

Mutations in a Dominant-Negative Isoform Correlate with Phenotype in Inherited Cardiac Arrhythmias

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Summary

The long QT syndrome is characterized by prolonged cardiac repolarization and a high risk of sudden death. Mutations in the *KCNQ1* gene, which encodes the cardiac KvLQT1 potassium ion (K⁺) channel, cause both the autosomal dominant Romano-Ward (RW) syndrome and the recessive Jervell and Lange-Nielsen (JLN) syndrome. JLN presents with cardiac arrhythmias and congenital deafness, and heterozygous carriers of JLN mutations exhibit a very mild cardiac phenotype. Despite the phenotypic differences between heterozygotes with RW and those with JLN mutations, both classes of variant protein fail to produce K⁺ currents in cultured cells. We have shown that an N-terminus-truncated KvLQT1 isoform endogenously expressed in the human heart exerts strong dominant-negative effects on the full-length KvLQT1 protein. Because RW and JLN mutations concern both truncated and full-length KvLQT1 isoforms, we investigated whether RW or JLN mutations would have different impacts on the dominant-negative properties of the truncated KvLQT1 splice variant. In a mammalian expression system, we found that JLN, but not RW, mutations suppress the dominant-negative effects of the truncated KvLQT1. Thus, in JLN heterozygous carriers, the full-length KvLQT1 protein encoded by the unaffected allele should not be subject to the negative influence of the mutated truncated isoform, leaving some cardiac K⁺ current available for repolarization. This is the first report of a genetic disease in which the impact of a mutation on a dominant-negative isoform correlates with the phenotype.

Introduction

Mutations in the *KCNQ1* gene are the most frequent cause of the autosomal dominant Romano-Ward (RW) syndrome (MIM 192500), which is characterized by prolonged cardiac repolarization, cardiac arrhythmias, and a high risk of sudden death (Roden et al. 1996; Li et al. 1998). *KCNQ1* encodes a pore-forming potassium ion (K⁺) channel subunit termed “KvLQT1” (Wang et al. 1996), which, in association with its regulatory β -subunit Isk, produces the slow component of the delayed-rectifier cardiac K⁺ current (Barhanin et al. 1996; Sanguinetti et al. 1996). Reduction in K⁺ current in the heart of a patient with RW prolongs repolarization and provokes arrhythmias.

Biallelic *KCNQ1* mutations cause the Jervell and Lange-Nielsen (JLN) syndrome (MIM 220400), in which inherited cardiac arrhythmias are associated with congenital deafness because of abnormal homeostasis of the inner ear endolymph (Neyroud et al. 1997; Tyson et al. 1997; Duggal et al. 1998). However, in contrast with patients who have true RW, heterozygous carriers of JLN mutations exhibit a very mild or even normal cardiac phenotype (Fraser et al. 1964; Neyroud et al. 1997, 1998; Schulze-Bahr et al. 1997). Thus, heterozygous RW and JLN mutations of the *KCNQ1* gene provoke very different cardiac phenotypes, although expression of either RW or JLN K⁺ channel mutants usually produces no K⁺ current (Chouabe et al. 1997; Shalaby et al. 1997).

We (Demolombe et al. 1998) and others (Jiang et al. 1997) have shown that an N-terminus truncated KvLQT1 splice variant (isoform 2) constitutively expressed in the human heart exerts strong dominant-negative effects on the full-length KvLQT1 protein (isoform 1). Here we demonstrate that JLN, but not RW, mutations suppress dominant-negative effects of the truncated KvLQT1, thereby explaining the different phenotypes found in RW and JLN mutation heterozygous carriers. This is the first report of a genetic disease in which the impact of a mutation on a dominant-negative isoform directs the phenotype.

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Material and Methods

KvLQT1 Plasmids

Experiments were conducted with the human KvLQT1–isoform 1 clone identified by Chouabe et al. (1997) (Genbank accession number AF000571). Mutated KvLQT1–isoform 1 plasmids were prepared by mutagenesis with the Transformer site-directed mutagenesis kit (Clontech). Wild-type–isoform 2 cDNA subcloned in pCI vector was digested with *EcoRI* and *AfIII*. The digestion product corresponding to the 5' end of isoform 2 was ligated into mutated pCI-KvLQT1 isoform 1, which was also digested with *EcoRI* and *AfIII*. All constructs were sequenced before expression studies.

Functional Expression

COS-7 cells (African green monkey kidney cells transformed with SV40), obtained from the American Type Culture Collection, were microinjected into the nucleus with plasmids at day 1 after plating. Our protocol to microinject cultured cells by means of the Eppendorf ECET microinjector 5246 system has been reported elsewhere (Mohammad-Panah et al. 1998). A Green Fluorescence Protein pCI plasmid was used as an inert plasmid to ensure that cells were always injected with a constant 15- μ g/ml plasmid concentration. Whole-cell currents were recorded as described elsewhere (Demolombe et al. 1998; Mohammad-Panah et al. 1998). Cells were continuously superfused with an extracellular solution containing 145 mM NaCl; 4 mM KCl; 1 mM MgCl₂; 1 mM CaCl₂; 5 mM HEPES; and 5 mM glucose; with the pH adjusted to 7.4 with NaOH. Patch pipettes with a tip resistance of 2.5–5 Ω were filled with 145 mM K-gluconate, 5 mM HEPES, 2 mM EGTA, 2 mM 1/2 Mg-gluconate (free-Mg²⁺: 0.1), and 2 mM K₂ATP, at pH 7.2 with KOH, whereas the extracellular medium used to record K⁺ currents contained 145 mM Na-gluconate, 4 mM K-gluconate, 7 mM 1/2 Ca-gluconate (free-Ca²⁺: 1), 4 mM 1/2 Mg-gluconate (free-Mg²⁺: 1), 5 mM HEPES, and 5 mM glucose, at pH 7.2 with NaOH. Stimulation, data recording, and analysis were performed through an A/D converter (Labmaster). A microperfusion system allowed local application and rapid change of the different experimental solutions warmed at 37°C. Patch-clamp measurements are presented as the mean \pm SEM.

KvLQT1 Mutation Carriers

All *KCNQ1* mutations reported here have been described elsewhere (Donger et al. 1997; Neyroud et al. 1997, 1998), with the exception of R243H. The R243H mutation has been identified in a nonconsanguineous French JLN family. The proband is a 44-year-old deaf

woman who carries the R243H mutation and another still undetermined KvLQT1 mutation. She has a corrected QT interval (QTc) of 498 ms and has experienced several syncopes since the age of 2 years. The R243H mutation has also been detected at the heterozygous state in her father and her aunt, who are both asymptomatic (QTc = 434 ms and 467 ms, respectively).

Results

Functional Expression of Mutated Isoform 1 KvLQT1

Human KvLQT1–isoform 1 cDNA plasmids comprising either RW (R555C, Y315S, G314S) or JLN (R243H, W305S, Δ 544; see fig. 1a) mutations were coinjected together with a human IsK cDNA plasmid, into the nucleus of COS-7 cells. Twenty-four hours after injection, the cells were analyzed for K⁺ channel expression. As reported elsewhere (Chouabe et al. 1997), all the RW- or JLN-mutated constructs, except for R555C-KvLQT1 isoform 1 (an RW mutation) and R243H-KvLQT1 isoform 1 (a novel JLN mutation), failed to induce detectable K⁺ current.

R555C-KvLQT1 isoform 1 produced a K⁺ current with a reduced amplitude (tail current at –40 mV elicited by a test pulse to +60 mV for 3 s: 2.03 \pm 0.89 pA/pF versus 10.86 \pm 0.97 pA/pF with wild-type isoform 1; n = 6 and 14 cells, respectively; P < .001) and a strong shift in the activation curve (half-maximal potential 40.7 \pm 4.3 mV; n = 6; fig. 1b) when compared to wild-type isoform 1 (–0.5 \pm 1.6 mV; n = 8 cells). R243H-KvLQT1 isoform 1 produced a very small K⁺ current (tail current at –40 mV: 0.35 \pm 0.27 pA/pF; n = 6 cells) also with a shift in the activation voltage (half-maximal potential at around +20 mV). Thus, on the basis of isoform 1 expression alone, RW mutations such as Y315S or G314S, which induce a severe phenotype in heterozygous carriers (Donger et al. 1997), cannot be distinguished from JLN mutations such as W305S or Δ 544, which induce a mild phenotype in heterozygous carriers (Neyroud et al. 1997, 1998). Furthermore, the RW mutation R555C, which led to a sizable K⁺ current, still induces a more-severe cardiac phenotype in heterozygous carriers (Donger et al. 1997) than does JLN mutations.

Functional Expression of Mutated Isoform 2

We next explored whether RW and JLN mutations, which are all situated in exons common to isoforms 1 and 2, would also affect the dominant-negative activity of isoform 2. Because the RW syndrome is inherited as an autosomal dominant trait, affected individuals possess one normal and one mutant *KCNQ1* allele. In the hearts of affected patients, the delayed rectifier K⁺ current available for repolarization therefore results from

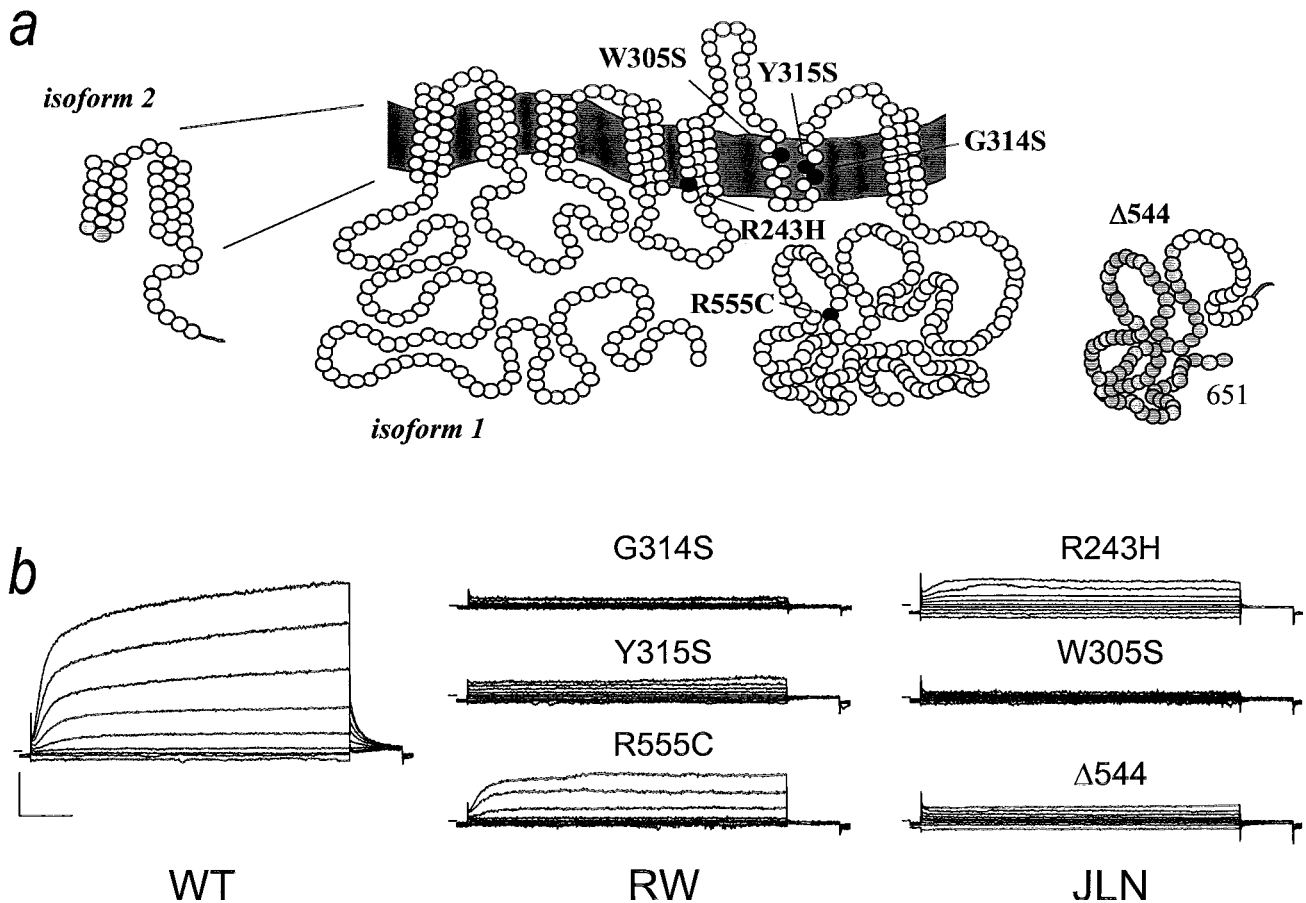
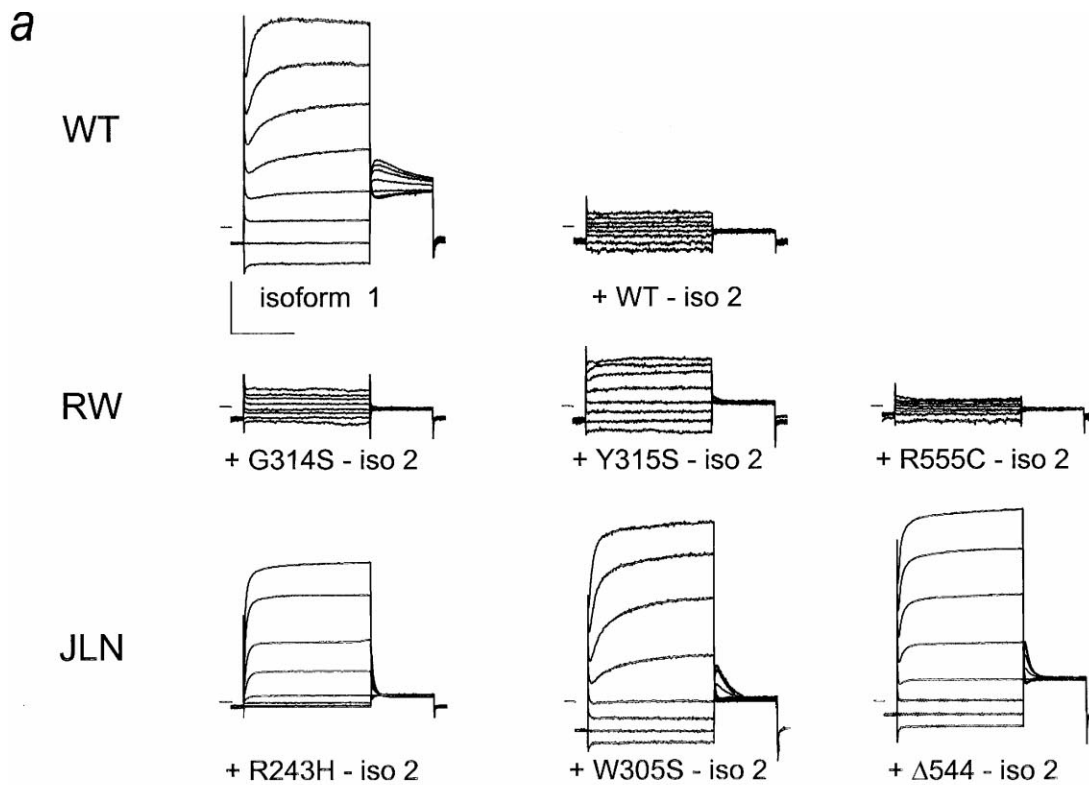


Figure 1 Expression of RW- and JLN-mutated KvLQT1 isoform 1. Schematic representation of KvLQT1 isoform 1 and isoform 2 proteins. *a*, Two amino acids shown in gray are specific for isoform 2. The mutated amino acids are shown in black. The JLN mutation $\Delta 544$ (D544, -7+8) is a deletion-insertion leading to a modification of the following 107 amino acid sequence and to a premature stop codon at position 651 (Neyroud et al. 1997). Superimposed whole-cell currents in COS-7 cells injected with wild-type (WT) isoform 1 cDNA and with RW- and JLN-mutated isoform 1 cDNA. *b*, Voltage steps applied in sequence from -80 mV to various voltages in the range of -100 to +60 mV, and then to -40 mV where tail currents were recorded. Current scale: 10 pA/pF; time scale: 500 ms. Injected pCI-KvLQT1 isoform 1, pCI-IsK and pCI-GFP plasmid concentrations: 5 μ g/ml.

the association between (1) mutated isoform 1, which produces no current (with the exception of R555C and R243H); (2) wild-type isoform 1; (3) mutated isoform 2; (4) wild-type isoform 2; and (5) IsK proteins. We thus investigated the dominant-negative properties of mutated isoform 2 constructs on the current produced by wild-type-isoform 1 expression. We have shown that isoform 2 dominant-negative properties are more pronounced in the absence than in the presence of IsK (Demolombe et al. 1998). A first set of experiments was thus conducted in the absence of IsK to exacerbate the potential effects of a mutation on isoform 2 dominant-negative properties. An isoform 2/isoform 1 ratio of 2/5 was used, corresponding to the expression ratio found in the adult human heart (Demolombe et al. 1998). As shown in figure 2, coinjection of wild-type isoform 2 markedly reduced the K⁺ current amplitude related to

wild-type-isoform 1 expression. RW-mutated isoform 2 still exerted strong dominant-negative effects. By contrast, JLN mutations abolished the dominant-negative properties of isoform 2.

As visible on current traces shown in figure 3, IsK expression (5 μ g/ml) markedly slowed the activation kinetics of KvLQT1 K⁺ current. In the presence of IsK (fig. 3), all RW mutations, but not JLN mutations, abolished the functional negative effects of isoform 2. The effects of wild-type isoform 2 were not restricted to a strong reduction in the amplitude of the K⁺ current. Indeed, wild-type isoform 2 also shifted to more depolarized potential the activation curve of the KvLQT1 current (not illustrated, but see Demolombe et al. 1998). A shift in the activation curve was also produced by isoform 2 comprising RW mutations such as R555C and G314S, and to a lesser extent, Y315S. By contrast, expression



b

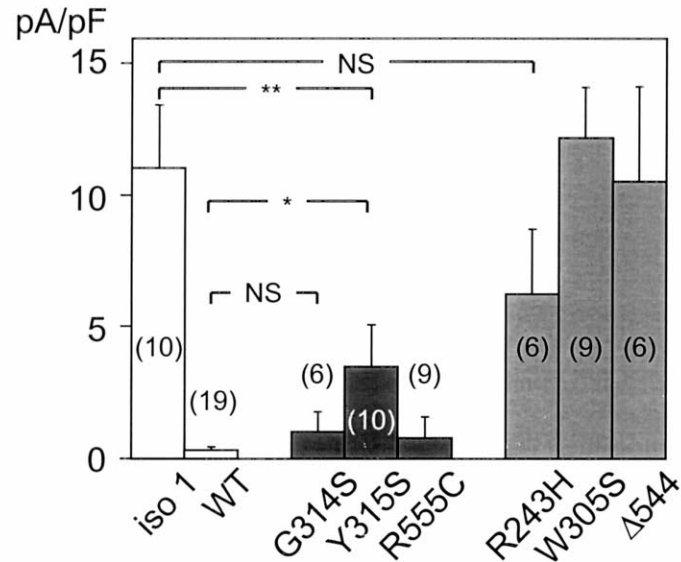


Figure 2 Expression of RW- and JLN-mutated KvLQT1 isoform 2 in the absence of IsK. *a*, K^+ currents recorded in COS-7 cells injected with wild-type (WT) isoform 1 ($5 \mu\text{g/ml}$) in the absence or presence of various isoform 2 constructs ($2 \mu\text{g/ml}$). *Upper panel*, Representative current tracings in cells injected with wild-type isoform 1 alone (isoform 1) and with wild-type isoform 1 plus wild-type isoform 2 (+ WT - iso 2) cDNA. *Middle panel*, Currents recorded with wild-type isoform 1 plus mutated RW (G314S, Y315S, R555C) isoform 2 cDNA. *Lower panel*, Currents recorded with wild-type isoform 1 plus mutated JLN (R243H, W305S, Δ544) isoform 2 cDNA. Voltage steps applied in sequence from -80 mV to various voltages in the range of -100 to $+60 \text{ mV}$, and then to -40 mV where tail currents were recorded. Pulse duration was $1,000 \text{ ms}$. Current scale: 10 pA/pF ; time scale: 500 ms . *b*, Averaged tail current amplitude in cells injected with isoform 1 ($5 \mu\text{g/ml}$) in the absence (iso 1) and presence of wild-type (WT) or mutated RW (G314S, Y315S, R555C; dark gray bars) and JLN (R243H, W305S, Δ544; light gray bars) isoform 2 plasmids ($2 \mu\text{g/ml}$). Numbers between brackets indicate the number of cells tested. NS = nonsignificant; * $P < .05$; ** $P < .01$ with standard *t*-test. In all experiments, injected plasmid concentration maintained constant at $15 \mu\text{g/ml}$ with pCI-GFP.

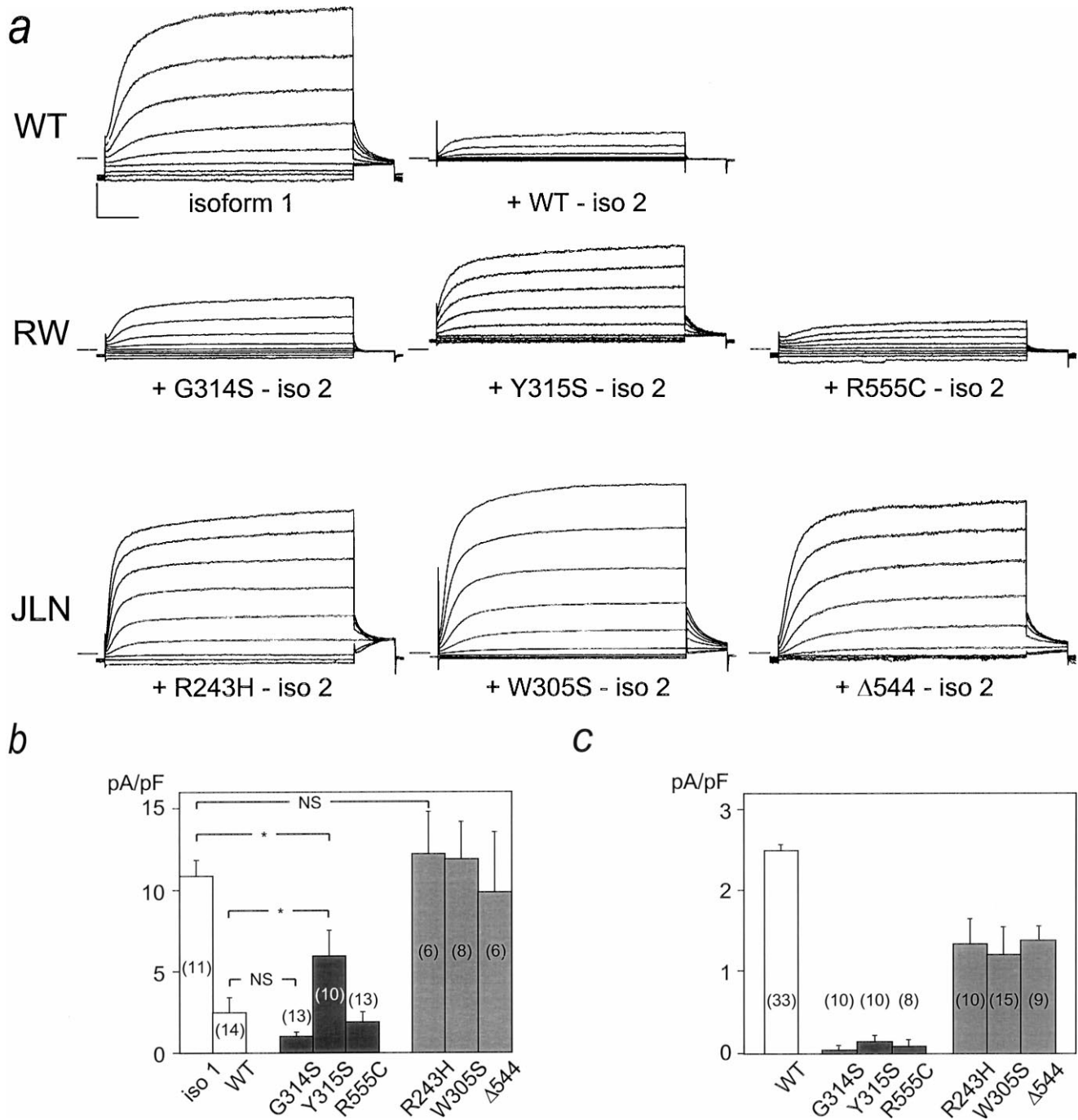


Figure 3 Expression of RW- and JLN-mutated KvLQT1 isoform 2 in the presence of IsK. *a*, K⁺ currents recorded in COS-7 cells injected with wild-type (WT) isoform 1 (5 μg/ml) in the absence or presence of various isoform 2 constructs (2 μg/ml). *Upper panel*, Representative current tracings in cells injected with wild-type isoform 1 alone (isoform 1) and with wild-type isoform 1 plus wild-type isoform 2 (+ WT - iso 2) cDNA. *Middle panel*, Currents recorded with wild-type isoform 1 plus mutated RW (G314S, Y315S, R555C) isoform 2 cDNA. *Lower panel*, Currents recorded with wild-type isoform 1 plus mutated JLN (R243H, W305S, Δ544) isoform 2 cDNA. Voltage steps applied in sequence from -80 mV to various voltages in the range of -100 to +60 mV, and then to -40 mV where tail currents were recorded. Pulse duration was 3,000 ms. Current scale: 10 pA/pF; time scale: 500 ms. *b*, Averaged tail current amplitude in cells injected with isoform 1 (5 μg/ml) in the absence (iso 1) and presence of wild-type (WT) or mutated RW (G314S, Y315S, R555C) and JLN (R243H, W305S, Δ544) isoform 2 plasmids (2 μg/ml). Numbers between brackets indicate the number of cells tested. NS = nonsignificant; * *P* < .05 with standard *t*-test. *c*, Averaged tail current amplitude in cells coinjected with a plasmid combination made of wild-type isoform 1 (2.5 μg/ml), RW- or JLN-mutated isoform 1 (2.5 μg/ml), wild-type isoform 2 (1 μg/ml), and mutated RW or JLN isoform 2 (1 μg/ml). The open bar (WT) indicates current amplitude in cells injected with 5 μg/ml wild-type isoform 1 plus 2 μg/ml wild-type isoform 2. The dark gray bars indicate RW-mutated constructs, whereas the light gray bars indicate JLN-mutated constructs. Differences in the tail current amplitude between WT and RW- or JLN-mutated constructs or between RW and JLN constructs reached significance, with *P* < .01. In *a*, *b*, and *c*, all cells were coinjected with pCI-IsK (5 μg/ml). Injected plasmid concentration maintained constant at 15 μg/ml with pCI-GFP.

of JLN-mutated isoform 2 did not shift the half-maximum activation potential. Expression of mutated JLN isoform 2 slowed the deactivation kinetics of the KvLQT1 current: deactivation time constant increased from 63.6 ± 15.1 ms in control to 108.7 ± 12.0 ms or 111.2 ± 12.8 ms in the presence of W305S isoform 2 or $\Delta 544$ isoform 2, respectively ($P < .05$ in both cases). These latter findings suggest that the JLN-mutated proteins were processed to the cell membrane.

Coexpression of Mutated and Wild-Type Isoforms

Finally, COS-7 cells were coinjected with a plasmid combination mimicking the KvLQT1 cDNA composition in heterozygous carriers. This combination was made of wild-type and mutated isoform 1 (each at $2.5 \mu\text{g/ml}$), and wild-type and mutated isoform 2 (each at $1 \mu\text{g/ml}$) cDNAs in the presence of IsK cDNA ($5 \mu\text{g/ml}$). As illustrated in figure 3c, cells injected with RW-mutated cDNAs produced a very small K^+ current, whereas cells injected with JLN-mutated cDNAs produced a sizable K^+ current with a reduced amplitude, as compared to control.

Discussion

In the normal heart, the amplitude of the delayed rectifier K^+ current available for repolarization depends on the relative expression of KvLQT1 isoform 1 and 2 encoded by both alleles (fig. 4). In the absence of the regulatory β -subunit IsK, the dominant-negative effects produced by isoform 2 are strong, and almost no current results from the expression of a physiological ratio isoform 2/isoform 1 (see fig. 2). In the presence of IsK, as in the adult human heart, the dominant-negative effects produced by isoform 2 are markedly reduced, and large K^+ currents can be recorded in expression system. In heterozygous carriers of *KCNQ1* mutations, the severity of the phenotype results from a complex interplay between wild-type and mutated proteins produced by the unaffected and affected allele, respectively (fig. 4). We show that RW and JLN mutations cannot be functionally discriminated on the basis of KvLQT1-isoform 1 (the channel pore) monomer expression, since both RW and JLN mutations abolish or strongly reduce the K^+ current amplitude related to isoform 1 expression. This is in agreement with findings by others (Chouabe et al. 1997; Shalaby et al. 1997; Wollnik et al. 1997). Here, we also show that JLN, but not RW, mutations suppress the dominant-negative properties of KvLQT1 isoform 2. Therefore, in cardiac cells from RW heterozygous carriers, wild-type KvLQT1 isoform 1 encoded by the unaffected allele should undergo not only the dominant-negative effects of wild-type isoform 2, but also those of mutated isoform 2 (fig. 4). Therefore, the cardiac cells

from RW patients should express almost no delayed rectifier K^+ current (see fig. 3c). In contrast, in cardiac cells from JLN heterozygous carriers, the K^+ current carried by wild-type isoform 1 from the unaffected allele should undergo the dominant-negative effects of wild-type isoform 2 only, leaving a consistent current available for repolarization (see fig. 3c). This may explain the almost normal phenotype observed in JLN heterozygous carriers: in the families where the JLN mutations reported here were identified, all heterozygous carriers were asymptomatic, and their average corrected QT duration was 430 ± 34 ms ($n = 16$); that is, below the normal value (Denjoy I, personal communication). Furthermore, it has been shown that RW-, but not JLN-mutated, KvLQT1 isoform 1 exerts by itself dominant-negative effects on wild-type KvLQT1 isoform 1 (Chouabe et al. 1997; Shalaby et al. 1997; Wollnik et al. 1997). The novel R243H JLN mutation reported herein also produced no dominant-negative effects on wild-type isoform 1 (current tail at -40 mV: 10.13 ± 3.13 pA/pF; i.e., not different from control). This should also help to reduce the K^+ current in RW heterozygotes. However, the dominant-negative effects of RW-mutated isoform 1 are weaker (coexpression of wild-type and mutated isoform 1 with a 1/1 ratio reduced the current by only 50%: see Chouabe et al. 1997) than that of wild-type or RW-mutated isoform 2 (coexpression of wild-type isoform 1 and wild-type or RW-mutated isoform 2 with a 1/0.4 ratio reduced the current amplitude by $\sim 90\%$). We thus propose that the amplitude of the KvLQT1 current remaining in heterozygous carriers depends mostly on whether or not mutated isoform 2 retains dominant-negative properties. This is a novel mechanism whereby a phenotype can be related to the functional consequences of mutations on an alternatively spliced dominant-negative isoform.

From a theoretical standpoint, studies in heterologous expression systems cannot be expanded to genotype/phenotype correlations with in vivo phenotypes. Direct recording of K^+ currents in cardiac myocytes would be a valuable step to fill the gap between heterologous systems and in vivo data. Although patch-clamp experiments of native human cardiac myocytes have been achieved, such studies cannot be performed in the context of the long QT syndrome. Another possibility would be to use transgenic or knock-out animals. It should be realized, however, that the cardiac electrophysiology of mice is very different from that of humans and that this approach has its own limits and flaws. Finally, it is possible that the JLN mutants are expressed at a low level or that the protein is unstable, resulting in a diminished dominant-negative potential. This may be because of the heterologous expression system in cells that do not normally express KvLQT1. In addition, it remains unclear whether the JLN-mutated proteins are correctly ad-

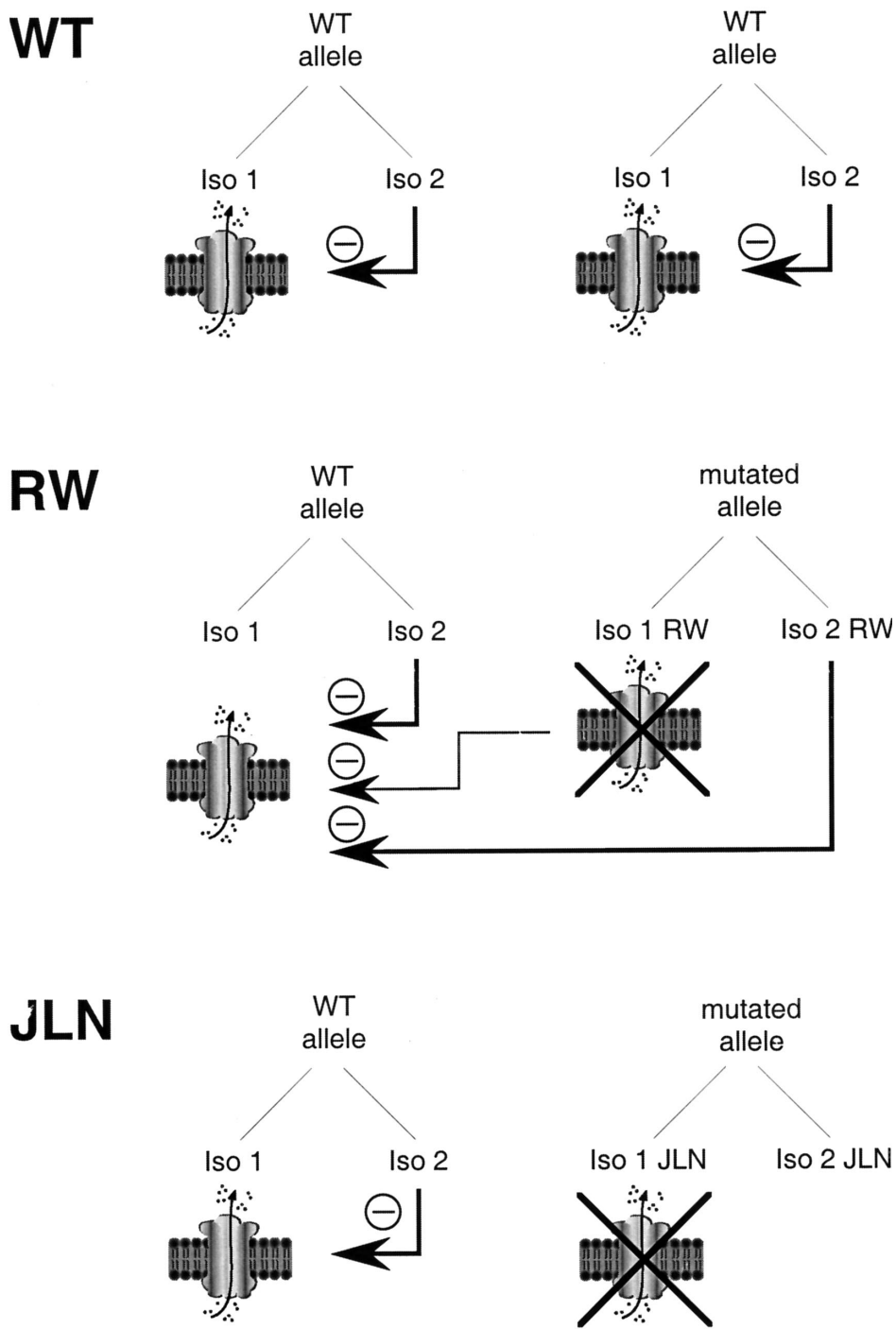


Figure 4 Schematic representation of the molecular basis of the effect on the potassium ion channel pore for dominant (RW) and recessive (JLN) phenotypes, in comparison with the wild-type (WT) phenotype. See Discussion section for more detail.

dressed to the cell membrane. The slight modification in the deactivation kinetics that we observed suggests, but does not prove, that JLN-mutated isoform 2 was indeed processed to the cell membrane. Specific experiments with tagged wild-type and mutated isoform 2 are cur-

rently being performed in our laboratory to clarify this important point.

KCNQ1 is not the only gene that generates dominant-negative spliced variants. Other dominant-negative isoforms have been reported that concern, among other

proteins, the transcription factor STAT6 (Patel et al. 1998), the human glucocorticoid receptor hGR (Oakley et al. 1997), the vitamin D receptor (Ebihara et al. 1996), the human growth factor hormone receptor GHR (Ross et al. 1997), the thyroid hormone receptor TR (Zhu et al. 1997), the prolactin receptor, and the human estrogen receptor (Wang and Miksicek 1991). In the future, these genes may be ascribed to a genetic disease (e.g., resistance to thyroid hormone disorders), and the potential effects of mutations on the dominant-negative isoforms should be evaluated.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Genbank, <http://www.ncbi.nlm.nih.gov/Web/Genbank> (for full-length KvLQT1 cDNA [AF000571])
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (for RW syndrome [MIM 192500] and JLN syndrome [MIM 220400])

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