces). Currents were evoked by depolarizing pulses at +40 mV for 1000 ms from a holding potential of −80 mV (the peak current amplitudes were normalized to 1). The superimposed dashed traces show the inactivation time course of the relevant wild-type channels for comparison.

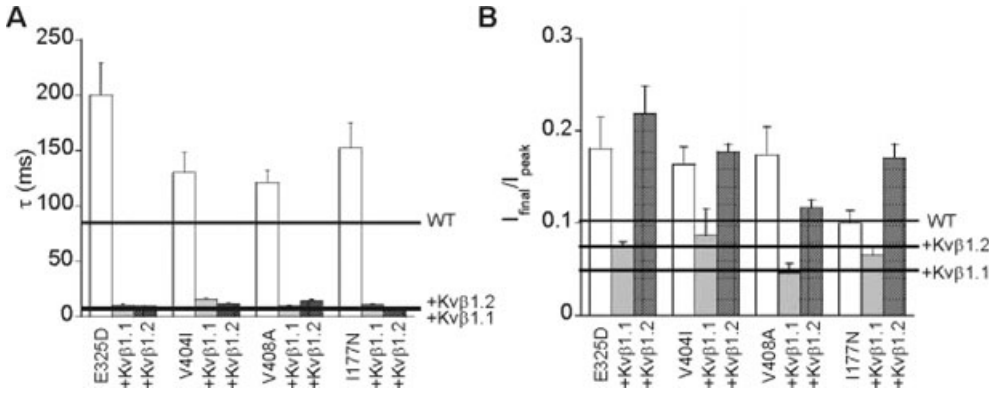

FIG. 6. Episodic ataxia type 1 mutations slow the inactivation kinetics and decrease the amount of current undergoing inactivation. (A) Bar graph of the inactivation time constant calculated by fitting the decaying current traces recorded at +60 mV (200-ms step) with a single exponential for the mutated dimer expressed by itself or coexpressed with the auxiliary subunits (+ Kvβ1.x). The currents resulting from the coexpression with Kvβ1.x required a double exponential for best fitting and the fast component was plotted. (B) Bar graph of the ratio of final to peak current determined at +60 mV for the indicated channels. The horizontal solid lines indicate the mean values for the Kv1.4-1.1 wild-type channels (WT) alone and coexpressed with Kvβ1.1 or Kvβ1.2 (+ Kvβ1.x). Data are means ± SEM of 6–12 cells.

details). Figure 6B shows that the EA1 mutations significantly decreased the amount of current undergoing inactivation, both in the presence and absence of auxiliary subunits.

We then investigated the effects of the EA1 mutations on the voltage dependence of the steady-state inactivation of Kv1.4-1.1 channels with or without the Kvβ1.1 and Kvβ1.2 subunits (Fig. 7; Table 1). The results showed that the E325D, V404I and I177N mutations shifted the half-maximal inactivation voltage by several millivolts to more positive potentials (Fig. 7; Table 1). By contrast, the V408A mutation did not alter this parameter (Fig. 7; Table 1).

A double-pulse protocol was used, as detailed above, to assess the recovery from inactivation of Kv1.4-1.1E325D, Kv1.4-1.1V404I, Kv1.4-1.1V408A and Kv1.4-1.1I177N channels expressed with and without the auxiliary subunits. Figure 8 shows the relative recovery from inactivation plotted as a function of the interpulse interval for each channel type and fitted with single exponential functions. The recovery time constants for the mutated Kv1.4-1.1 channels expressed singly were not significantly different from those of wild-type channels (Fig. 8; Table 1). By contrast, in the presence of the Kvβ1.1 and Kvβ1.2 subunits, all mutations, except Kv1.4-1.1V404I/Kvβ1.2, speeded the recovery from inactivation of the channel (Fig. 8; Table 1). These results suggest that EA1 mutations alter the recovery from the inactivation induced by ancillary subunits more than that induced by Kv1.4 subunits. All of the EA1 mutations also greatly affected the cumulative inactivation properties of the channel determined by delivering a train of depolarizing pulses, as described above. In fact, they significantly increased the decay time constants of Kv1.4-1.1, Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.1 channels (Fig. 9).

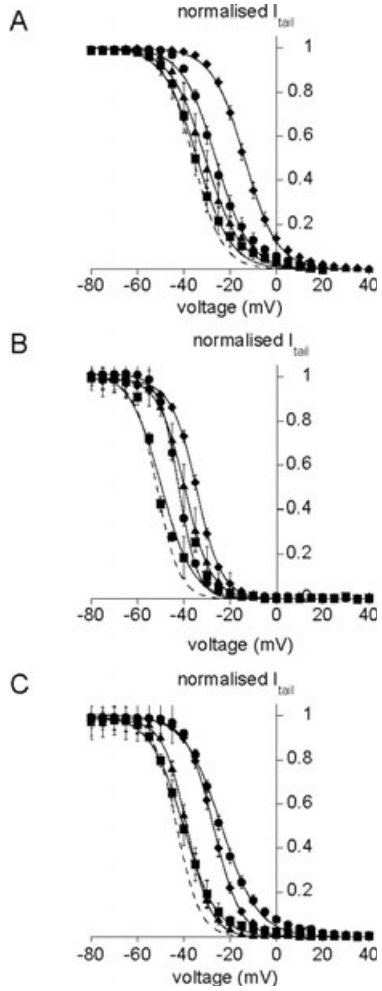

FIG. 7. The episodic ataxia type 1 mutations E325D, V404I and I177N positively shift the voltage dependence of heteromeric channel inactivation. The voltage dependence of the inactivation for the Kv1.4-1.1 channels either by themselves (A) or coexpressed with Kvβ1.1 (B) and Kvβ1.2 subunits (C). The dashed curves indicate the steady-state inactivation relationship of the relevant wild-type channels for direct comparison with those of E325D (circles), V404I (triangles), V408A (squares) and I177N (diamonds). The data were obtained as described in Fig. 2F and are plotted as means ± SEM of 6–12 cells.

Discussion

Here we have sought to characterize the functional properties of channels composed of the human Kv1.1 and Kv1.4 subunits in the absence and presence of auxiliary subunits such as human Kvβ1.1 and Kvβ1.2. We have demonstrated that the hetero-oligomeric assemblies of these subunits generate channels with distinct gating properties that are profoundly modified by several mutations identified in the *KCNA1* gene of EA1-affected patients.

Neurophysiological and disease implications

Kv1.1, Kv1.4 and Kvβ1.x subunits form the A-type potassium channel. These channel types control the firing properties of neurones and their response to input stimuli (Connor & Stevens, 1971). The functional properties of Kv1.4-1.1/Kvβ1.1 showed some similarities with the potassium currents characterized by Geiger & Jonas (2000) in hippocampal mossy fibre boutons. However, some biophysical parameters such as the inactivation $V_{1/2}$ and refractory period are not exactly identical. These discrepancies may result from different methodological approaches, temperatures at which the recordings are carried out (34 vs. 20 °C), channels with different molecular identities (rat vs. human potassium channel; association with Lgi1 protein; Schulte *et al.*, 2006) and channel modulation occurring within the boutons, etc. In fact, N-type inactivation can be modulated by the redox state of the cell (Ruppersberg *et al.*, 1991), phosphorylation or microfilaments via direct interactions with cytoskeletal associated proteins and via G-protein βγ subunits (Levin *et al.*, 1996; Jing *et al.*, 1997). Some of these modulatory pathways may be initiated by neurotransmitter receptors, such as β1-adrenergic receptors and mGluR1 metabotropic glutamate receptors (Levy *et al.*, 1998). Despite these discrepancies, a growing body of evidence indicates that Kv1.1, Kv1.4 and Kvβ1.1 subunits do form heteromeric channels in hippocampal axons and terminals (Sheng *et al.*, 1992; Rhodes *et al.*, 1997; Roeper *et al.*, 1997; Monaghan *et al.*, 2001; Trimmer & Rhodes, 2004; Schulte *et al.*, 2006). Moreover, it has been shown that Kv1.1, Kv1.4 and Kvβ1.1 subunits are the most likely molecular counterpart of the A-current recorded from hippocampal mossy fibre boutons, which regulates the action potential duration and frequency-dependent action potential broadening of the synaptic terminal (Geiger & Jonas, 2000). Our study provides a detailed biophysical characterization of potassium currents that regulate the activity-dependent spike broadening, synaptic strength and, probably, LTP processes occurring within the hippocampus.

A-type potassium channels with altered gating properties may exert a remarkable impact on the integration properties and firing patterns of the neurons in which they are expressed. In particular, our results demonstrate that EA1 mutations slow down the N-type inactivation of heteromeric channels composed of Kv1.1, Kv1.4 and Kvβ1.1 subunits. Thus, it is expected that the action potential propagation within hippocampal axons and the glutamate release from these terminals might be modified by the mutations. As a consequence, the cognitive symptoms reported by some kindreds of patients with EA1 during attacks of ataxia may result from altered activity-dependent spike broadening and hippocampal synaptic strength (Maylie *et al.*, 2002).

Kv1.1, Kv1.4 and Kvβ1 subunits may also form heteromeric channels in the substantia nigra, globus pallidus and cortical interneurones (Rhodes *et al.*, 1997). Therefore, EA1 mutations probably alter the electrical properties of the neurones residing within these brain structures that regulate the motor activity of human beings. In particular, the propagation of axonal action potentials and their back propagation in dendrites are probably affected by these mutations, as A-type potassium currents regulate these specific nerve cell properties (Debanne *et al.*, 1997; Hoffman *et al.*, 1997).

The accelerated recovery from inactivation and attenuated cumulative inactivation with repeated depolarizations represent a gain of function. However, the increased voltage threshold of activation, faster C-type inactivation (V408A and E325D), increased Zn^{2+} sensitivity and reduced expression levels caused by most EA1 mutations represent a loss of function. In areas of the brain where Kv1.1 coassembles with Kvα1.2 and Kvβ2, such as the axons and terminals of cerebellar basket cells and juxtaparanodal regions in the cerebellar white matter (Rhodes *et al.*, 1997), N-type inactivation does not occur so loss of function is likely to be the dominant phenomenon. These areas are implicated in the genesis of ataxia. Impaired repolarization in basket cell terminals may disturb the normal regulation of Purkinje cell output (D'Adamo *et al.*, 1999; Herson *et al.*, 2003). How paroxysms of ataxia are triggered is unclear but a phenomenon akin to spreading depression (spreading acidification of the cerebellar cortex) has been suggested (Chen *et al.*, 1999; Ebner & Chen, 2003; Ebner

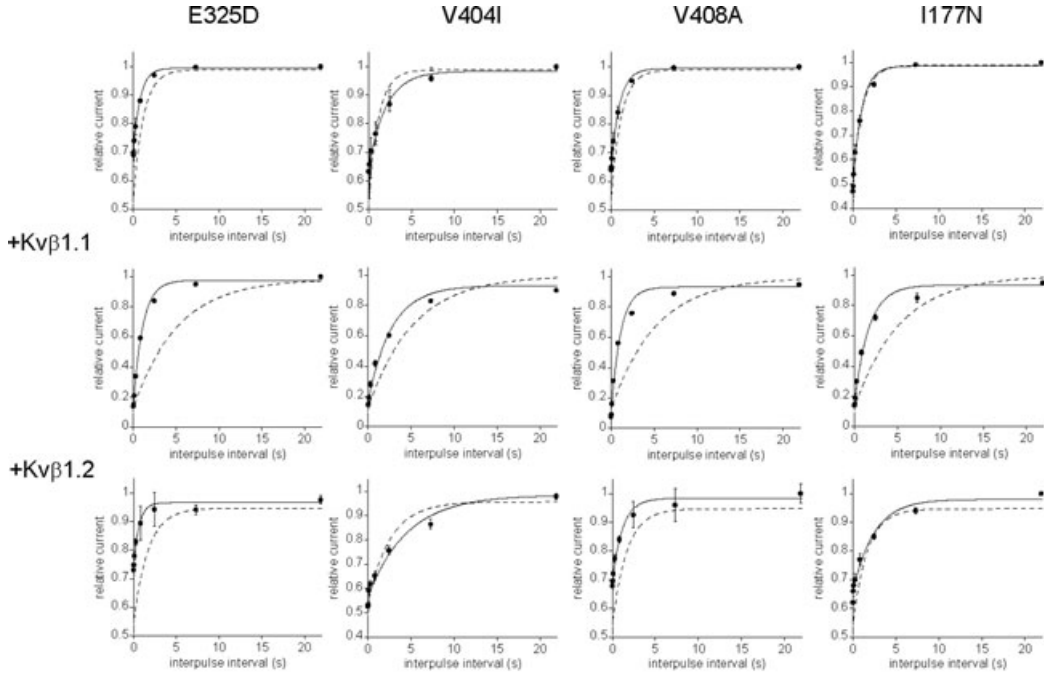

FIG. 8. The episodic ataxia type 1 mutations E325D, V404I, V408A and I177N shorten the refractory period of heteromeric channels. Recovery from inactivation of the mutated dimers expressed alone (top panels) and in the presence of either Kvβ1.1 (central panels) or Kvβ1.2 subunits (bottom panels). The data were obtained and plotted as described in Fig. 3 and fitted with a single exponential function. The dashed traces indicate the recovery from inactivation of the relevant wild-type channels for direct comparison. The data points are means ± SEM of 6–12 cells.

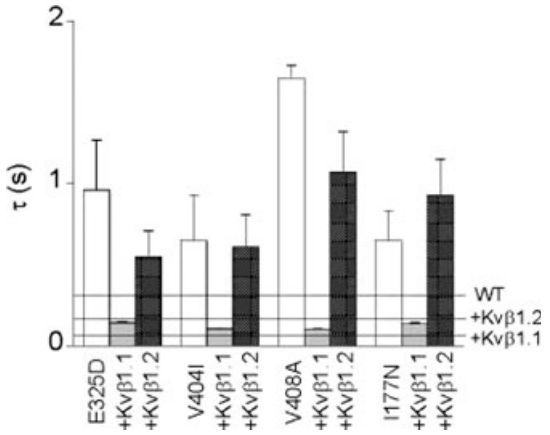

FIG. 9. Episodic ataxia type 1 mutations slow the cumulative inactivation of heteromeric channels. Bar graph of the mean decay time constants for Kv1.4-1.1, Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2 channels determined as described in Fig. 4. The decay time constants were calculated by fitting the decaying peak current amplitudes with a single exponential function. Data are means ± SEM of 6–12 cells. WT, wild-type.

et al., 2005). In contrast, in areas where Kv1.1 coassembles with Kvα1.4 and Kvβ1, the impairment of inactivation may partially mask the loss of potassium channel function. However, the relative weight of these opposite effects is not easy to assess and therefore it is difficult to predict specifically how they will affect the electrical properties of distinct neurons. Direct evidence on how EA1 mutations alter each electrical event occurring within the hippocampus and other brain areas will be provided by the *in vitro* and *in vivo* analysis of wild-type and knock-in mice harbouring EA1 point mutations.

Structure–function effects of episodic ataxia type 1 mutations

The analysis of homomeric single channels carrying either the E325D or V408A mutation showed that they possess reduced open probability, reduced open duration, and unstable open state of the channel (D'Adamo *et al.*, 1998, 1999). Here we found that the heteromeric channels harbouring these mutations are characterized by slower inactivation kinetics and increased rates of recovery from inactivation. It is known that N-type inactivation is due to an open channel block and that the transitions between the open–inactivated–closed state regulate the kinetics of fast inactivation (Zagotta *et al.*, 1990; Rasmusson *et al.*, 1995, 1998; Morales *et al.*, 1996; De Biasi *et al.*, 1997; Jiang *et al.*, 2003; Li *et al.*, 2003; Bett & Rasmusson, 2004; Glenna *et al.*, 2004). Therefore, if the mutated channel possesses an unstable open state, it is reasonable to conclude that the inactivation particle reaches its final binding site, located in the central cavity, with increased difficulty. The expected overall effect would be a slower rate of N-type inactivation, which we observed from macroscopic currents recorded from cells expressing the mutated channels. However, an increased stability in the closed state would favour the release of the inactivation particle and speed up the rate of recovery from inactivation. Indeed, the unbinding kinetics, estimated by measuring the extent of recovery of the mutated channels from inactivation as a function of time between paired pulses, were much faster than the wild-type. Therefore, the impaired inactivation and recovery properties of Kv1.4-1.1/Kvβ1 channels carrying E325D or V408A mutations are probably due to unstable open and inactivated states of the channel (Maylie *et al.*, 2002).

The I177N mutation, which is located within the S1 segment, causes similar defects in fast inactivation. In a previous study, we showed that the I177N mutation accelerates approximately fourfold the deactivation kinetics of the channel, shifts the voltage dependence of activation by ~60 mV to more depolarized potentials and reduces

~2.6-fold the mean open duration of the channel (Imbrici *et al.*, 2003). These results indicate that I177N channels possess a destabilized open state. Thus, the I177N mutation may also affect the inactivation properties of heteromeric channels by altering the open–inactivated–closed transitions. Nevertheless, we cannot exclude that the I177N mutation alters fast inactivation through allosteric modifications of the side portals, altering the predocking site for the inactivation particle before insertion into the inner vestibule, or of the inner vestibule itself. Alternatively, EA1 mutations could alter the 'permeation and allosteric mechanisms' underlying N-type and C-type inactivation coupling (Rasmusson *et al.*, 1995, 1998). Indeed, Rasmusson and co-workers proposed that the EA1 mutation V408A, at an equivalent position of the Kv1.4 channel, modulates inactivation through membrane-spanning mechanisms involving S6 (Li *et al.*, 2003). The S6 helices of the human Kv1.1 contain the amino acid sequence Pro-Val-Pro, which is highly conserved among the *Shaker* family of voltage-gated potassium channels. The Pro-X-Pro motif produces a bend in the S6 helices of *Shaker*-like channels and provides a flexible element within this domain that regulates channel gating (Tieleman *et al.*, 2001; Webster *et al.*, 2004; Long *et al.*, 2005a, b; Sands *et al.*, 2005). Moreover, functional studies indicate that the proline residues are essential for channel gating (Labro *et al.*, 2003). The V404I mutation changes the sequence of the Pro-Val-Pro motif into Pro-Ile-Pro. Our data suggest that the altered inactivation properties of the heteromeric Kv1.4-1.1/Kvβ1 channels harbouring V404I probably result from impaired conformational changes, occurring within the S6 segments, during channel opening and closing. Interestingly, an hKv1.1 RNA editing event that changes Ile400, located upstream of the PXP motif, into a valine also alters the fast inactivation properties of the channel (Bhalla *et al.*, 2004).

In conclusion, the results of this and our previous studies strongly suggest that EA1 mutations in the *KCNA1* gene impact differently depending on which current type is generated by the nerve cell. If homomeric Kv1.1 and heteromeric Kv1.1/Kv1.4/Kvβ1.x coexist in the same neuron then the opposite biophysical effects of the mutation on the delayed-rectifier and A-type current probably impair the distinct nerve cell property regulated by each current type.

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Abbreviations

EA1, episodic ataxia type 1.

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