Stilbenes and fenamates rescue the loss of IKS channel function induced by an LQT5 mutation and other IsK mutants

Ilane Abitbol, Asher Peretz, Christian Lerche1, Andreas E.Busch2 and Bernard Attali3

Department of Neurobiology, The Weizmann Institute of Science, 76100 Rehovot, Israel, 1Eberhard-Karls-University, 72076 Tubingen and 2Hoechst Marion Roussel Deutschland GmbH, 65926 Frankfurt, Germany

3 Corresponding author e-mail: bnattali@weizmann.weizmann.ac.il

Genetic and physiological studies have established a link between potassium channel dysfunction and a number of neurological and muscular disorders. Many ‘channelopathies’ are accounted for by a dominant-lethal suppression of potassium channel function. In the cardiac IKS channel complex comprising the α and β subunits, KvLQT1 and IsK, respectively, several mutations lead to a dominant-negative loss of channel function. These defects are responsible for a human cardiovascular disease called long QT (LQT) syndrome. Here we show that binding of IKS channel activators, such as stilbenes and fenamates, to an extracellular domain flanking the human IsK transmembrane segment, restores normal IsK channel gating in otherwise inactive IsK C-terminal mutants, including the naturally occurring LQT5 mutant, D76N. Our data support a model in which allosteric interactions exist between the extracellular and intracellular boundaries of the IsK transmembrane segment as well as between domains of the α and β subunits. Disruption of this allosteric interplay impedes slow activation gating, decreases current amplitude and restores channel inactivation. Owing to allosteric interactions, stilbene and fenamate compounds can rescue the dominant-negative suppression of IKS produced by IsK mutations and thus, may have important therapeutic relevance for LQT syndrome.

Keywords: IKS/IsK/LQT/Mink/potassium channels

Introduction

The IKS potassium channel complex underlies the slowly activating outwardly-rectifying K+ current that plays a major role in repolarizing the cardiac action potential (Noble and Tsien, 1969; Sanguinetti and Jurkiewicz, 1990). Recently, it was demonstrated that IKS consists of the heteromeric assembly of two structurally distinct α and β subunit proteins called KvLQT1 and IsK, respectively (Barhanin et al., 1996; Sanguinetti et al., 1996). Mutations in KvLQT1 and IsK genes produce the long QT (LQT) syndrome, a genetically heterogeneous human cardiovascular disease, for which four ion channel subunit genes have been identified so far (Russell et al., 1996; Wang et al., 1996; Ackerman and Clapham, 1997; Neyroud et al., 1997; Schulze-Bahr et al., 1997; Splawski et al., 1997a,b; Tyson et al., 1997; Wollnik et al., 1997; Duggal et al., 1998). The LQT syndrome is characterized by abnormal ventricular repolarization, as reflected by a prolonged QT interval on a surface electrocardiogram. It causes syncope, seizures and sudden death from ventricular arrhythmias, known as ‘torsade de points’. Both autosomal dominant (Romano-Ward syndrome) and autosomal recessive (Jervell and Lange-Nielsen syndrome) forms of LQT are known, with the latter form also including bilateral deafness. Mutations in KvLQT1 and IsK genes were found to be associated with both dominant and recessive forms of LQT (Neyroud et al., 1997; Schulze-Bahr et al., 1997; Splawski et al., 1997a,b; Tyson et al., 1997; Wollnik et al., 1997; Duggal et al., 1998).

KvLQT1 belongs to a newly characterized K+ channel family, KCNQ, whose members are widely expressed in epithelial and excitable tissues, such as the brain, the heart, the kidney or the inner ear (Barhanin et al., 1998; Bievert et al., 1998; Charlier et al., 1998; Singh et al., 1998; Kubisch et al., 1999). The importance of the KCNQ channel family is documented by the loss of channel function produced by mutations of the KCNQ genes, which have been identified as causes of LQT, syndromic and non-syndromic deafness and neonatal epilepsy. The KvLQT1 α subunit is a typical member of the voltage-gated K+ channel superfamily with six putative transmembrane segments and a P loop domain bearing the K+ selectivity filter signature. The IsK β subunit (also called Mink) has a single putative transmembrane segment and cannot form K+ channels on its own (Takumi et al., 1988; Attali et al., 1993; Lesage et al., 1993; Attali, 1996; Busch and Suessbrich, 1997; Kaczmarek and Blumenthal, 1997). When expressed alone, KvLQT1 elicits a rapidly activating K+ current (Barhanin et al., 1996; Sanguinetti et al., 1996). However, coexpression of KvLQT1 with the β subunit IsK leads to a dramatic slowing of the activation kinetics and a marked increase in macroscopic K+ current amplitude, thus reproducing the biophysical features of the native cardiac IKS (Noble and Tsien, 1969; Sanguinetti and Jurkiewicz, 1990).

Functional studies showed that many naturally occurring mutations in KvLQT1 and IsK lead to a dominant-negative loss of channel function, which accounts for the most severe forms of LQT (Chouabe et al., 1997; Splawski et al., 1997b; Wollnik et al., 1997). Previous mutagenesis work has identified the intracellular IsK C-terminus as an important determinant of IKS channel function (Takumi et al., 1991; Attali et al., 1993; Wang and Goldstein, 1995; Ben-Efraim et al., 1996). Several IsK C-terminal mutant proteins, though expressed and incorporated efficiently in the plasma membrane of Xenopus oocytes, were shown to exhibit a drastic reduction in channel activity,
resulting in virtually undetectable K⁺ currents (Takumi et al., 1991). Furthermore, many of these mutants had a strong dominant-negative effect (Takumi et al., 1991; Attali et al., 1993; Wang and Goldstein, 1995). This feature was underscored by a recent study showing that a mutation in the human IsK C-terminus (D76N), causes LQT syndrome and suppresses IsK channel function in a dominant-negative fashion (Splawski et al., 1997b).

We hypothesized that the loss of function produced by the IsK C-terminal mutants, results from the locking of the IsK cytoplasmic domain into inactive conformations, though preserving a tight but inhibitory interaction with KvLQT1. In this study, we tested whether activators of the IsK channel complex (Busch et al., 1994, 1997) such as 4,4′-disothiocyanostilbene-2,2′-disulfonic acid (DIDS) or mefenamic acid, could rescue the loss of function induced by human IsK C-terminal mutants. Our results show that binding of DIDS or mefenamic acid to the extracellular N-terminal boundary of the IsK transmembrane segment, can rescue the functional defect produced by various IsK C-terminal mutants, including a naturally occurring LQT5 mutant, D76N.

Results

Effects of DIDS on wild-type KvLQT1 and IsK currents

Stilbene and fenamate compounds have been shown to activate the K⁺ channel activity induced by the expression of IsK in Xenopus oocytes (Busch et al., 1994, 1997). Before investigating the effect of these molecules on IsK C-terminal mutants, we first checked the effects of stilbenes such as DIDS on wild-type (WT) homomultimeric mouse KvLQT1 channels, expressed in Xenopus oocytes. KvLQT1 K⁺ currents were elicited by membrane depolarization above ~70 mV (Figure 1A). Although DIDS superfusion (25–100 µM) did not affect the current amplitude of homomultimeric KvLQT1 channels (Figure 1A, D and E), it produced a significant leftward shift (~16.7 mV) in the voltage-dependence of activation (Figure 1C; from V₅₀ = −28.1 ± 1.5 mV, s = −17.8 ± 1.2 mV/e-fold to V₅₀ = −44.8 ± 2.8 mV, s = −18.9 ± 0.8 mV/e-fold for control and DIDS (100 µM)-treated oocytes, respectively; n = 11, p < 0.01). DIDS accelerated the activation kinetics of KvLQT1 (Figure 1A; A.Peretz, I.Abitbol and B.Attali, manuscript in preparation) and increased the instantaneous current (Figure 1A and E). Following a 5 min superfusion with 100 µM DIDS, the instantaneous current measured at 50 ms subsequent to a test pulse to +30 mV, increased from 0.27 ± 0.03 µA to 0.43 ± 0.5 µA (n = 9, p < 0.01). DIDS markedly slowed (>2.6-fold) the KvLQT1 deactivation kinetics (Figure 1B).

Similar results were obtained with another stilbene molecule, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (100 µM SITS) and with a fenamate compound, 100 µM mfenamic acid (data not shown). The onset of DIDS action was at ~1 min, then the effects developed progressively to reach steady-state within 10 min. The washout was partial and slow (>20 min). Internally applied, DIDS had no effect (when microinjected at up to 300 µM).

DIDS action was next examined on WT heteromultimeric IsK channels which were expressed in Xenopus oocytes by co-injecting cRNAs encoding the mouse WT KvLQT1 α subunit (2 ng cRNA/oocyte) and the human WT IsK protein (1 ng cRNA/oocyte). The effects of DIDS were far more spectacular on IsK currents than on homomultimeric KvLQT1 channels (Figure 2). The sigmoidal delay in IsK activation kinetics disappeared and instead, a major quasi-instantaneous current became evident, together with an ~2.4-fold increase in maximal current amplitude (Iₘₐₓ) (Figure 2A, D and F). The quasi-instantaneous current rose within the settling range of the capacitance transient (~5 ms) which obscured the initial few milliseconds of current activation (Figure 2A, inset). Following this fast rising phase, the IsK current activation could be described by a single exponential, with τₑ₅₀ = 4.14 ± 0.51 s (6 s pulse at +30 mV; n = 7). As for homomultimeric KvLQT1 channels, DIDS was active on IsK externally and was ineffective when microinjected into Xenopus oocytes, at up to 300 µM. As illustrated by the isochronal current/voltage relations (Figure 2D) and the relative tail current/voltage relations (Figure 2B), DIDS also produced a pronounced leftward shift in the voltage dependence of activation of IsK. Control IsK activated above a threshold of ~−30 mV, while DIDS-treated IsK channels opened at much more negative potentials (threshold above ~−60 mV). Following DIDS superfusion (3 min), IsK deactivation kinetics were markedly slowed down, with time constants increasing from τₑ₅₀ = 1.069 ± 0.122 s to τₑ₅₀ = 6.304 ± 1.259 s (Figure 2E; from a 6 s step at +30 mV and tail at −80 mV, n = 8, p < 0.01). Remarkably, upon prolonged DIDS incubation (>5 min), a substantial fraction of the current did not deactivate and a positive holding current appeared at −80 mV (Figure 2A and C). Consequently, during repetitive stimulations, DIDS produced a progressive accumulation of open states at low frequency (0.03 Hz) of stimulation (Figure 2C). The same feature is seen in control IsK, but at >60-fold higher frequency (2 Hz) of stimulation (Romey et al., 1997). Taken together, these results suggest that although stilbenes and fenamates act on homomultimeric KvLQT1 channels, their spectacular effects on WT IsK channels appear to be crucially dependent on the presence of the IsK β subunit. The data also suggest that DIDS acts externally, on both KvLQT1 and IsK domains, to cause a drift of IsK channels towards the open state.

Effects of DIDS and mfenamic acid on human IsK C-terminal mutants coexpressed with WT KvLQT1

Several human IsK C-terminal mutants were generated (see Figure 4D), including deletion mutants (Δ80–129 IsK, Δ73–129 IsK, Δ73–79 IsK) and point mutants (S68T, S74A, D76N, N79D, Y81F). Each human IsK mutant (1 ng cRNA/oocyte) was coexpressed with WT mouse KvLQT1 α subunit (2 ng cRNA/oocyte). All IsK C-terminal mutants exhibited slower activation kinetics than homomultimeric WT KvLQT1 channels (Figures 3A, 4A and 5A). However, in contrast to WT IsK channels, no sigmoidal delay in activation was observed (Figure 3A).

There was also a more than +40 mV rightward shift of the current–voltage relationships, when compared with that of WT IsK (Figures 3B and 4B) and an acceleration of the deactivation kinetics (Figure 3A; for Δ80–129 IsK, τₑ₅₀ = 0.185 ± 0.011 s, as compared with τₑ₅₀ = 1.115 ± 0.109 s for WT IsK; from a 6 s step at +30 mV and
Rescue of IKS channel function

**Fig. 1.** Effects of DIDS on WT homomultimeric KvLQT1. (A) KvLQT1 currents were recorded from 2.5 s depolarizing pulses to potentials of –70 to +30 mV, in 10 mV increments from a holding potential of –80 mV, before (left) and following 10 min superfusion with 100 µM DIDS (right). Tail currents were recorded at –60 mV. (B) DIDS slowed deactivation of KvLQT1 with $\tau_{\text{deact}} = 0.321 \pm 0.029$ s and $\tau_{\text{deact}} = 0.841 \pm 0.122$ s for control (empty bars) and DIDS-treated oocytes (solid bars), respectively, as measured at +30 mV step and tail at –80 mV, $n = 6$, $p < 0.01$. (C) Normalized conductances of KvLQT1 steady-state currents (2.5 s) before (solid squares) and following 10 min superfusion with 100 µM DIDS (empty squares). Curves were fitted to a Boltzmann distribution (for parameters, see Results). Note that the error bars are smaller than the symbols. (D) Train of KvLQT1 currents, recorded at +20 mV for 2.5 s at 0.03 Hz stimulation in the presence of 100 µM DIDS. (E) Following a 5 min superfusion with 100 µM DIDS, the instantaneous current ($I_{\text{inst}}$) measured at 50 ms subsequent to a test pulse to +30 mV, increased from 0.27 ± 0.03 µA (control, empty bar) to 0.43 ± 0.05 µA (DIDS-treated, solid bar) ($n = 9$, $p < 0.01$). Maximal current amplitude ($I_{\text{max}}$) was measured following a 2.5 s step to +30 mV in control (empty bar) and DIDS-treated oocytes (solid bar).

Except for S74A IsK, all these mutants led to a loss of channel function with very low levels of K+ currents. They generated a lower K+ current amplitude than that induced by homomultimeric KvLQT1 channels (Figures 3–5) and displayed a strong dominant-negative effect on IKS channel activity when coexpressed with WT IsK and WT KvLQT1. For example, the current produced by expression of D76N IsK (1 ng cRNA/oocyte) with WT KvLQT1 (2 ng cRNA/oocyte) was barely detectable above that found in uninjected oocytes ($I_{\text{max}} = 0.12 \pm 0.05$ µA, at +30 mV; $n = 6$). In addition, expression of D76N IsK (1 ng cRNA/oocyte) with WT IsK (1 ng cRNA/oocyte) and WT KvLQT1 (2 ng cRNA/oocyte) produced 10-fold less K+ current (Figure 4A; $I_{\text{max}} = 0.24 \pm 0.06$ µA) than that induced by WT IKS ($I_{\text{max}} = 2.32 \pm 0.6$ µA, at +30 mV; $n = 7$, $p < 0.01$; with WT IsK and WT KvLQT1, each at 2 ng cRNA/oocyte). In agreement with previous reports (Takumi et al., 1991; Wang and Goldstein, 1995; Splawski et al., 1997b), the very low percentage of current level (~10% of WT IKS) produced by the D76N IsK mutation indicates a strong dominant-negative effect.

Since we postulated that the loss of function produced by IsK C-terminal mutants may result from the locking of the IsK cytoplasmic domain into inactive conformations, we tested whether the potent activators of IKS, DIDS or mefenamic acid, could possibly unlock the defective IsK C-terminus and rescue the loss of channel function. Within 1 min of external superfusion with DIDS (100 µM) or mefenamic acid (100 µM), the oocytes coexpressing the
IsK C-terminal mutants and the WT KvLQT1 displayed a progressive recovery of large K⁺ currents. Using a train protocol (0.03 Hz), we could show that the rescuing process induced by DIDS, developed progressively following repeated stimulations, in oocytes coexpressing Δ80–129 IsK and WT KvLQT1 (Figure 3D). At steady-state (10–15 min), the various mutant channels displayed a 2.5- to 15-fold increase in maximal current amplitude (Figures 3 and 4). The current characteristics of DIDS-treated IsK C-terminal mutants were very similar to those exhibited by the WT IKS channels (see Δ80–129 IsK and D76N IsK; Figures 3 and 4). These include a sigmoidal delay in activation, slow activation and slow deactivation kinetics, K⁺ selectivity as well as similar current–voltage relationships (Figures 3B, 3E and 4B). When measured from tail currents, V₅₀ = −3.6 ± 1.1 mV, s = −14.8 ± 0.5 mV/e-fold (n = 11), V₅₀ = −7.6 ± 3.1 mV, s = −12.5 ± 0.7 mV/e-fold (n = 16) and V₅₀ = −28.2 ± 0.8 mV, s = −12.2 ± 0.3 mV/e-fold (n = 4) for WT IKS, DIDS-treated Δ80–129 IsK and for homomultimeric WT KvLQT1, respectively (Figure 3E).

When the naturally occurring LQT5 (Schulze-Bahr et al., 1997; Splawski et al., 1997b; Duggal et al., 1998), D76N IsK, was coexpressed with WT KvLQT1, a 4- and 3-fold current stimulation was obtained upon DIDS and mefenamic acid superfusion, respectively (not shown). Remarkably, a very potent relief of the dominant-lethal suppression of IKS was obtained (~10-fold current stimulation), when oocytes coexpressing D76N IsK, WT IsK and WT KvLQT1 were perfused with 100 µM mefenamic acid.
Rescue of IKS channel function

**Fig. 3.** Effects of DIDS on the Δ80–129 IsK C-terminal mutant. (A) Currents were recorded from Δ80–129 IsK mutant coexpressed with WT KvLQT1, as in Figure 1A. (B) Current–voltage relations (6 s) of Δ80–129 IsK (n = 14) in the absence (solid squares) or presence (empty squares) of 100 µM DIDS. (C) Currents were recorded from Δ80–129 IsK as in (A), before (left) and after 4 min superfusion with 100 µM DIDS (right). (D) Train of currents recorded from Δ80–129 IsK, in oocytes perfused with 100 µM DIDS and stimulated, as in Figure 2C. (E) Normalized conductances of DIDS-treated Δ80–129 IsK (solid triangles, n = 16) and for comparison, that of WT IKS (solid squares, n = 11) and WT KvLQT1 (empty squares, n = 4), as deduced from tail currents (for parameters, see Results). Note that the error bars are smaller than the symbols.

Acid (Figure 4C). Similar results were obtained with 100 µM DIDS (Figure 4D). This finding is of particular significance, knowing that some LQTs affect individuals possessing one normal and one mutant IsK gene allele (Splawski et al., 1997b). The washout of DIDS or mefenamic acid actions was partial and slow (up to 30 min). Rescue of IKS current by DIDS was relatively modest in the Δ73–129 IsK mutant (1.5 ± 0.1-fold increase in maximal current amplitude, n = 8; see Figures 4D, 5B and 5C). Similar results were obtained with the Δ73–79 IsK mutant. Coexpression of Δ73–79 IsK with WT KvLQT1 produced a K+ current of lower amplitude than that of WT IKS and even that of WT KvLQT1 (I_max = 0.69 ± 0.07 µA at +30 mV, n = 6; Figures 4D and 5D). DIDS increased by 2.6 ± 0.1-fold (n = 6) the maximal current flowing through Δ73–79 IsK mutant channels (Figures 4D, 5E and 5F). The relatively modest action of DIDS on Δ73–129 IsK and Δ73–79 IsK mutants suggests that the IsK residues 73–79 are important for preserving a functionally active C-terminal conformation necessary for the rescue of IKS current.

**Mapping the DIDS-binding site on IsK**

Knowing that DIDS and mefenamic acid act externally, we attempted to map their binding domain at the extracellular N-terminus of IsK. Coexpression of the N-terminal deletion mutant, Δ11–38 IsK, with WT KvLQT1 produced large K+ currents whose gating characteristics were very similar to those of WT IKS, including a sigmoidal delay and slow kinetics of activation and deactivation (Figure 6A). However, Δ11–38 IsK opened at more hyperpolarized potentials (V_50 = −18.6 ± 3.0 mV, s = −12.7 ± 1.4 mV/e-fold; from tail currents, n = 13) than WT IKS (V_50 = −3.6 ± 1.1 mV, s = −14.8 ± 0.5 mV/e-fold; from tail currents, n = 11) (Figure 6B). The effects of DIDS on Δ11–38 IsK were very similar to those observed for WT IKS, with a major instantaneous current, accompanied by a 2.4-fold increase in maximal current amplitude, a leftward shift in the voltage dependence of activation and a marked slowing of deactivation (Figures 6A, 6C and 7E). Similar results were obtained with mefenamic acid (not shown). This suggests that the IsK residues 11–38 are not involved in the action of DIDS and mefenamic acid. The
**Fig. 4.** Effects of DIDS and mefenamic acid on the LQT5 D76N IsK mutation and other IsK C-terminal mutants. (A) Currents were recorded as in Figure 1A, from oocytes coexpressing D76N IsK (1 ng cRNA/oocyte), WT IsK (1 ng cRNA/oocyte) and WT KvLQT1 (2 ng cRNA/oocyte). (B) Current–voltage relations (6 s) of D76N IsK coexpressed with WT IsK and WT KvLQT1 (n = 9) in the absence (solid squares) or presence (empty squares) of 100 µM mefenamic acid. (C) Currents were recorded as in (A) from D76N IsK coexpressed with WT IsK and WT KvLQT1, before (left) and after 5 min superfusion with 100 µM mefenamic acid (right). (D) Maximal current amplitudes (6 s, at +30 mV) of the various IsK C-terminal mutants (n = 5–21, *p < 0.01) were measured in the absence (empty bars) or presence of 100 µM DIDS (solid bars). For the D76N IsK mutant, the currents were recorded from oocytes coinjected with D76N IsK, WT IsK and WT KvLQT1.

Δ39–43 IsK mutant, whose deletion is at the N-terminal boundary of the IsK transmembrane segment, produced a K⁺ current which is smaller than that of WT KvLQT1 and WT IKS (Iₘₐₓ = 0.32 ± 0.02 µA, at +30 mV, n = 18; Figures 6D and 7E). The activation kinetics of Δ39–43 IsK were slow, although without a sigmoidal delay and with a significant instantaneous component (at +30 mV, τₜₐₜₚ = 0.114 ± 0.010 s and τₜₑₛₖ = 3.365 ± 0.568 s; n = 7). Like the IsK C-terminal mutants, the current flowing through Δ39–43 IsK did not reach steady-state following 3 s depolarizing pulses (Figures 6D and 7A). However, in contrast to IsK C-terminal mutants and even to WT IKS, the voltage-dependence of activation of Δ39–43 IsK was shifted to more negative potentials, with a threshold of activation above ~60 mV (compare Figures 2D and 3E with Figures 6E and 7B, respectively; from tail currents, Vₜₐₜₚ = −24.9 ± 7.7 mV, s = −11.9 ± 0.5 mV/e-fold; n = 4). Interestingly, the K⁺ current produced by the Δ39–43 IsK mutant was completely insensitive to either 100 µM DIDS or 100 µM mefenamic acid (Figures 6E, 7A and 7E). Moreover, neither compound could rescue the loss of function in the double-deletion mutant Δ39–43/D80–129 IsK (Figure 7E). These findings suggest that the IsK residues 39–43 are crucially involved in the action of DIDS and mefenamic acid on WT IKS and on their rescuing effect on the loss of channel function produced by the IsK C-terminal mutants. In order to narrow down the binding domain of stilbenes and fenamates on IsK, we created additional mutations within the IsK domain 39–43. Deletion of leucine 42 (ΔL42 IsK coexpressed with WT KvLQT1), produced K⁺ currents that were 2.3-fold larger than those of WT IKS, with gating characteristics very similar to those of WT IKS. The currents generated by ΔL42 IsK were still sensitive to DIDS (Figure 7E). The D39N IsK mutant (coexpressed with WT KvLQT1) led to K⁺ currents smaller than WT IKS (Iₘₐₓ = 0.63 ± 0.07 µA, at +30 mV, n = 6), with gating characteristics similar to those of WT IKS and which were modestly sensitive to 100 µM DIDS (1.4-fold current stimulation, only; Figure 7E). Finally, E43N IsK coexpressed with WT KvLQT1, produced K⁺ currents that were very similar to those displayed by Δ39–43 IsK, both in terms of amplitude
Rescue of IKS channel function

Fig. 5. Effects of DIDS on the Δ73–129 and Δ73–79 IsK C-terminal mutants. (A) Currents were recorded from Δ73–129 IsK mutant coexpressed with WT KvLQT1, as in Figure 1A. (B) Current–voltage relations (6 s) of Δ73–129 IsK (n = 4) in the absence (solid squares) or presence (empty squares) of 100 µM DIDS. (C) Train of currents recorded from Δ73–129 IsK, in oocytes perfused with 100 µM DIDS and stimulated, as in Figure 2C. (D) Currents were recorded from Δ73–79 IsK mutant coexpressed with WT KvLQT1, as in Figure 1A. (E) Current–voltage relations (6 s) of Δ73–79 IsK (n = 7) in the absence (solid squares) or presence (empty squares) of 100 µM DIDS. Note that the error bars are smaller than the symbols. (F) Train of currents recorded from Δ73–79 IsK, in oocytes perfused with 100 µM DIDS and stimulated, as in Figure 2C.

(I_{max} = 0.34 ± 0.05 µA, at +30 mV, n = 12) and in terms of gating characteristics, with a threshold of activation above –60 mV, a lack of sigmoidal delay and instead, an instantaneous current component (Figure 7C and D). Like Δ39–43 IsK, the current flowing through E43N IsK did not reach steady-state following 3 s depolarizing pulses and was totally insensitive to DIDS (Figure 7D and E).

Δ39–43 IsK and E43N IsK mutants restore channel inactivation

Regarding the pivotal role played by the IsK residues 39–43, in channel gating and in rescuing the loss of I_{KS} channel function produced by the IsK C-terminal mutants, it was important to evaluate their impact on the inactivation process. Recent studies have shown that KvLQT1 channels undergo a voltage-dependent inactivation (Pusch et al., 1998; Tristani-Firouzi and Sanguinetti, 1998). Inactivation of KvLQT1 is incomplete, develops with a delay and is greatly prevented by coexpression with IsK (Pusch et al., 1998; Tristani-Firouzi and Sanguinetti, 1998). Using a triple-pulse protocol (Tristani-Firouzi and Sanguinetti, 1998), we could measure the inactivation process (Figure 8A–E). A conditioning prepulse to +20 mV was applied to activate and inactivate channels, then a brief (20 ms) hyperpolarizing interpulse allowed recovery from inactivation before a test pulse (150 ms) to various potentials was applied to reactivate and reinactivate the channels. Under these conditions, the onset of KvLQT1 inactivation was quasi immediate (Figure 8A). The decay of current during the third test pulse (reinduction of inactivation) was best fitted by a single exponential function (at P3 = +20 mV, τ = 20.7 ± 1.2 ms, n = 8). From the amplitude of the instantaneous inactivating current which is estimated by extrapolating the fitted curve, one could deduce the relative percentage of inactivation (see Materials and methods and Figure 8). Homomultimeric KvLQT1 channels produced a time- and voltage-dependent partial inactivation (Figure 8A and D). Following a 2 s prepulse to +20 mV, KvLQT1 inactivated by 15.2 ± 1.1% (n = 6) when reinduction of inactivation was measured at P3 = –10 mV (Figure 8D). In contrast to WT IsK, which virtually eliminated I_{KS} channel inactivation (Figure 8B), we found that the Δ39–43 IsK mutant (coexpressed with WT KvLQT1) regains a significant
time- and voltage-dependent inactivation (Figure 8C–E), with characteristics intermediate between those of WT \(I_{KS}\) (virtually no inactivation) and WT KvLQT1. The extent of inactivation produced by \(\Delta 39–43\) IsK is smaller than that induced by WT KvLQT1; \(\Delta 39–43\) IsK inactivated by 8.1 ± 0.1% \((n = 5)\) under the same conditions as above. There was a rightward shift (by +20 mV) in the voltage-dependence of \(\Delta 39–43\) IsK inactivation when compared with that of WT KvLQT1 (not shown; A.Peretz, I.Abitbol and B.Attali, manuscript in preparation). The \(\Delta 39–43\) IsK inactivation developed with a longer delay than that produced by WT KvLQT1 (Figure 8D and E). The delays are −550 and 120 ms for \(\Delta 39–43\) IsK and WT KvLQT1, respectively (at +20 mV prepulse of varying duration and with reinduction of inactivation measured at P3 = −10 mV, see Figure 8E). Similar results were obtained with the E43N IsK mutant which inactivated by 8.2 ± 0.3% \((n = 4)\) at 2 s prepulse to +20 mV and reinduction of inactivation measured at P3 = −10 mV).

**Discussion**

The slowly activating outwardly-rectifying \(K^+\) current, \(I_{KS}\), is essential for controlling the repolarization phase of cardiac action potentials and for \(K^+\) homeostasis in the inner ear (Noble and Tsien, 1969; Sanguinetti and Jurkiewicz, 1990; Marcus and Shen, 1994; Vetter et al., 1996). \(I_{KS}\) is formed by the heteromeric assembly of two distinct transmembrane proteins, KvLQT1 and IsK (Barhanin et al., 1996; Sanguinetti et al., 1996). The unusually slow kinetics and the very different structures of the \(\alpha\) and \(\beta\) subunits of the \(I_{KS}\) channel complex provide a unique framework for exploring the structural determinants of ionic channel function. Mutations in either \(KvLQT1\) or \(IsK\) genes produce the LQT syndrome. Functionally, several mutations produce a dominant-negative loss of channel function (Chouabe et al., 1997; Splawski et al., 1997b; Wollnik et al., 1997). In this work, we show that binding of \(I_{KS}\) channel activators such as stilbenes or fenamates to the extracellular N-terminal domain of human IsK transmembrane segment, restores normal \(I_{KS}\) channel gating in otherwise inactive IsK C-terminal mutants, including the LQT5 mutant (D76N) responsible for LQT syndrome.

The present findings map the residues 39–43 of human IsK as the domain to which stilbenes and fenamates can bind. DIDS or mefenamic acid also act, though subtly, on homomultimeric KvLQT1 channels to open them at more
Rescue of IKS channel function

Fig. 7. Mapping the DIDS-binding site. (A) Train of currents recorded from Δ39–43 IsK, in oocytes perfused with 100 µM DIDS and stimulated, as in Figure 2C. (B) Normalized conductances from tail currents of Δ39–43 IsK in the absence (solid squares) or presence of 100 µM DIDS (empty squares). V50 = −24.9 ± 7.7 mV, s = −11.9 ± 0.5 mV/e-fold and V50 = −23.7 ± 6.5 mV, s = −13.3 ± 0.7 mV/e-fold for control and DIDS-treated oocytes, respectively (n = 4). (C) Currents were recorded, as in Figure 1A, from E43N IsK mutant coexpressed with WT KvLQT1. (D) Current–voltage relations (3 s) of E43N IsK (n = 9) in the absence (solid squares) or presence (empty squares) of 100 µM DIDS. (E) Maximal current amplitudes (3 s, at +30 mV) of the various IKS N-terminal mutants (n = 6–18, *p <0.01) were measured in the absence (empty bars) or presence of 100 µM DIDS (solid bars).

hyperpolarized potentials as well as to speed up and slow down their activation and deactivation kinetics, respectively. Although the DIDS-binding domain on KvLQT1 is not yet known, our results clearly identify the residues 39–43 of human IsK, and notably the residue E43, as a pivotal interface between IsK and KvLQT1 into which stilbenes or fenamates can dock in order to drive and stabilize the IKS channel complex to the open state. Stilbenes, such as DIDS, were previously shown to reversibly block the anion exchanger AE1, by competing with anions for the external anion-binding site (Lambert and Lowe, 1978). In addition, DIDS can, over extended incubations, bind covalently to one of two neighboring lysines (539 or 542) in human AE1 (Lux et al., 1989). Indeed, DIDS can react with nucleophilic sites on the external membrane surface, including lysyl ε-amino groups (Maldonado and Cala, 1994). Likewise, DIDS could possibly react with a lysine residue, either on IsK (K41) or on KvLQT1 in the external vestibule of the pore (K220, K253 or K261) or in the S3–S4 external loop (K153). This feature may, at least partially, account for the relatively slow onset of DIDS action (~1 min).

Our data suggest an allosteric interplay between the extracellular and the intracellular boundaries of the IsK transmembrane segment as well as between domains of the α and β subunits. In the IsK C-terminal mutants, binding of stilbenes or fenamates to the extracellular IsK domain unlocks the defective C-terminal intracellular segment, and drives it to a functionally active conformation which in turn, leads to a conformational change of KvLQT1 channel α subunit. Removing the N-terminal residues 39–43 of IsK renders the channel complex insensitive to DIDS or mfenamic acid and disrupts the allosteric interplay between the N- and C-termini of IsK. The
Fig. 8. Inactivation characteristics of Δ39–43 IsK N-terminal mutant and model of allosteric interaction. (A) Inactivation of homomultimeric KvLQT1. A 2 s conditioning prepulse (P1) to +20 mV was applied to activate and inactivate KvLQT1 channels, then a brief (20 ms) hyperpolarizing interpulse to −130 mV (P2), allowed recovery from inactivation before a test pulse of 150 ms (P3) to varying potentials (from −70 to +20 mV) was applied to reactivate and reinactivate the channels. The decay of current during the P3 test pulse (reinduction of inactivation) was best-fitted by a single exponential function. (B) Inactivation of IsK, using the same protocol as in (A). The traces of P3 indicate that IsK virtually removed inactivation from KvLQT1 channels. (C) Inactivation characteristics of Δ39–43 IsK coexpressed with WT KvLQT1, using the same protocol as in (A). The decay of current during the P3 test pulse indicate that Δ39–43 IsK regains inactivation. (D) and (E) A +20 mV conditioning prepulse P1 was applied for various durations, followed by a 20 ms P2 to −130 mV and a P3 test pulse (to −10 mV for 150 ms) was evoked to reactivate and reinactivate channels (see traces in E). In (D) the percentage of inactivation of WT KvLQT1 (solid squares) and that of Δ39–43 IsK (empty squares) was calculated by dividing the current measured at the end of P3 to that extrapolated from a single exponential fit at the beginning of P3. (F) A speculative model for IsK gating. Co and Ci refer to activatable and inactivatable closed states. Cn refers to an unknown number of closed transitions through which the IsK channel complex should dwell before reaching the open state O. I refers to an inactivated state which is virtually not populated as a result of the interaction of WT IsK with WT KvLQT1. DIDS or mefenamic acid are able to shift the conformational equilibrium of the IsK C-terminal mutants, from the inactivatable closed state Ci to the activatable closed state Co, leading to the functional recovery of a WT IsK current phenotype.

Δ39–43 IsK mutation traps the cytoplasmic segment in a defective conformation which in turn, cannot produce a conformational change of KvLQT1 as efficiently as in WT IsK. The Δ39–43 IsK mutant generates a current phenotype that does not exhibit a sigmoidal delay and cannot, as with WT IsK, shift rightward the voltage-dependence of activation of KvLQT1.

Recently, we found that the intracellular C-terminus of IsK interacts with the internal vestibule of KvLQT1 and we speculated that this interaction may underlie the slowing of KvLQT1 activation gating (Romey et al., 1997). The mechanisms underlying the slowing of IsK gating have not been elucidated yet. For example, it is not clear whether IsK interaction affects, if at all, the S4 voltage sensor motion of KvLQT1. As suggested by a recent study (Wang et al., 1998), the slow kinetics of IsK are not due to a slow process of voltage-dependent subunit assembly in the membrane. Instead, voltage-dependent conformational changes of the IsK–KvLQT1 complex may account for the slow gating kinetics. Our data strongly suggest that the allosteric interplay between the N- and C-termini of IsK as well as the subsequent interaction of the IsK C-terminus with the internal vestibule of KvLQT1 are crucial determinants of the slow IsK gating. Several groups showed recently that IsK interacts with KvLQT1 to increase its unitary channel conductance (Pusch, 1998; Sesti and Goldstein, 1998; Yang and Sigworth, 1998). It will be important to check whether stilbenes and fenamates act to increase the unitary conductance of WT IsK and IsK C-terminal mutants, in addition to their effects on gating kinetics. Interestingly, these allosteric interactions also appear to be important for preventing inactivation of KvLQT1 channels. In contrast to WT IsK, which virtually eliminates channel inactivation, Δ39–43 IsK and E43N IsK mutants regain a significant time and voltage-dependent inactivation.

We suggest that IsK acts as an allosteric regulator of IsK gating and permeation. We propose a model in which the IsK channel complex exists in two discrete and interconvertible closed states (Figure 8F): an activatable closed
state Co and an inactivatable closed state Ci. For the IsK C-terminal mutants, the conformational equilibrium is shifted towards the inactivatable closed state, Ci, producing a current phenotype with marked rightward shift of the voltage-dependence of activation, faster deactivation and lower unitary conductance (Sesti and Goldstein, 1998).

This leads to a loss of channel function with a dominant-negative phenotype. DIDS and mefenamic acid shift the conformational equilibrium of these IsK C-terminal mutants towards the activatable closed state Co, leading to the functional recovery of a WT IsK current phenotype (Figure 8F). The transition into the activatable closed state, Co, as reflected by the sigmoidal delay, is a necessary step for an efficient conformational change of the IsK–KvLQT1 channel complex. Indeed, DIDS or mefenamic acid restore a sigmoidal delay and an IsK current phenotype in IsK C-terminal mutants (see Δ80–129 IsK and D76N IsK mutants).

In summary, our findings show for the first time that small organic molecules can restore the functional defect responsible for an LQT syndrome and should provide clues for the rational design of new anti-arrhythmic therapies. Our data support a model in which allosteric interactions exist between the extracellular and intracellular boundaries of the IsK transmembrane segment as well as between domains of the α and β subunits. This allosteric interplay leads to a slowing of activation gating and elimination of channel inactivation. However, several cardinal questions remain unresolved. Does the transmembrane domain of IsK modulate allosterically the permeation pathway of the KvLQT1 α subunit or does it directly line the pore of IsK (Wang, K.W. et al., 1996; Tai and Goldstein, 1998)? And if so, how does this IsK segment fit into ‘the inverted teepee’ structural constraints of a K+ channel pore (Doyle et al., 1998)? Does IsK affect the gating charge movement of the KvLQT1 voltage sensor? Future experiments should attempt to address these issues.

Materials and methods

**Molecular biology**

Site-directed and deletion mutants of human IsK were generated either on single-stranded or double-stranded DNA plasmids using the Trans-former™ mutagenesis kit (Clontech). For each mutant, DNA sequence analysis on both strands was performed prior to expression. Mouse KvLQT1 cDNA was a gift from Drs J.Barhanin and M.Lazdunski (France). Human IsK and its mutants as well as WT mouse KvLQT1 RNA injection into Xenopus oocytes were produced by injecting 2 ng cRNA/oocyte. cRNAs were quantified by UV spectroscopy and inspected for purity by gel electrophoresis.

**RNA injection into Xenopus oocytes**

*Xenopus laevis* frogs were purchased from C.R.B.M (Montpellier, France). Frogs were anesthetized with 0.2% tricaine (Sigma). Pieces of the ovary were surgically removed and digested with 2 mg/ml collagenase (type IA, Sigma) in CrCl2-free ND96 (in mM: 96 NaCl, 2 KCl, 1 MgCl2 and 5 HEPES titrated to pH 7.5 with NaOH) for 1.5 h, to remove follicular cells. Stage V and VI oocytes were used for cRNA injection and maintained at 18°C in ND96 (1.8 mM CaCl2), supplemented with 1 mM pynvate and 50 pg/ml gentamycin. Human IsK and its mutants were injected at 1 ng cRNA/oocyte together with WT KvLQT1 at 2 ng cRNA/oocyte. Homomultimeric expression of WT KvLQT1 was performed by injecting 2 ng cRNA/oocyte.

**Electrophysiology**

Standard two-electrode voltage-clamp measurements were performed 3–5 days following cRNA microinjection into oocytes. Oocytes were bathed in a modified ND96 solution containing (in mM): 96 NaCl, 2 KCl, 1 MgCl2, 0.1 CaCl2 and 5 HEPES titrated to pH 7.5 with NaOH under constant perfusion using a peristaltic pump (Gilson) at a flow rate of 0.4 ml/min. CaCl2 was reduced to 0.1 mM to virtually eliminate the contribution of endogenous Ca2+–activated Cl− currents. Whole-cell currents were recorded at room temperature (20–22°C) using a Gene-Clamp 500 amplifier (Axon Instruments). Glass microelectrodes (A-M systems, Inc) were filled with 3 M KCl and had tip resistances of 0.5–1.5 MΩ. Stimulation of the preparation, data acquisition and analyses were performed using the pCLAMP 6.02 software (Axon Instruments) and a 586 personal computer interfaced with a Digidata 1200 interface (Axon Instruments). Current signals were filtered at 0.2–0.5 kHz and digitized at 1–2 kHz. The holding potential was −80 mV. Leak subtraction was performed off-line, using the Clampfit program of the pCLAMP 6.02 software. Drugs such as DIDS, SITS and mefenamic acid (Sigma) were diluted from stock solutions (0.5 M in dimethyl sulfoxide) and applied either externally to the superfuse or internally (10 nl) with a PL1-100 microinjector (Medical Systems Corp) using an additional micropipette (2–4 μm diameter).

**Data analyses**

Data analysis was performed using the Clampfit program (pCLAMP 6.02, Axon Instruments), Microsot Excel 5.0 (Microsoft), Axograph 3.0 (Axon Instruments) and CA-Cricket Graph III (Computer Associates International). The simplex algorithm was used to fit exponential functions to current traces in order to determine the time constants and their amplitudes. To analyze the voltage dependence of IsK channel activation, a single exponential fit was applied to the tail currents and extrapolated to the beginning of the repolarizing step. Chord conductance (G) was calculated by using the following equation:

\[ G = \ln(V – V_m) \]

where J corresponds to the extrapolated tail current and V_m, the measured reversal potential assumed to be −90 mV (−90 ± 2 mV, n = 7). G was estimated at various test voltages V and then, normalized to a maximal conductance value, G_max, calculated at +30 mV. Activation curves were fitted by a Boltzmann distribution:

\[ G/G_{\max} = 1/[1+\exp((V\mu – V/V)/k)] \]

where V_μ is the voltage at which the current is half-activated and s is the slope factor. To analyze the voltage dependence of KvLQT1 channel activation, G was deduced either from steady-state currents or from tail currents as above. For quantification of inactivation, the decay of current during the third test pulse (reinduction of inactivation) was best fitted by a single exponential function. The relative percentage of inactivation was calculated by dividing the current measured at the end of the third pulse to that extrapolated from the single exponential fit at the beginning of the third pulse. All data were expressed as mean ± SEM. Statistically significant differences were assessed by Student’s t-test.

**Acknowledgements**

We thank Dr Eitan Reuveni, Professor Israel Silman and Professor Vivian Teichberg for helpful discussions and a careful reading of the manuscript. This work was supported by grants from the Minerva Foundation, the Israel Science Foundation, the Israel Ministry of Health and the German–Israel Foundation (to B.A.). B.A. is an incumbent of the Philip Harris and Gerald Ronson Career Development Chair.

**References**


Received May 6, 1999; revised and accepted June 8, 1999