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Stilbenes and fenamates rescue the loss of I_{KS} channel function induced by an LQT5 mutation and other IsK mutants

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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 I_{KS} 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 I_{KS} channel activators, such as stilbenes and fenamates, to an extracellular domain flanking the human IsK transmembrane segment, restores normal I_{KS} 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 I_{KS} produced by IsK mutations and thus, may have important therapeutic relevance for LQT syndrome.

Keywords: I_{KS}/IsK/LQT/Mink/potassium channels

Introduction

The I_{KS} 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 I_{KS} 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,Q. *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 pointes'. 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 I_{KS} (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 I_{KS} 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 I_{KS} 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 I_{KS} channel complex (Busch *et al*., 1994, 1997) such as 4,4′-diisothiocyanatostilbene-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 I_{KS} 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 V_{50} = –28.1 ± 1.5 mV, s = –17.8 ± 1.2 mV/e-fold to V_{50} = –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′-isothiocyanatostilbene-2,2′-disulfonic acid (100 μM SITS) and with a fenamate compound, 100 μM mefenamic 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 I_{KS} 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 I_{KS} currents than on homomultimeric KvLQT1 channels (Figure 2). The sigmoidal delay in I_{KS} activation kinetics disappeared and instead, a major quasi-instantaneous current became evident, together with an ~2.4-fold increase in maximal current amplitude (I_{max}) (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 I_{KS} current activation could be described by a single exponential, with τ_{act} = 4.14 ± 0.51 s (6 s pulse at +30 mV; n = 7). As for homomultimeric KvLQT1 channels, DIDS was active on I_{KS} 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 I_{KS}. Control I_{KS} activated above a threshold of ~ –30 mV, while DIDS-treated I_{KS} channels opened at much more negative potentials (threshold above ~ –60 mV). Following DIDS superfusion (3 min), I_{KS} deactivation kinetics were markedly slowed down, with time constants increasing from τ_{deact} = 1.069 ± 0.122 s to τ_{deact} = 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 I_{KS}, 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 I_{KS} 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 I_{KS} channels towards the open state.

Effects of DIDS and mefenamic 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 I_{KS} 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 I_{KS} (Figures 3B and 4B) and an acceleration of the deactivation kinetics (Figure 3A; for Δ80–129 IsK, τ_{deact} = 0.185 ± 0.011 s, as compared with τ_{deact} = 1.115 ± 0.109 s for WT I_{KS}; from a 6 s step at +30 mV and

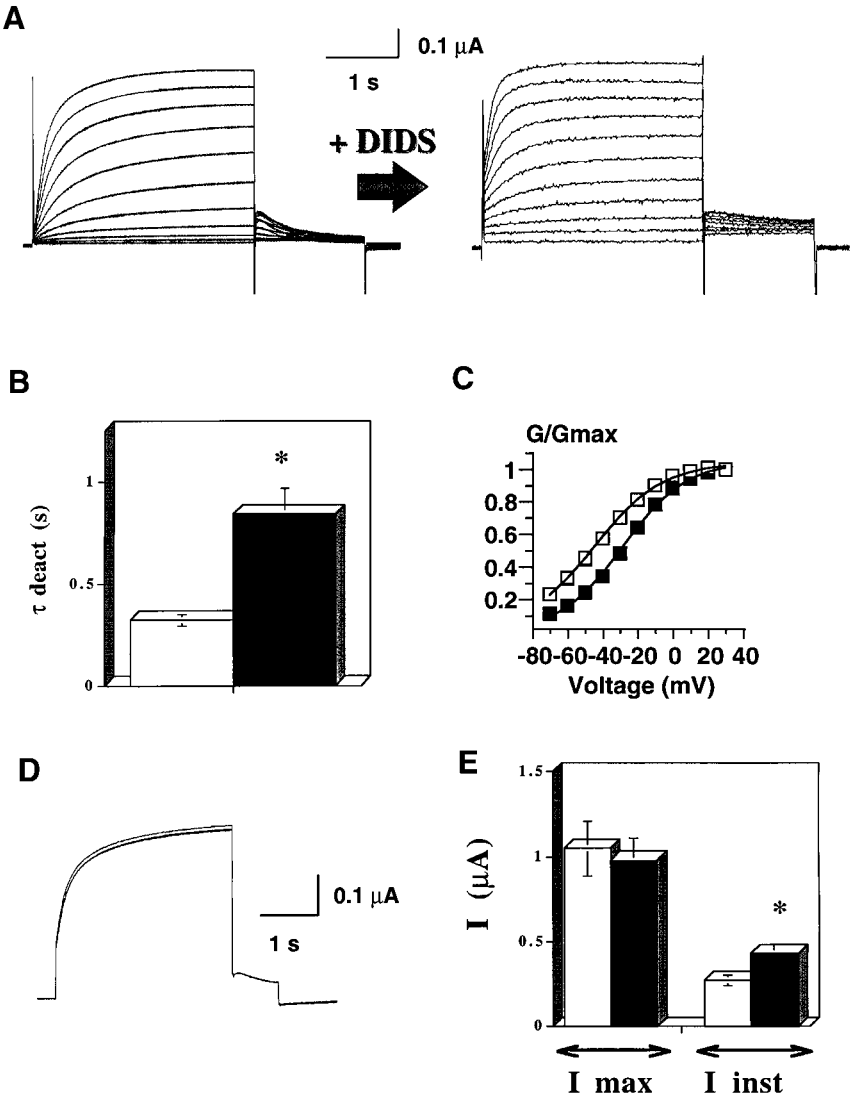

Fig. 1. Effects of DIDS on WT homomultimeric KvLQT1. (**A**) KvLQT1 currents were recorded from 2.5 s depolarizing pulses to potentials of –70 to +30 mV, in 10 mV increments from a holding potential of –80 mV, before (left) and following 10 min superfusion with 100 μM DIDS (right). Tail currents were recorded at –60 mV. (**B**) DIDS slowed deactivation of KvLQT1 with τ_{deact} = 0.321 ± 0.029 s and τ_{deact} = 0.841 ± 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_{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 ± 0.5 μA (DIDS-treated, solid bar) (n = 9, p <0.01). Maximal current amplitude (I_{max}) was measured following a 2.5 s step to +30 mV in control (empty bar) and DIDS-treated oocytes (solid bar).

tail at –80 mV, n = 6, p <0.01). 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 I_{KS} 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_{max} = 0.12 ± 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_{max} = 0.24 ± 0.06 μA) than that induced by WT I_{KS} (I_{max} = 2.32 ± 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 I_{KS}) 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 I_{KS}, 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

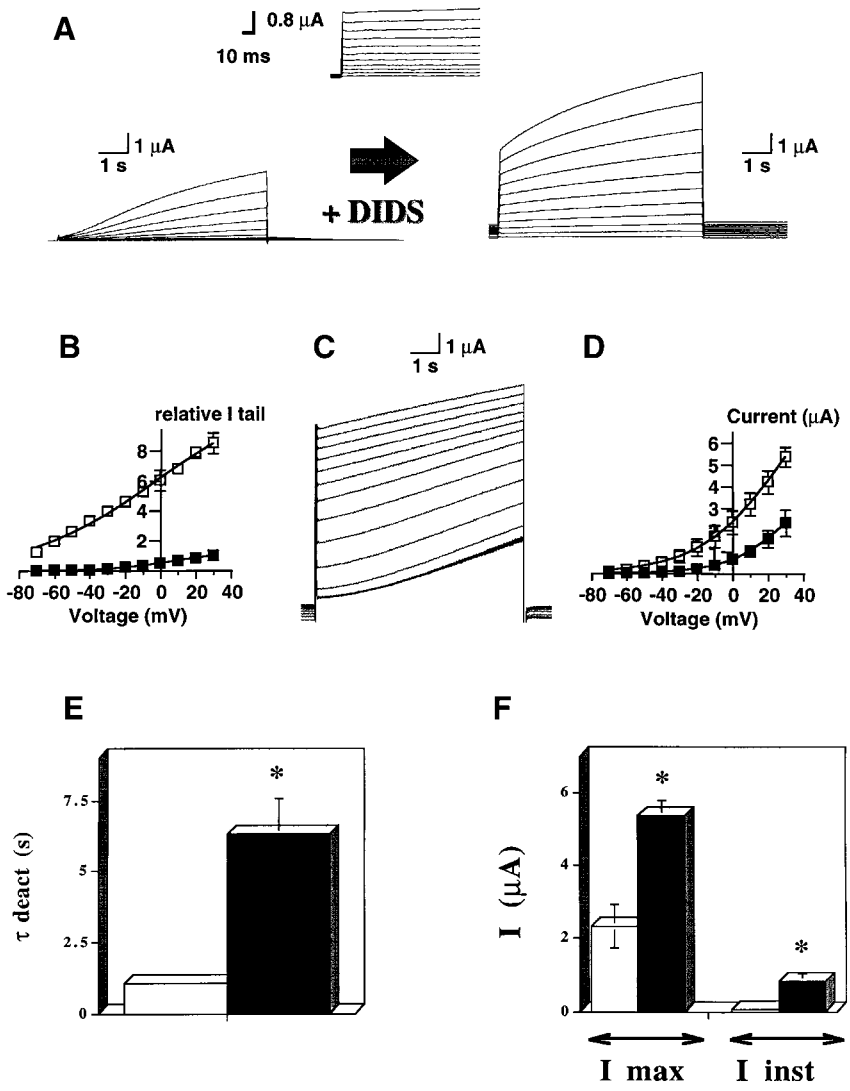

Fig. 2. Effects of DIDS on WT I_{KS}. (**A**) I_{KS} currents were recorded as in Figure 1A for 6 s pulses before (left) and following 10 min superfusion with 100 μM DIDS (right). Note, the appearance of a holding positive current that did not deactivate at –80 mV. Inset shows the effect of DIDS on I_{KS} rising phase, at the millisecond time range. (**B**) Relative tail current–voltage relations. In 3 min DIDS-treated oocytes (empty squares), tail currents at –80 mV were normalized, relative to those of control I_{KS} (solid squares). Control I_{tail} = 117.5 ± 6.4 nA, n = 9, as measured from a 6 s step to +30 mV. (**C**) Train of I_{KS} currents were recorded at +20 mV for 6 s at 0.03 Hz stimulation in the presence of 100 μM DIDS (added after the third pulse). (**D**) Current–voltage relations (6 s) of I_{KS} (n = 9) in the absence (solid squares) or presence of 100 μM DIDS (empty squares). (**E**) When compared with the control (empty bar), deactivation time constants of I_{KS} significantly increased (n = 8, p <0.01) following 3 min superfusion with 100 μM DIDS (solid bar), as measured from a 6 s step at +30 mV and tail at –80 mV (for parameters, see results). (**F**) When compared with the control (empty bars), the maximal current amplitude (I_{max}, measured following a 6 s pulse to +30 mV, n = 13, *p <0.01) and the instantaneous current (I_{inst}, measured as in Figure 1E, n = 8, *p <0.01) of I_{KS} were significantly increased following a 10 min superfusion with 100 μM DIDS (solid bars).

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 I_{KS} 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– and conductance–voltage relationships (Figures 3B, 3E and 4B). When measured from tail currents, V_{50} = –3.6 ± 1.1 mV, s = –14.8 ± 0.5 mV/e-fold (n = 11), V_{50} = –7.6 ± 3.1 mV, s = –12.5 ± 0.7 mV/e-fold (n = 16) and V_{50} = –28.2 ± 0.8 mV, s = –12.2 ± 0.3 mV/e-fold (n = 4) for WT I_{KS}, 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 I_{KS} was obtained (~10-fold current stimulation), when oocytes coexpressing D76N IsK, WT IsK and WT KvLQT1 were perfused with 100 μM mefenamic

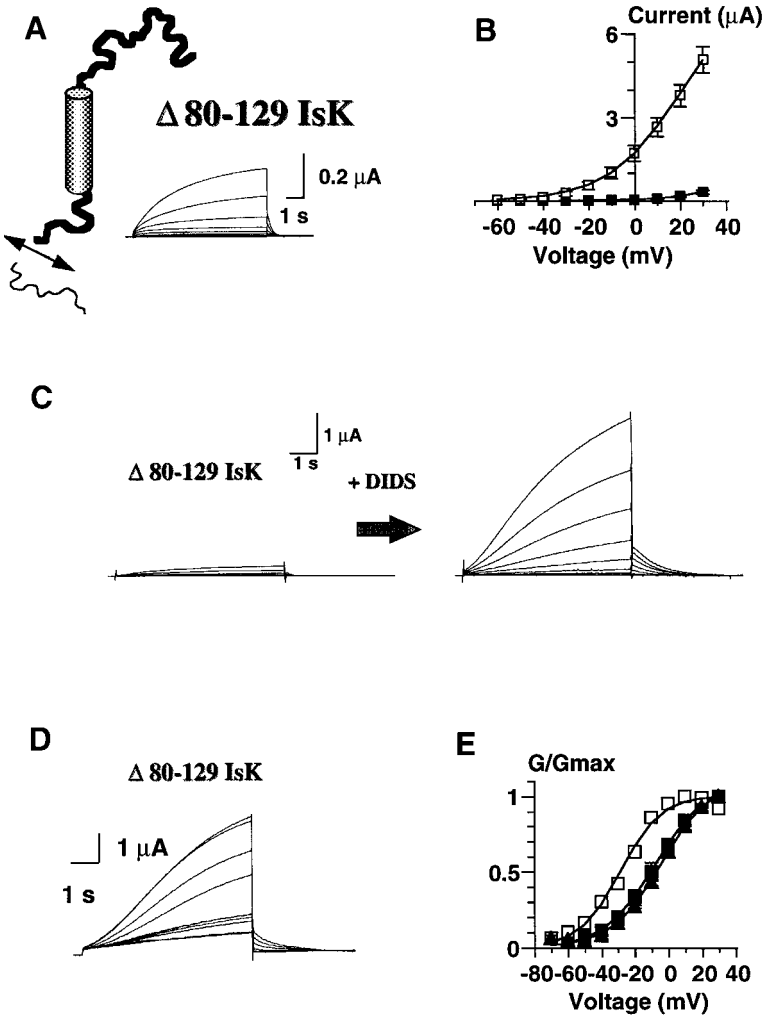

Fig. 3. Effects of DIDS on the Δ80–129 IsK C-terminal mutant. (**A**) Currents were recorded from Δ80–129 IsK mutant coexpressed with WT KvLQT1, as in Figure 1A. (**B**) Current–voltage relations (6 s) of Δ80–129 IsK (n = 14) in the absence (solid squares) or presence (empty squares) of 100 μM DIDS. (**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 I_{KS} (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 I_{KS} 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 I_{KS} 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 I_{KS} 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 I_{KS}, 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 I_{KS} (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 I_{KS}, 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

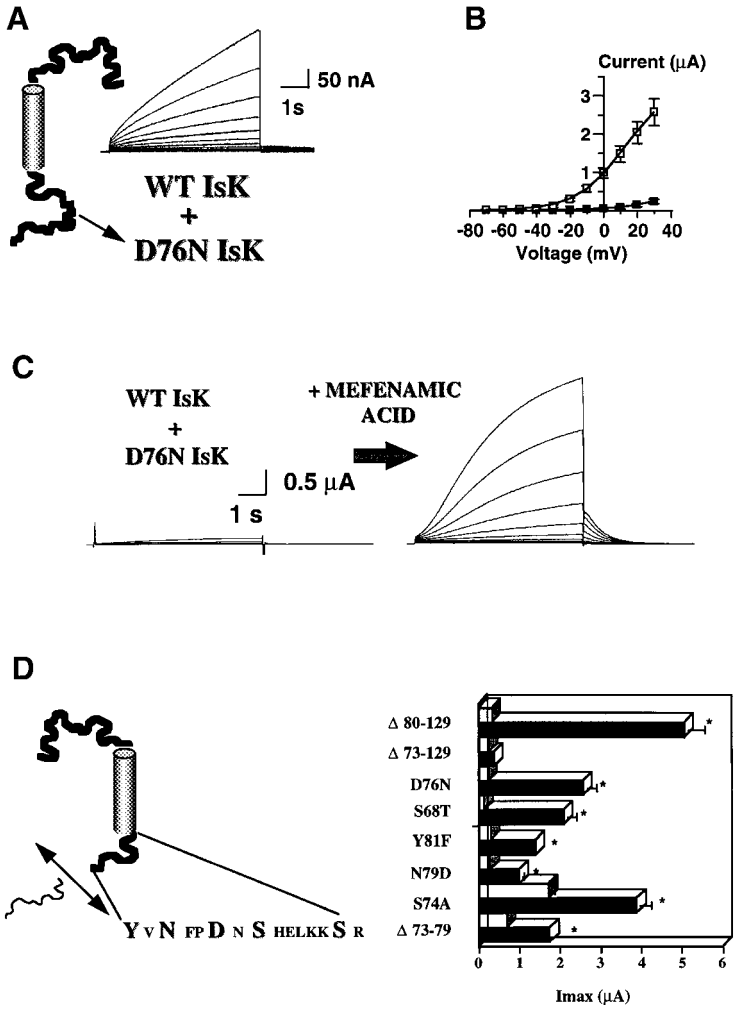

Fig. 4. Effects of DIDS and mefenamic acid on the LQT5 D76N IsK mutation and other IsK C-terminal mutants. (**A**) Currents were recorded as in Figure 1A, from oocytes coexpressing D76N IsK (1 ng cRNA/oocyte), WT IsK (1 ng cRNA/oocyte) and WT KvLQT1 (2 ng cRNA/oocyte). (**B**) Current–voltage relations (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 I_{KS} (I_{max} = 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, τ_{act1} = 0.114 ± 0.010 s and τ_{act2} = 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 I_{KS}, 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, V_{50} = –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/Δ80–129 IsK (Figure 7E). These finding suggest that the IsK residues 39–43 are crucially involved in the action of DIDS and mefenamic acid on WT I_{KS} 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 I_{KS}, with gating characteristics very similar to those of WT I_{KS}. 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 I_{KS} (I_{max} = 0.63 ± 0.07 μA, at +30 mV, n = 6), with gating kinetics similar to those of WT I_{KS} 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

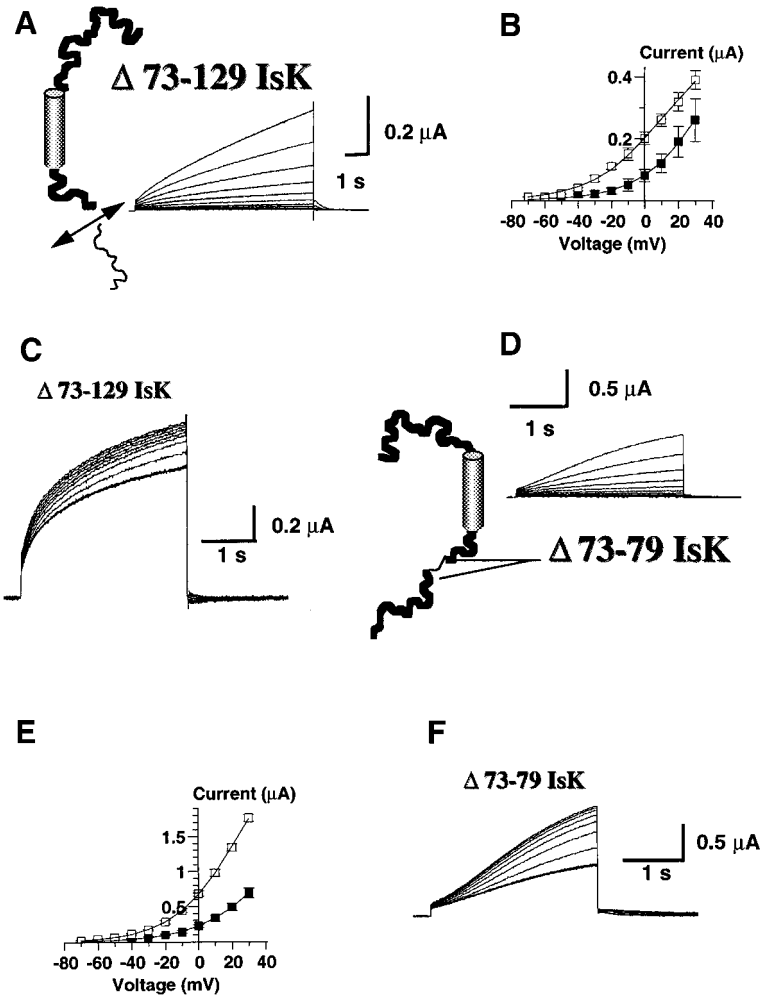

Fig. 5. Effects of DIDS on the Δ73–129 and Δ73–79 IsK C-terminal mutants. (**A**) Currents were recorded from Δ73–129 IsK mutant coexpressed with WT KvLQT1, as in Figure 1A. (**B**) Current–voltage relations (6 s) of Δ73–129 IsK (n = 4) in the absence (solid squares) or presence (empty squares) of 100 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

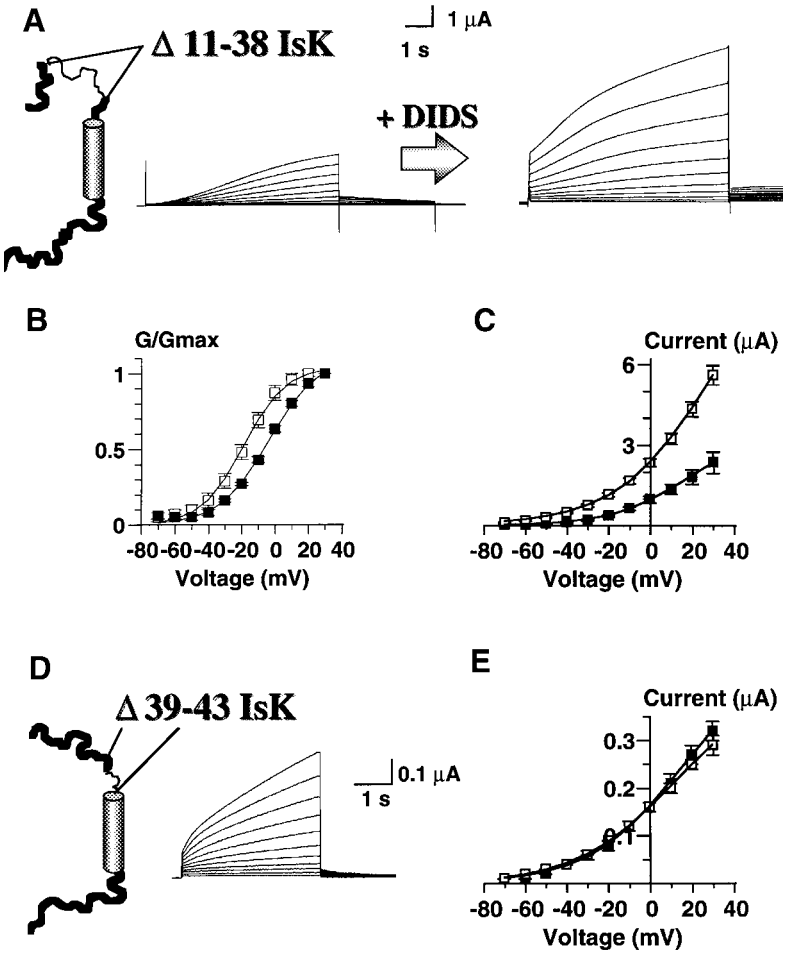

Fig. 6. Effects of DIDS on IsK N-terminal mutants. (**A**) Currents were recorded, as in Figure 1A, from Δ11–38 IsK mutant coexpressed with WT KvLQT1, before (left) and after 5 min superfusion with 100 μM DIDS (right). (**B**) Normalized conductances of Δ11–38 IsK (empty squares, $n = 13$) and for comparison, that of WT I_{KS} (solid squares, $n = 11$), as deduced from tail currents (for parameters, see Results). Note that the error bars are smaller than the symbols. (**C**) Current–voltage relations (6 s) of Δ11–38 IsK ($n = 4$) in the absence (solid squares) or presence (empty squares) of 100 μM DIDS. (**D**) Currents were recorded, as in Figure 1A, from Δ39–43 IsK mutant coexpressed with WT KvLQT1. (**E**) Current–voltage relations (3 s) of Δ39–43 IsK ($n = 10$) in the absence (solid squares) or presence (empty squares) of 100 μM DIDS.

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 Δ39–43 IsK is smaller than that induced by WT KvLQT1; Δ39–43 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 Δ39–43 IsK inactivation when compared with that of WT KvLQT1 (not shown; A.Peretz, I.Abitbol and B.Attali, manuscript in preparation). The Δ39–43 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 Δ39–43 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 α and β 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 boundary 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

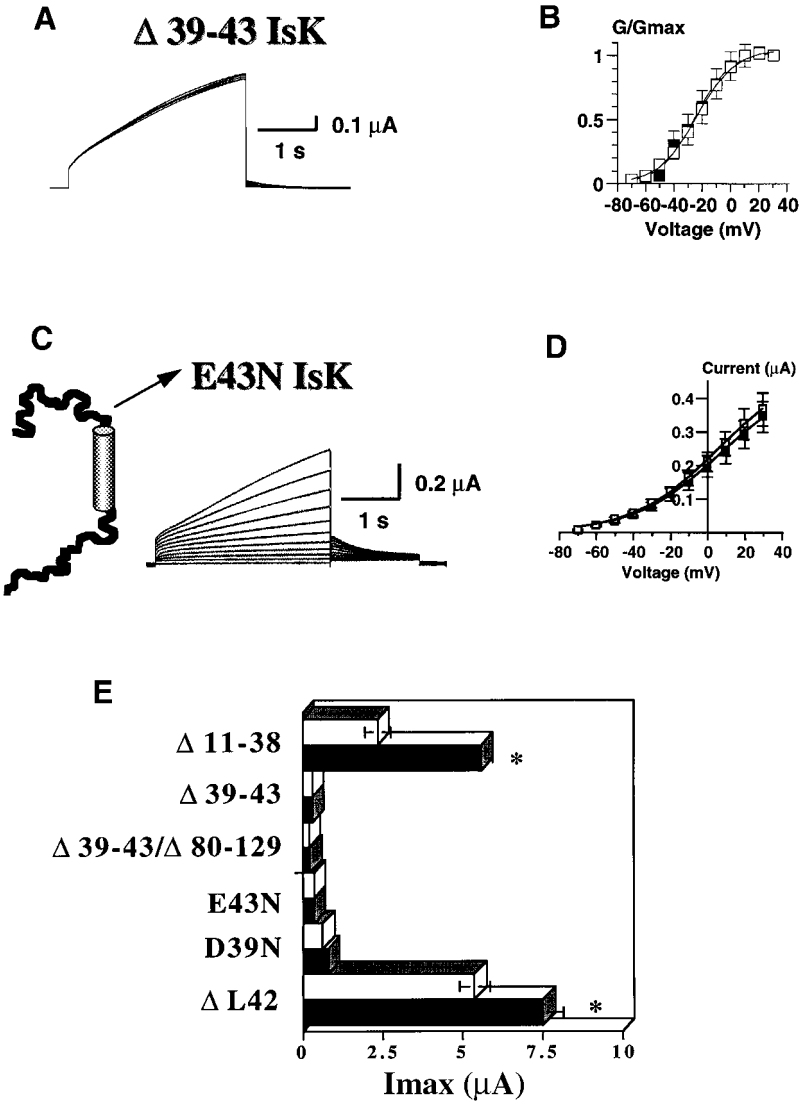

Fig. 7. Mapping the DIDS-binding site. (**A**) Train of currents recorded from Δ39–43 IsK, in oocytes perfused with 100 μM DIDS and stimulated, as in Figure 2C. (**B**) Normalized conductances from tail currents of Δ39–43 IsK in the absence (solid squares) or presence of 100 μM DIDS (empty squares). V_{50} = –24.9 ± 7.7 mV, s = –11.9 ± 0.5 mV/e-fold and V_{50} = –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 IsK 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 I_{KS} 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 mefenamic acid and disrupts the allosteric interplay between the N- and C-termini of IsK. The

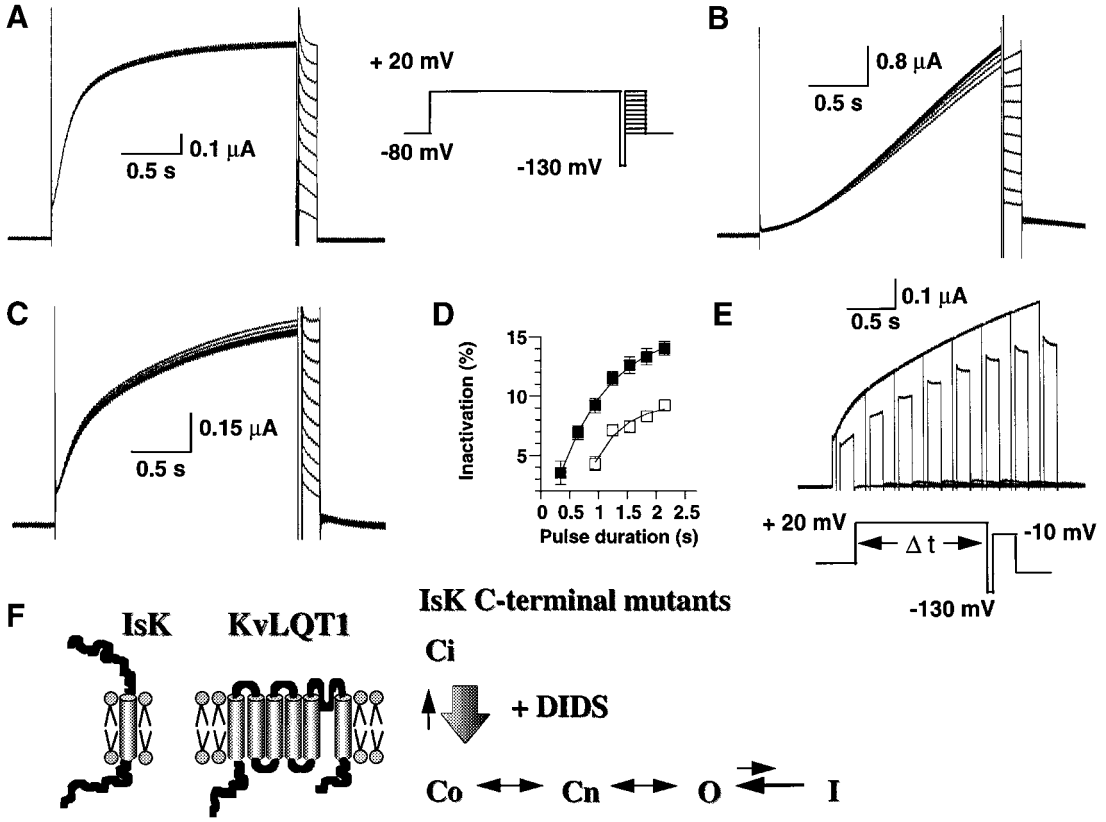

Fig. 8. Inactivation characteristics of Δ39–43 IsK N-terminal mutant and model of allosteric interaction. (**A**) Inactivation of homomultimeric KvLQT1. A 2 s conditioning prepulse (P1) to +20 mV was applied to activate and inactivate KvLQT1 channels, then a brief (20 ms) hyperpolarizing interpulse to –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 I_{KS}, 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 I_{KS} gating. Co and Ci refer to activatable and inactivatable closed states. Cn refers to an unknown number of closed transitions through which the I_{KS} 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 I_{KS} 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 I_{KS}. 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 I_{KS} 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 I_{KS} 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 I_{KS} 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 I_{KS} 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 I_{KS} gating and permeation. We propose a model in which the I_{KS} 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 I_{KS} 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 I_{KS} 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 I_{KS} (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 Transformer™ 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 were linearized by *Bam*HI. Capped complementary RNAs (cRNAs) were transcribed from WT and mutants of human IsK and WT mouse KvLQT1 by T3 and T7 RNA polymerases (Stratagene), respectively. 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 Ca^{2+}-free ND96 (in mM: 96 NaCl, 2 KCl, 1 $MgCl_2$ 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 $CaCl_2$), supplemented with 1 mM pyruvate and 50 μg/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 $MgCl_2$, 0.1 $CaCl_2$ 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. $CaCl_2$ was reduced to 0.1 mM to virtually eliminate the contribution of endogenous Ca^{2+}-activated Cl^- currents. Whole-cell currents were recorded at room temperature (20–22°C) using a GeneClamp 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 superfusate or internally (10 nl) with a PLI-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), Microsoft 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 I_{KS} 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:

$$G = I/(V-V_{rev})$$

where I corresponds to the extrapolated tail current and V_{rev}, 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:

$$G/G_{max} = 1/\{1+\exp[(V_{50}-V)/s]\}$$

where V_{50} 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.

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