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Episodic ataxia type-1 mutations in the hKv1.1 cytoplasmic pore region alter the gating properties of the channel

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Episodic ataxia type-1 is a rare human neurological syndrome which occurs during childhood and persists through the whole life of affected patients. Several heterozygous point mutations have been found in the coding sequence of the voltage-gated potassium channel gene hKv1.1 of different affected families. V408A and E325D mutations are located in the cytoplasmic putative pore region of hKv1.1 channels and profoundly alter their gating properties. V408A channels showed increased kinetic rates of activation, deactivation and C-type inactivation. Expression of E325D channels in *Xenopus* oocytes led to an ~13-fold current amplitude reduction and to a 52.4 mV positive shift in the voltage dependence of activation. Moreover, the E325D mutation altered the kinetics of activation, deactivation, C-type inactivation and channel open probability. Heteromeric channels composed of two wild-type and two mutated subunits, linked as dimers, showed gating properties intermediate between channels formed from four normal or four mutated subunits. The results demonstrate that the highly conserved residues Val408 and Glu325 play a pivotal role in several gating processes of a human potassium channel, and suggest a pathogenetic mechanism by which the impairment of the delayed-rectifier function of affected neurons is related to the type and number of mutated subunits which make up the hKv1.1 channels.

Keywords: episodic ataxia type-1/gating/hKv1.1 channels/mutation/potassium channel

Introduction

Episodic ataxia type-1 (EA-1) is a rare, autosomal dominant neurological disorder first described in 1975 by Van Dyke and co-workers. Affected patients present constant muscle rippling movements (myokymia) and episodic attacks of ataxia. Attacks are characterized by imbalance, with jerking movements of the head, arms and legs, and can be triggered by emotional stress or fatigue. Therefore, EA-1 patients tend to avoid abrupt movements or sport activity (Van Dyke *et al.*, 1975; Bouchard *et al.*, 1984; Gancher and Nutt, 1986; Brunt and van Weerden, 1990). The first symptoms manifest during childhood, persist through the whole life and can vary from severe to no detectable neurological abnormalities. Some patients undergo >15 attacks a day of severe ataxia, lasting from minutes to hours; in contrast, other individuals show attack frequencies of <1 per month (Van Dyke *et al.*, 1975). Linkage studies in several EA-1 families led to the discovery of a number of point mutations in the voltage-dependent potassium channel gene *KCNA1* (Kv 1.1), on chromosome 12p13 (Browne *et al.*, 1994, 1995; Litt *et al.*, 1994; Comu *et al.*, 1996). The amino acid residues mutated in EA-1 patients are at positions highly conserved amongst the delayed-rectifier potassium channel genes of several species from *Drosophila melanogaster* to humans (Browne *et al.*, 1994). Patients affected by EA-2, another acetazolamide-responsive disease, present truncating mutations in the *CACNL1A4* gene. Such mutations result in a premature stop codon and aberrant splicing in the P/Q-type calcium channel α1 subunit gene (Ophoff *et al.*, 1996).

By expressing, in *Xenopus* oocytes, hKv1.1 channels (Ramaswami *et al.*, 1990) bearing some EA-1 point mutations, Adelman and co-workers found that V408A channels display faster kinetics of activation, deactivation and C-type inactivation while F184C channels display altered voltage dependence of activation. In contrast, E325D and other mutants did not give rise to functional channels (Adelman *et al.*, 1995).

Immunocytochemical studies have shown that Kv1.1 channel proteins are present in the node of Ranvier of myelinated axons, on the dendrites, synaptic zones and soma of neurons found in the cerebellum and in other mouse brain areas (Wang *et al.*, 1993, 1994). In the peripheral nervous system RCK1 channels, a rat homolog, have been localized in the dorsal root ganglia, sciatic nerves, head nerves, etc. (Beckh and Pongs, 1990). These results suggest that Kv1.1 channels have a role in the saltatory conduction of impulses, repolarizing phase of action potentials, release of neurotransmitter from the terminals and firing pattern of central and peripheral neurons located in regions important for the control of movements.

Voltage-dependent potassium channels are formed by the assembly of four subunits, each an independent polypeptide. These subunits may be the same, generating homomeric channels, or in some cases different subunits may associate, giving rise to heteromeric channels (Christie *et al.*, 1990; Ruppersberg *et al.*, 1990). EA-1-affected individuals are heterozygous at the Kv 1.1 locus, possessing a normal and a mutant allele which may be expressed equally. Therefore, heteromeric channels composed of wild-type and mutated subunits might be formed. In our previous study, we reported that the co-injection into *Xenopus* oocytes of wild-type and V408A mRNAs in a 1:1 ratio resulted in delayed-rectifying potassium currents, with deactivation and C-type inactivation time

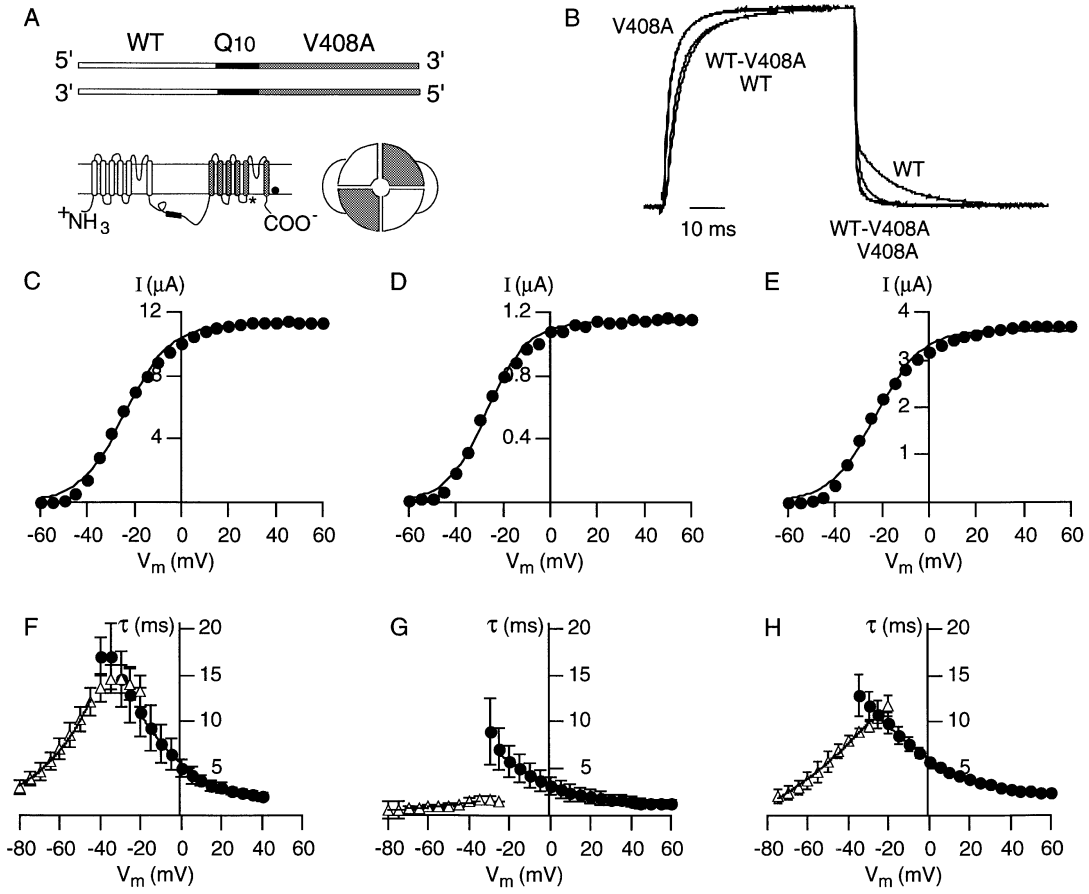

Fig. 1. Kinetics of activation and deactivation for wild-type (WT), V408A and WT–V408A channels. (**A**) Schematic representation of a WT–V408A dimeric construct. Top: wild-type hKv1.1 (open) and V408A (shaded) cDNAs were linked by 30 nucleotides which encoded 10 glutamines (Q_{10}; black). Bottom left: membrane topology of a WT–V408A dimer in which the wild-type (open) C-terminal domain and V408A (shaded) N-terminal domain were linked by 10 glutamine residues (bold). The positions of V408A and E325D mutations are marked (●) and (*) respectively. Bottom right: proposed representation of a tetrameric channel in which two wild-type and two V408A subunits are tandemly assembled. **(B)** Superimposed and normalized current traces recorded at +20 mV from *Xenopus* oocytes expressing hKv1.1 WT, V408A and WT–V408A (middle trace) channels. The peak tail currents of wild-type (**C**), V408A (**D**) and WT–V408A dimer (**E**) were plotted as a function of membrane pre-pulse potentials and fitted with a Boltzmann function: $I = 1/[1+\exp^{-(V-V_{1/2})/k}]$ from which the $V_{1/2}$ and k values were computed. Wild-type (**F**), V408A (**G**) and WT–V408A (**H**) activating and deactivating current traces were fitted with double and single exponential functions, respectively, and the time constants were plotted as a function of membrane potentials and fitted with the equation: $\tau = \tau_{V_{1/2}} \exp^{(V-V_{1/2})/k}$, where $\tau_{V_{1/2}}$ is the time constant at the $V_{1/2}$ of the channels and k is the slope factor for the voltage dependence of the time constants (●: fast Tau of activation; △: Tau deactivation; the data points are mean ± SD of 6–7 cells).

constants different from homomeric wild-type and V408A channels. These results suggested that wild-type and mutated subunits might be assembled to form heteromeric channels with distinct properties (Adelman *et al.*, 1995). To study the gating properties of channels composed of two wild-type and two V408A or two E325D subunits, the relevant cDNAs were linked as dimers. The functional analysis of these molecules expressed in *Xenopus* oocytes demonstrated that V408A and E325D, two EA-1 mutations located in the cytoplasmic putative pore region of Kv1.1 channels (Slesinger *et al.*, 1993; Lopez *et al.*, 1994), markedly altered several gating properties of the channel. In addition, the results suggest that the severity of the delayed-rectifier function impairment in EA-1 patients depends on both the type of mutation and the number of mutant subunits within the tetrameric hKv1.1 channels.

Results

To determine the biophysical parameters of a presumed homogeneous population of heterotetrameric channels which will have two subunits of each type, we concatenated the wild-type and the mutated subunits with a flexible glutamine linker (Figure 1A). The injection into *Xenopus* oocytes of mRNA encoding a dimeric construct in which hKv1.1 wild-type was followed by hKv1.1 V408A (WT–V408A; Figure 1A) resulted in functional delayed-rectifying potassium currents (Figure 1B). To determine the voltage dependence of wild-type, V408A and WT–V408A channels, current families were evoked from a holding potential of –80 mV, by 500 ms depolarizing commands from +60 to –70 mV in –5 mV decrements. The membrane potential was then repolarized to –50 mV (wild-type) and –45 mV (V408A and WT–V408A) to record tail currents. The peak amplitudes of tail currents were plotted as a function of the pre-pulse potential and fitted with a Boltzmann function from which the $V_{1/2}$ and k (slope factor) values were computed (Figure 1C–E; Table I). These results confirmed that V408A channels have a normal voltage dependence of activation, as is also true for the WT–V408A dimer. Our previous study showed that the rates of activation and deactivation of V408A

Table I. Kinetics and voltage-dependent parameters of wild-type, V408A and E325D homomeric and heteromeric channels

	WT	WT–WT	V408A	WT–V408A	E325D	WT–E325D
Voltage dependence						
$V_{1/2}$ (mV)	–28.5 ± 2.3	–27.0 ± 2.9	–27.1 ± 1.1	–26.7 ± 1.2	29.4 ± 1.9	–0.7 ± 3.6
k (mV)	6.0 ± 0.5	5.8 ± 0.6	6.3 ± 0.8	6.6 ± 0.7	24.0 ± 1.0	12.7 ± 1.4
Activation						
τ $V_{1/2}$ (ms)	14.0	12.2	6.9	10.8	2.2	8.5
k (mV)	29.9	30.5	39.6	47.7	72.5	50.0
Deactivation						
τ $V_{1/2}$ (ms)	23.7	22.7	1.8	12.6	2.3	10.5
k (mV)	26.3	25.8	43.0	27.8	59.1	34.2
C-type inactivation						
τ (s)	9.6 ± 0.9	10.9 ± 1.5	2.2 ± 0.2	7.9 ± 1.3	1.9 ± 0.1	6.1 ± 0.7
I_{final}/I_{peak}	0.64 ± 0.03	0.56 ± 0.06	0.14 ± 0.02	0.46 ± 0.06	0.52 ± 0.02	0.42 ± 0.03

The voltage-dependent parameters were computed as described in Figure 1. The C-type inactivation time constants and amount, expressed as the ratio of I_{final}/I_{peak}, were computed at +20 mV. The data are the mean ± SD of 5–7 cells.

channels were faster compared with wild-type channels (Adelman *et al.*, 1995). To compare the kinetics of activation and deactivation for wild-type, V408A and WT–V408A channels, activating and deactivating current traces were fitted with double and single exponential functions, respectively. The plots of time constants as a function of membrane potential (Figure 1F–H) confirm that the activation and deactivation of V408A channels are faster, and clearly show that the kinetics of activation for WT–V408A channels were very similar to those for wild-type channels. These results suggest that the presence of two wild-type subunits within a tetramer almost completely normalizes the kinetics of channel activation (Figure 1B). On the other hand, the deactivation kinetics were intermediate between wild-type and V408A channels (Table I).

Val408 resides in the C-terminal region of transmembrane domain 6 (TM6), which is thought to comprise part of the cytoplasmic vestibule of the pore (Choi *et al.*, 1993; Lopez *et al.*, 1994). V408A channels undergo a much faster and voltage-independent C-type inactivation process than wild-type channels (Figure 2; Adelman *et al.*, 1995). Single exponential fits to inactivating currents, evoked by 10 s depolarizing pulses between +40 and –20 mV, demonstrated that V408A time constants are markedly faster than those of the wild-type. In addition, >80% of the V408A current inactivates at all tested voltages, compared with <40% of wild-type currents (Table I, Adelman *et al.*, 1995). The C-type inactivation time constants and the amount of inactivating current for WT–V408A channels were intermediate between the homotetrameric wild-type and V408A channels (Figure 2; Table I). These results suggest that the effects of V408A mutation on the C-type inactivation process is proportional to the number of mutated subunits within the tetrameric channel.

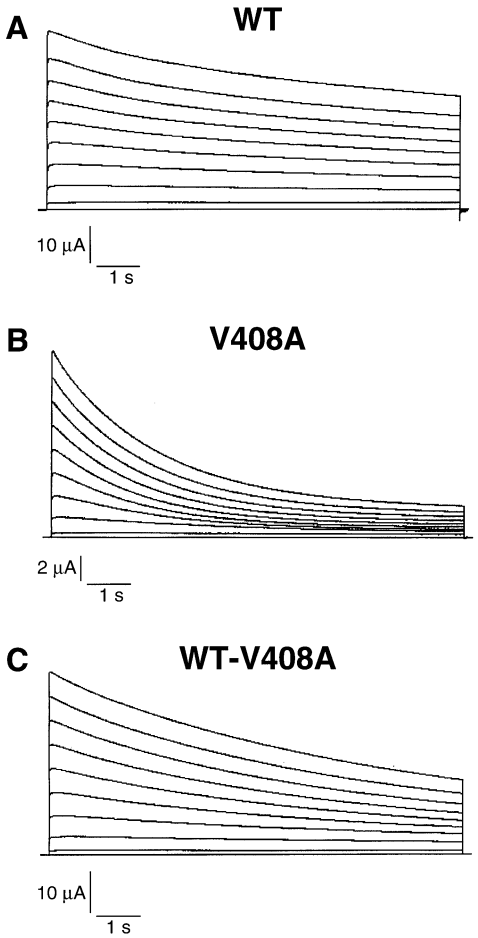

Fig. 2. Kinetics of C-type inactivation for wild-type, V408A and WT–V408A channels. Representative current families were recorded from oocytes injected with wild-type (**A**), V408A (**B**) and WT–V408A (**C**) mRNAs and evoked by 10 s depolarizing commands from +40 to –20 mV, every 10 mV.

Expression and kinetic properties of hKv1.1 WT–WT dimer

To determine whether the process of concatenating two subunits affects the biophysical parameters of the channel, we linked wild-type subunits as a dimer (WT–WT). The expression of WT–WT mRNA in *Xenopus* oocytes produced potassium channels with voltage dependence and kinetics of activation, deactivation and C-type inactivation indistinguishable from those of wild-type channels (Table

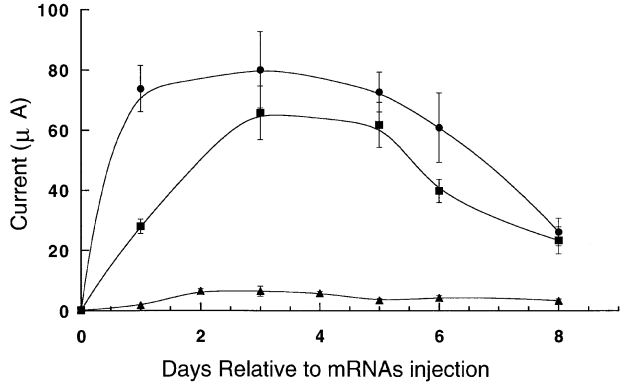

Fig. 3. Expression kinetics for wild-type, E325D and WT:E325D channels in *Xenopus* oocytes. Steady-state whole-cell currents amplitudes, recorded at +30 mV, were plotted as a function of days after the injections of wild-type (●), E325D (▲) and WT:E325D (■) mRNA into *Xenopus* oocytes drawn from the same frog (data points are mean ± SEM; $n = 6$).

I). These results demonstrated that the gating properties of the channel were not altered by the flexible 10 glutamine residues joining the subunits.

E325D mutation reduces current amplitudes and alters the gating of hKv1.1 channels

Glu325 resides in the loop linking the TM4 to TM5, which is thought to comprise part of the cytoplasmic mouth of the ion-conducting pore (Figure 1A; Slesinger *et al.*, 1993). hKv1.1 channels bearing the E325D mutation previously were reported not to form functional channels (Adelman *et al.*, 1995); in contrast, with *Shaker* channels from *Drosophila* the same mutation does form functional channels (Isacoff *et al.*, 1991; Slesinger *et al.*, 1993). To increase mRNA stability and translation efficiency, wild-type and E325D cDNAs were subcloned into the oocyte expression vector pBF (courtesy of Dr B.Fakler), which provides the 5′- and 3′-untranslated regions from the *Xenopus* β-globin gene. The injection into *Xenopus* oocytes of 5 ng of E325D mRNA resulted in an average whole-cell current amplitude, recorded at the peak day of expression, ~13-fold smaller than of oocytes injected with the same amount of wild-type mRNA (Figure 3). The co-injection of 2.5 ng of E325D and 2.5 ng of wild-type mRNAs resulted in an ~1.2-fold current reduction. Moreover, the time course of wild-type channel expression was much faster than of E325D or of channels composed of wild-type and E325D subunits (Figure 3).

To determine the voltage dependence of E325D channel activation, tail current amplitudes were recorded at 0 mV and plotted as a function of the pre-pulse potentials. The analysis of Boltzmann fits to E325D current–voltage data points revealed that the $V_{1/2}$ for E325D channel activation was shifted by 52.4 mV to more positive potentials, and that the slope factor k was increased ~3-fold compared with that of wild-type (Figure 4B; Table I).

WT–E325D dimers were constructed to determine the gating properties of channels composed of two wild-type and two E325D subunits. The injection of WT–E325D mRNA produced functional channels, and the analysis of the current–voltage relationships revealed that the $V_{1/2}$ for activation of WT–E325D channels was shifted by 22.1 mV to more positive potentials and the slope factor k was increased ~2-fold, compared with that of wild-type (Figure 4B; Table I). These results demonstrate that E325D channels have an altered voltage dependence and that their activation requires much stronger depolarizations than wild-type channels. The presence of two wild-type subunits in the tetramer shifted its voltage dependence ~50% towards the wild-type values, suggesting that the effect of E325D mutation on this parameter is proportional to the relative number of mutated subunits within the channel.

To determine the kinetics of activation and deactivation of wild-type, E325D and WT–E325D channels, activating and deactivating current traces were best fitted with double and single exponential functions, respectively. The plots of the time constants as a function of membrane potential showed that the kinetics of activation and deactivation of E325D channels were ~10-fold faster than those of wild-type channels (Figure 4A and C). These results suggest that at the resting potential of a neuron, E325D channels close very rapidly. Concatemeric WT–E325D channels showed activation kinetics very similar to wild-type, suggesting that the presence of two wild-type subunits within the tetrameric channel is sufficient to normalize the activation kinetics, as also observed for WT–V408A channels (Figure 4A and D). On the other hand, deactivation kinetics were intermediate between homotetrameric wild-type and E325D channels (Table I).

To ascertain whether the E325D mutation affects the kinetics of C-type inactivation, oocyte membranes were depolarized to potentials between +40 and –20 mV for 10 s. C-type inactivation time constants, obtained from single exponential fits to E325D current decays, were ~5-fold faster than those of wild-type (Figure 5, Table I). However, C-type inactivation time constants for WT–E325D channels were intermediate between those of homotetrameric wild-type or E325D channels (Figure 5, Table I). These observations suggest that Glu325 may play an important role in the C-type inactivation process of hKv1.1 channels and that the number of mutant subunits within the channel affects this inactivation proportionally.

The much smaller current amplitudes observed for E325D channels might be due to an effect of the mutation on the single channel properties or to an altered processing of the mutated protein and impaired targeting to the plasma membrane. To investigate the effects of E325D mutation on single channel activity, patch–clamp experiments were performed with oocytes expressing wild-type and E325D channels (Figure 6). The slope conductances for wild-type and E325D channels were 10.4 ± 0.4 and 10.2 ± 0.6 pS, respectively (mean ± SD, $n = 4$). The voltage-dependent parameters $V_{1/2}$ and k of the E325D channel open probability were –12.3 and 19.8 mV, respectively, compared with –53.2 and 6.1 mV for wild-type channels (Figure 6D). These results demonstrate that the E325D mutation does not affect the single channel conductance but markedly alters the voltage dependence of the channel open probability. The single channel properties of the WT–E325D dimer were also determined in oocytes injected with the relevant concatemeric mRNA (Figure 6). WT–E325D channels had a slope conductance of 10.6 ± 0.8 pS (mean ± SD, $n = 4$) and the voltage-dependent parameters $V_{1/2}$ and k of the channel open probability were –33.1 and 10.9 mV, respectively.

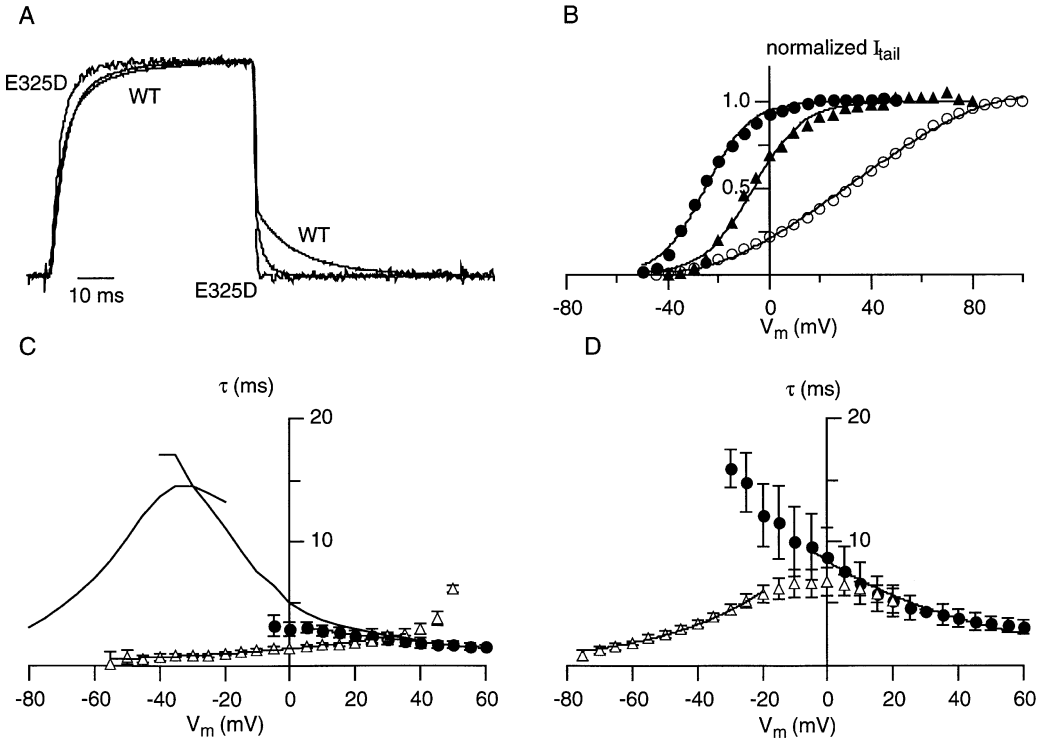

Fig. 4. Kinetics of activation, deactivation and voltage dependence for homomeric and heteromeric E325D channels. (**A**) Wild-type, E325D and WT–E325D (middle trace) superimposed and normalized current traces evoked by a +20 mV depolarizing pulse from a holding potential of –80 mV. (**B**) Wild-type (●), E325D (○) and WT–E325D (▲) normalized peak tail currents, recorded at –50, 0 and –40 mV respectively, were plotted as a function of 500 ms pre-pulse potentials and fitted with Boltzmann functions. The time constants of activation (fast; ●) and deactivation (△) for E325D (**C**) and WT–E325D (**D**) were plotted as a function of membrane potential and fitted with the equation reported in Figure 1. For comparison, wild-type values are shown in (C) as solid lines. Data points represent the mean ± SD of 6–7 cells.

By using the limiting slope method, we computed from the Boltzmann fits to the conductance–voltage and open probability–voltage relationships the product of the gating charge *z*, multiplied by the fraction of the field that they traverse δ (zδ value) for wild-type and E325D channels. These values were: $z_{wt} \times \delta_{wt} = 4.2$ and $z_{E325D} \times \delta_{E325D} =$ 1.1–1.3. The E325D mutation does not alter the total charge of the protein; therefore, if $z_{wt} = z_{E325D}$, the ratio $\delta_{wt}/\delta_{E325D} = 3.2–3.8$. This result suggests that the E325D mutation affects the voltage dependence of the channel by reducing ~3.5-fold the total charge displacement within the membrane electric field.

Discussion

The functional analysis of homomeric and heteromeric V408A channels clearly demonstrates that the kinetics of activation, deactivation and C-type inactivation are largely affected by the number of mutated subunits assembled in a functional channel. Moreover, our results show that the EA-1 mutation E325D alters the voltage dependence and the kinetics of activation, deactivation and C-type inactivation of the hKv1.1 channel. Specifically, E325D channels require a much stronger depolarization for activation, open and close very rapidly and undergo a faster C-type inactivation process. In addition, E325D current amplitudes are markedly smaller than wild-type. The slower time course of expression displayed by E325D channels, rather than the effect on the channel open probability, suggests that the current amplitude reduction might be caused by an altered processing of the mutated protein and placement into the plasma membrane. In fact, significant differences in expression level were still detected between wild-type and E325D channels even at strong depolarized potentials where the open probabilities of the two channels are similar (data not shown). These observations suggest that the nerve cells of EA-1 patients expressing E325D channels have delayed-rectifier currents with reduced amplitudes. Furthermore, E325D channels remain open for only a short period of time and display marked alterations in the voltage dependence of activation. The data suggest that the effects of the E325D mutation on the voltage dependence may be caused by a reduced mobility of the voltage sensor which requires stronger depolarizations to activate the channel. Therefore, the Glu325 residue may play an important role in the voltage-dependent gating of the hKv1.1 channels.

The N- and C-type inactivation processes of delayed-rectifier potassium channels control the firing properties of neurons and their response to input stimuli (Aldrich *et al.*, 1979; Hille, 1992). Fast, N-type, inactivation is caused by a 'ball-and-chain' occlusion mechanism (Armstrong and Bezanilla, 1977; Hoshi *et al.*, 1990; Zagotta *et al.*, 1990). In contrast, the slower C-type inactivation is thought to involve a conformational modification of the extracellular mouth of the pore (Grissmer and Cahalan, 1989; Stühmer *et al.*, 1989; Hoshi *et al.*, 1991; Pardo *et al.*, 1992; Kavanaugh *et al.*, 1992; López-Barneo *et al.*, 1993; Baukrowitz and Yellen, 1995; Molina *et al.*, 1997). The finding that the E325D mutation accelerates the C-type inactivation process clearly demonstrates that not only the C-terminal region of the protein is

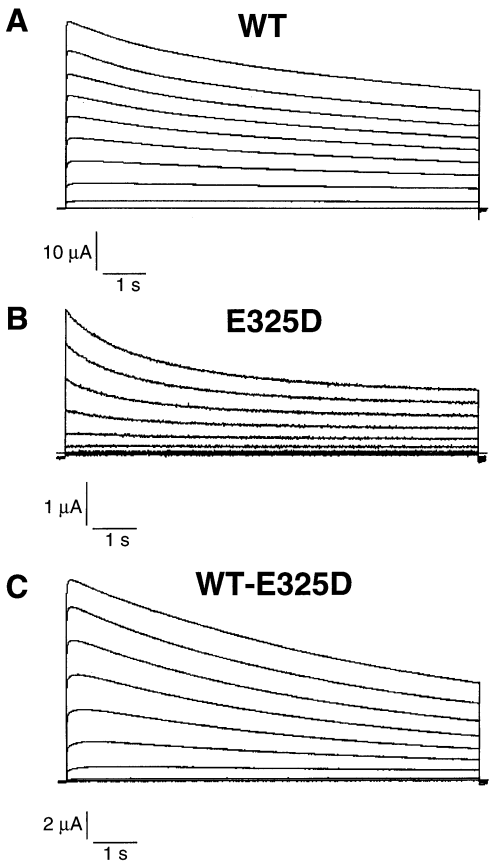

Fig. 5. C-type inactivation kinetics for wild-type, E325D and WT–E325D channels. Representative current traces evoked from oocytes expressing wild-type (**A**), E325D (**B**) and WT–E325D (**C**) channels. The membrane potential was held at –80 mV and stepped from +40 to –20 mV, every 10 mV for 10 s.

involved in C-type inactivation but that the S4–S5 loop also contributes to this inactivation.

During intense neuronal activity, C-type inactivation can accumulate, modifying both the firing rate and the shape of the action potential (Aldrich *et al.*, 1979). Our data demonstrate that E325D and V408A channels undergo C-type inactivation with faster time courses. These findings suggest a mechanism by which faster accumulation of C-type inactivation, during the repetitive firings caused by physical or emotional stress, would be responsible at least in part for triggering the attacks of episodic ataxia in patients expressing V408A and E325D channels.

All families affected by EA-1 are heterozygous, possessing both a normal and a mutant allele. If both alleles are expressed, the potassium channels formed would be composed of both normal and mutated subunits assembled according to a binomial distribution (MacKinnon, 1991; Sanguinetti and Keating, 1997). We determined the biophysical parameters of channels composed of two wild-type and two mutated subunits by expressing linked WT–V408A and WT–E325D mRNAs, and found that they were mostly intermediate between those of channels composed of four normal or four mutated subunits. These findings suggest that the effects of V408A and E325D on the gating parameters of the channels are proportional to the number of mutated subunits within the channel. We showed that E325D mutation markedly impairs several gating properties of the channel. In contrast, the V408A mutation undoubtedly has less severe effects on channel function; in fact, it does not alter the voltage dependence of the channel and had smaller effects on current amplitudes (Adelman *et al.*, 1995). Taken together, these results suggest that the impairment caused by such mutations on channel gating is proportional to the number of mutated subunits within the channel and also depends upon the type of mutation. Therefore, if the degree of allele expression dictates the relative composition of the channel, consequently, it would also affect the severity of EA-1 symptoms.

The data suggest that the overall effect of the V408A and E325D mutations is a reduction in the outward flow of potassium ions which becomes insufficient to repolarize the membrane potential. Therefore, the action potential of neurons expressing mutated hKv1.1 channels might be prolonged, resulting in increased influx of calcium ions through the voltage-dependent Ca^{2+} channels. Therefore, the prolonged neurotransmitter release from the terminals may cause attacks of ataxia. The cellular localization of the Kv1.1 channels and their involvement in EA-1 syndrome confirm the important role of this channel type in the control of movements (Wang *et al.*, 1994). The delayed-rectifier potassium channel is the main class of channels expressed in axons, where they regulate the overall excitability of the membrane (Hille, 1992). Kv 1.1 channels are expressed in myelinated and unmyelinated axons where impaired delayed-rectifier function may also increase the neuronal excitability at rest. Spontaneous discharges may result, causing the myokymic activity displayed by EA-1 patients during and between attacks.

The similarities between EA-1 neurological symptoms and the abnormalities found in *Shaker Drosophila melanogaster* (Kaplan and Trout, 1969) are striking. The A-type potassium currents are abolished in the homozygous *Shaker* phenotype, and are reduced in the heterozygous phenotype. The action potentials in axons of mutant flies are prolonged, exhibiting multiple firings and prolonged neurotransmitter release (Jan *et al.*, 1977; Salkof and Wyman, 1981; Tanouye and Ferrus, 1985; Kamb *et al.*, 1987; Adelman *et al.*, 1995).

Materials and methods

Electrophysiology

Xenopus laevis frogs were anaesthetized deeply with an aerated solution containing 5 mM 3-aminobenzoic acid ethyl ester methanesulfonate salt and 60 mM sodium bicarbonate, pH = 7.3. The ovary was dissected and the oocytes digested in OR-2 solution containing 0.5 U/ml of collagenase A (Sigma). *In vitro* transcribed mRNAs were microinjected into the oocytes 24 h later using a WPI nanoliter injector and incubated at 16°C. The amount of the mRNAs injected was determined by spectrophotometer and ethidium bromide stain.

Two-electrode voltage-clamp recording

The oocyte currents were recorded 1–8 days later with a GeneClamp 500 amplifier (Axon Instruments) interfaced to a Power Macintosh 7200/90 computer with an ITC-16 computer interface (Instrutech Corp., NY). The recording solution contained (mM): NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, pH = 7.4. Data acquisition and analysis were performed using Pulse+PulseFit (HEKA elektronik GmbH, Germany), KaleidaGraph (Synergy Software, USA) and IGOR (Wavemetrics) software. Leak and capacitative currents were subtracted using a P/4 protocol.

Patch–clamp recording

Patch–clamp recordings were performed with an Axopatch 200 amplifier (Axon Instruments). The pipette solution contained (mM): NaCl 120,

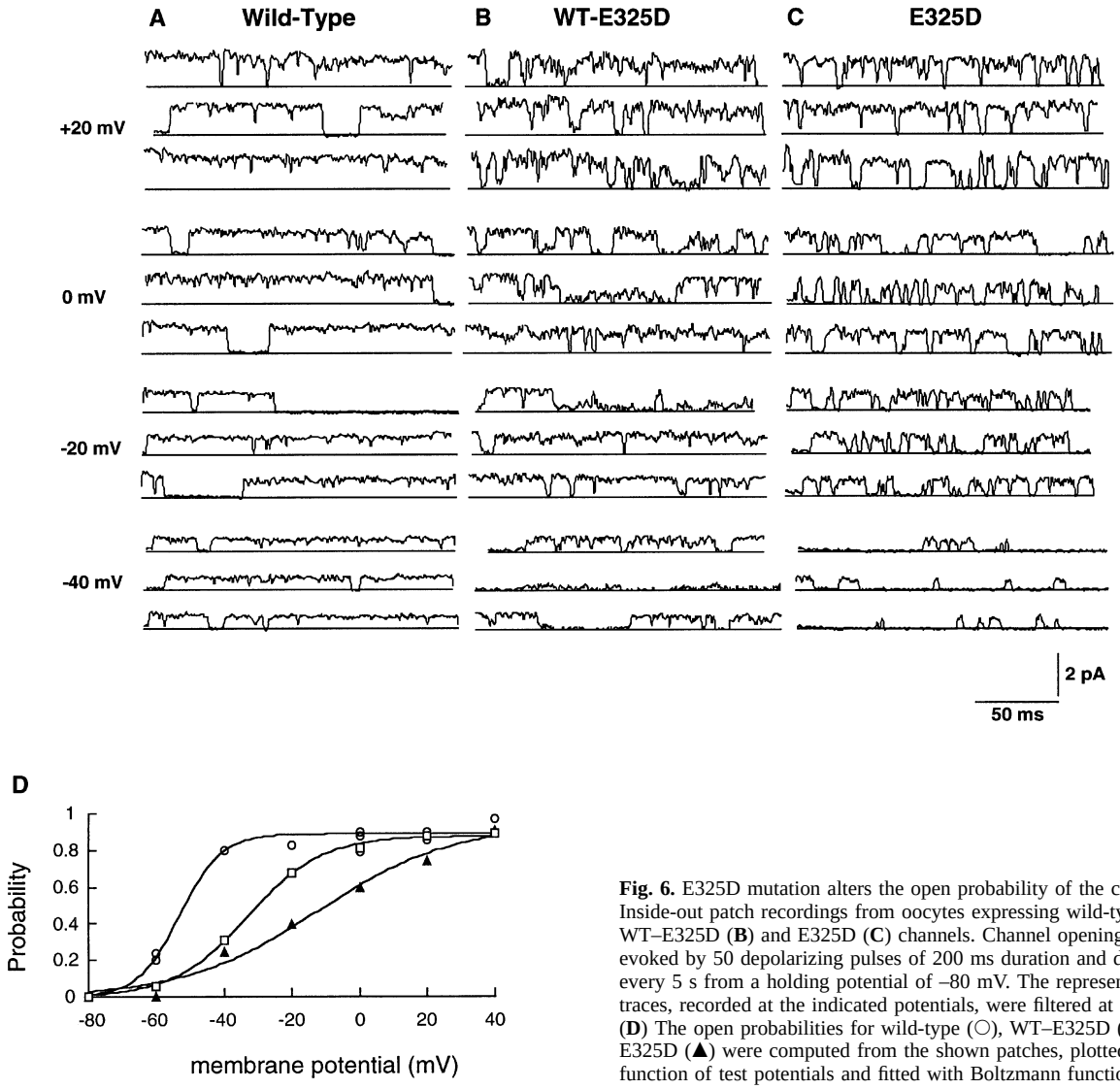

Fig. 6. E325D mutation alters the open probability of the channel. Inside-out patch recordings from oocytes expressing wild-type (**A**), WT–E325D (**B**) and E325D (**C**) channels. Channel openings were evoked by 50 depolarizing pulses of 200 ms duration and delivered every 5 s from a holding potential of –80 mV. The representative traces, recorded at the indicated potentials, were filtered at 0.5 kHz. (**D**) The open probabilities for wild-type (○), WT–E325D (□) and E325D (▲) were computed from the shown patches, plotted as a function of test potentials and fitted with Boltzmann functions.

KCl 2, HEPES 5, pH = 7.4 whereas the cytoplasmic solution contained (mM): KCl 120, EGTA 1, HEPES 5, pH = 7.4. Recording electrodes were pulled from borosilicate glass, coated with Sylgard and had resistances of 5–15 MΩ. The excised inside-out patch recordings were low-pass filtered at 0.5–2 kHz with an 8-pole bessel filter (Frequency Devices, MA) and acquired with a Pulse+PulseFit program (HEKA elektronik GmbH, Germany). Channel activity was analyzed with a TAC-TACfit program (Bruxton Co. WA), inspecting all transitions, and the slope conductance of the channels was computed at different potentials from all events histograms.

Molecular biology

To concatenate the subunits as dimers, 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 using a sequential PCR protocol modified from Horton (Horton *et al.*, 1989). Briefly, junctions were generated by overlap extension of the PCR primers which also encoded the glutamine linker. The constructed dimers were in pGEMA, a modified version of pGEM9zf– (Promega) with a stretch of 40 A–T base pairs inserted just upstream of the *Tth*111I site. The nucleotide sequences of all linked subunits were determined across the joined segments by automated sequencing. Oligonucleotides were obtained from EUROBIO, France. Wild-type, V408A and E325D hKv1.1 were subcloned into the oocyte expression vector pBF (courtesy of Dr B.Fakler), which provides 5′- and 3′-untranslated regions from the *Xenopus* β-globin gene, flanking a polylinker containing multiple restriction sites. *In vitro* mRNAs were generated using SP6 and T7 polymerase.

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