Differential Activation of Vascular Smooth Muscle Kv7.4, Kv7.5, and Kv7.4/7.5 Channels by ML213 and ICA-069673[§]

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ABSTRACT

Recent research suggests that smooth muscle cells express Kv7.4 and Kv7.5 voltage-activated potassium channels, which contribute to maintenance of their resting membrane voltage. New pharmacologic activators of Kv7 channels, ML213 (*N*-mesitylbicyclo[2.2.1]heptane-2-carboxamide) and ICA-069673 (*N*-(6-chloropyridin-3-yl)-3,4-difluorobenzamide), have been reported to discriminate among channels formed from different Kv7 subtypes. We compared the effects of ML213 and ICA-069673 on homomeric human Kv7.4, Kv7.5, and heteromeric Kv7.4/7.5 channels exogenously expressed in A7r5 vascular smooth muscle cells. We found that, despite its previous description as a selective activator of Kv7.2 and Kv7.4, ML213 significantly increased the maximum conductance of homomeric Kv7.4 and Kv7.5, as well as heteromeric Kv7.4/7.5 channels, and induced a negative shift of their activation curves. Current deactivation rates decreased in the

presence of the ML213 (10 μ M) for all three channel combinations. Mutants of Kv7.4 (W242L) and Kv7.5 (W235L), previously found to be insensitive to another Kv7 channel activator, retigabine, were also insensitive to ML213 (10 μ M). In contrast to ML213, ICA-069673 robustly activated Kv7.4 channels but was significantly less effective on homomeric Kv7.5 channels. Heteromeric Kv7.4/7.5 channels displayed intermediate responses to ICA-069673. In each case, ICA-069673 induced a negative shift of the activation curves without significantly increasing maximal conductance. Current deactivation rates decreased in the presence of ICA-069673 in a subunit-specific manner. Kv7.4 W242L responded to ICA-069673-like wild-type Kv7.4, but a Kv7.4 F143A mutant was much less sensitive to ICA-069673. Based on these results, ML213 and ICA-069673 likely bind to different sites and are differentially selective among Kv7.4, Kv7.5, and Kv7.4/7.5 channel subtypes.

Introduction

Members of the KCNQ (Kv7) voltage-gated potassium channel family are differentially expressed throughout the body. The five known Kv7 channel subtypes (Kv7.1–7.5 encoded by KCNQ1–KCNQ5 genes) play major roles in the regulation of membrane potential and cell excitability within different tissues (Barhanin et al., 1996; Biervert et al., 1998; Charlier et al., 1998; Kubisch et al., 1999; Lerche et al., 2000; Schroeder et al., 2000; Brueggemann et al., 2007; Yeung et al., 2007; Mackie et al., 2008; Jepps et al., 2009; McCallum et al., 2011; Ng et al., 2011). The channels are formed as tetrameric complexes (homotetramers or heterotetramers) of the individual gene products (Schwake et al., 2003). In neurons, channels formed by combinations of Kv7.3 with Kv7.2 or Kv7.5 underlie M= currents that are the targets of novel antiepileptic drugs, such as retigabine (*N*-(2-amino-4-[fluorobenzylamino]-phenyl) carbamic acid; trade name: Ezogabine) (Wickenden and McNaughton-Smith, 2009; Grunnet et al., 2014). In other tissues, Kv7 channel composition differs (e.g., homomeric Kv7.4 channels are found in the inner ear,

and homomeric Kv7.1 channels are expressed in the heart). However, to the extent that drugs such as retigabine do not discriminate among the different channel configurations (retigabine is an activator of channels formed from all known combinations of Kv7.2, Kv7.3, Kv7.4, or Kv7.5 subunits), there is a risk of unwanted off-target effects. Development of drugs that exhibit selectivity for specific channel configurations could provide great potential for therapeutic targeting with fewer adverse side effects.

The first evidence that Kv7 channels are expressed in smooth muscle came from electrophysiologic measurements of M= currents in the gastric smooth muscle of toads (Sims et al., 1985). More recently, expression of Kv7.4 and Kv7.5 was detected in smooth muscle from multiple arterial beds in mice, rats, and humans (Yeung et al., 2007; Joshi et al., 2009; Ng et al., 2011; Chadha et al., 2012), as well as in nonvascular smooth muscle: from the gastrointestinal tract (Ohya et al., 2002; Jepps et al., 2009; Ipavec et al., 2011), the bladder (Afeli et al., 2013; Svalo et al., 2013), the uterus (McCallum et al., 2009; McCallum et al., 2011), and the airway (Brueggemann et al., 2012, 2014a; Evseev et al., 2013). There have been suggestions that smooth muscle Kv7 channels may serve as important therapeutic targets, although research to date has used drugs that do not discriminate between vascular and neuronal Kv7 channels, raising questions about unwanted off-target effects.

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ABBREVIATIONS: ANOVA, analysis of variance; GFP, green fluorescent protein; ICA-27243, *N*-(6-chloropyridin-3-yl)-3,4-difluorobenzamide; IV, current-voltage relationship; ML213, *N*-mesitylbicyclo[2.2.1]heptane-2-carboxamide.

Development of the Kv7 channel pharmacopoeia has been directed primarily toward activators of neuronal M = channels in hopes of finding new drugs to treat epilepsy and neuropathic pain. However, recent reports have refocused attention on smooth muscle isoforms of Kv7 channels, noting that side effects of the M-channel activator retigabine, such as urinary retention and constipation, are likely due to off-target actions on smooth muscle Kv7 channels (French et al., 2011; Brickel et al., 2012; Jepps et al., 2013). Recent evidence suggests that Kv7.4 and Kv7.5 are the predominant functional isoforms in vascular smooth muscle, with expression of heteromeric Kv7.4/7.5 channels detected in rat mesenteric and middle cerebral arterial myocytes (Brueggemann et al., 2014b; Chadha et al., 2014).

A few pharmacologic Kv7 channel activators display selectivity among Kv7.2–7.5 channel complexes. ICA-27243 (*N*-(6-chloropyridin-3-yl)-3,4-difluorobenzamide) was found to be 20-fold more potent as an activator of heteromeric Kv7.2/7.3 channels than homomeric Kv7.4 channels, unable to activate homomeric Kv7.3, and only a weak activator of heteromeric Kv7.3/7.5 channels (Wickenden et al., 2008; Padilla et al., 2009; Blom et al., 2010). Unfortunately, the efficacy of ICA-27243 on homomeric Kv7.5 and heteromeric Kv7.4/7.5 was not evaluated. The only drug shown to distinguish among Kv7.4 and Kv7.5 channels was diclofenac (benzeneacetic acid, 2-[(2,6-dichlorophenyl) amino]-monosodium salt), which enhanced homomeric Kv7.4 channels, blocked homomeric Kv7.5 channels, and had intermediate effects on heteromeric Kv7.4/7.5 channels (Brueggemann et al., 2011). Identifying new drugs that act selectively on smooth muscle isoforms of Kv7 channels could be of great interest for recognition of functional channel composition, as well as to enable improved clinical therapies with fewer off-target effects. Here we compare effects of a new commercially available Kv7 channel activator, ML213 (*N*-mesitylbicyclo[2.2.1]heptane-2-carboxamide), which was reported to distinguish between Kv7.4 and Kv7.5 (Yu et al., 2011), with ICA-069673 (*N*-(2-chloro-pyrimidin-5-yl)-3,4-difluoro-benzamide), a structural analog of ICA-27243, on homomeric human Kv7.4, Kv7.5, and heteromeric Kv7.4/7.5 channels using A7r5 vascular smooth muscle cells as an expression system.

Materials and Methods

Construction of Mutants Kv7.5 W235L, Kv7.4 W242L, and Kv7.4 F143A. Retigabine-insensitive mutant Kv7.5 W235L was designed using QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. Using 50 ng of hKCNQ5 pIRES2-EGFP cDNAs, mutagenic oligonucleotide primer CAGCAAGGAATTAATCACAGCTTTGTACATAGGATTTTGGTTCTTA and its reverse complement (Integrated DNA Technologies, Inc.) were used to synthesize the mutated plasmid

Kv7.4

Fig. 1. ML213 induced a concentration-dependent enhancement of the Kv7.4 current accompanied by negative shift of the activation curve and prolonged Kv7.4 current deactivation. (A, B) Representative traces of Kv7.4 currents recorded with voltage-step protocol in the absence (A) and in the presence of 10 μM ML213 (B). (C) IV curves of normalized steady-state Kv currents recorded before (control, black circles) and after 10-minute treatment with increasing concentrations of ML213 as indicated ($n = 5$). (D) Averaged fractional conductance plots normalized to maximal conductance in control for each experiment fitted to a Boltzmann distribution (at increasing concentrations of ML213 as indicated in (C)). (E) Representative traces of tail currents recorded in the absence (black, current scale at left) and in the presence of 10 μM ML213 (red, current scale at right) scaled to size of the currents recorded at 0 mV in the absence of ML213 for visual comparison. (F) The time constants (τ) of current deactivation, calculated from the single exponential fits of tail currents measured at voltages ranging from -130 mV to -90 mV in the absence (black circles) and in the presence of 10 μM ML213 (red circles) fitted to straight lines. * $P < 0.05$, paired Student's t test, $n = 5$.

according to the manufacturer's protocol. Retigabine-insensitive mutant Kv7.4 W242L (Schenzer et al., 2005) was generously provided by Dr. Michael Schwake. The Kv7.4 F143A mutant was designed using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's protocol. To synthesize the mutated

TABLE 1

ML213 dose-dependence parameters based on negative shift of activation curve and increase in maximal conductance for homomeric Kv7.4, Kv7.5, and heteromeric Kv7.4/7.5 channels

Kv7 Isoforms	$G_{\max}$	EC ₅₀ Based on $G_{\max}$	$ \Delta V_{0.5} $	EC ₅₀ Based on $\Delta V_{0.5}$
	% of Control	μM	mV	μM
Kv7.4	212.3 ± 27.4	0.8 ± 0.3	25.0 ± 2.5	1.6 ± 0.2
Kv7.5	180.7 ± 16.9	0.7 ± 0.2	43.9 ± 7.7*	4.5 ± 2.0
Kv7.4/7.5	204.8 ± 11.1	1.1 ± 0.6	34.2 ± 3.3	3.8 ± 1.2

*Significant difference from Kv7.4, Student's t test, $P < 0.05$, $n = 5$.

plasmid, 10 ng of hKCNQ4 pIRES2-EGFP cDNA, mutagenic oligonucleotide primer CTTGGAATTCGTGATGATCGTGGTTGCCGCTTG-GAGTAC and its reverse complement (Integrated DNA Technologies, Coralville, IA) were used. The mutations were confirmed by DNA sequencing (ACGT, Inc., Wheeling, IL).

Cell Culture. A7r5 cells were cultured as described previously (Byron and Taylor, 1993). For overexpression studies, subcultured A7r5 cells at 70% confluence were infected with Adv-Kv7.4 or Adv-Kv7.5 or both at a multiplicity of infection of 100 and used for electrophysiologic experiments 2–12 days after infection as previously described (Brueggemann et al., 2011). For mutagenesis studies, subcultured A7r5 cells at 70% confluence were transfected with appropriate Kv7 channel mutants inserted into a pIRES2-EGFP (Kv7.5 W235L, Kv7.4 F143A) or pShuttle-IRES-hrGFP-2 (Kv7.4 W242L) vector using Lipofectamine transfection reagent according to the manufacturer's protocol. Cells expressing the exogenous channels were identified based on the detection of green fluorescence protein (GFP).

Patch Clamp. The whole-cell perforated patch configuration was used to measure membrane currents under voltage-clamp conditions. All experiments were performed at room temperature with continuous perfusion of bath solution as described previously (Brueggemann et al., 2009, 2011). The standard bath solution contained the following (in mM): 5 KCl, 130 NaCl, 10 HEPES, 2 CaCl₂, 1.2 MgCl₂, 5 glucose, pH 7.3. Standard internal (pipette) solution contained the following (in mM): 110 K gluconate, 30 KCl, 5 HEPES, 1 K₂EGTA, pH 7.2. Osmolality was adjusted to 270 mOsm/liter with D-glucose. Voltage-clamp command potentials were generated using an Axopatch 200B amplifier under control of PCLAMP9 software. Amphotericin B (120 µg/ml) in the internal solution was used for membrane patch perforation. Series resistance after amphotericin perforation was 8–15 MΩ and was compensated by 60%. Whole-cell currents were digitized at 2 kHz and filtered at 1 kHz. Stable currents were recorded for at least 15 minutes before drug application.

Fig. 2. ML213 induced a concentration-dependent enhancement of Kv7.5 current accompanied by negative shift of the activation curve and decreased Kv7.5 current deactivation rate. (A, B) Representative traces of Kv7.5 currents recorded with a voltage step protocol in the absence (A) and in the presence of 10 µM ML213 (B). (C) IV curves of normalized steady-state Kv currents recorded before (control, black circles) and after 10-minute treatment with increasing concentrations of ML213 as indicated ($n = 4$ to 5). (D) Averaged fractional conductance plots normalized to maximal conductance in control for each experiment fitted to a Boltzmann distribution (at increasing concentrations of ML213 as indicated in C). (E) Representative traces of tail currents recorded in the absence (black, current scale at left) and in the presence of 10 µM ML213 (red, current scale at right) scaled to size of the currents recorded at 0 mV in the absence of ML213 for visual comparison. (F) The time constants (τ) of current deactivation, calculated from the single exponential fits of tail currents measured at voltages ranging from -130 mV to -105 mV in the absence (black circles) and in the presence of 10 µM ML213 (red circles) fitted to straight lines. $*P < 0.05$, paired Student's t test, $n = 5$.

Fig. 3. ML213 induced a concentration-dependent enhancement of Kv7.4/7.5 current accompanied by negative shift of the activation curve and decreased Kv7.4/7.5 current deactivation rate. (A, B) Representative traces of Kv7.4/7.5 currents recorded with voltage step protocol in the absence (A) and in the presence of 10 µM ML213 (B). (C) IV curves of normalized steady-state Kv currents recorded before (control, black circles) and after 10-minute treatment with increasing concentrations of ML213 as indicated ($n = 3$ –5). (D) Averaged fractional conductance plots normalized to maximal conductance in control for each experiment fitted to a Boltzmann distribution (at increasing concentrations of ML213 as indicated in C). (E) Representative traces of tail currents recorded in the absence (black, current scale at left) and in the presence of 10 µM ML213 (red, current scale at right) scaled to size of the currents recorded at 0 mV in the absence of ML213 for visual comparison. (F) The time constants (τ) of current deactivation, calculated from the single exponential fits of tail currents measured at voltages ranging from -130 mV to -90 mV in the absence (black circles) and in the presence of 10 µM ML213 (red circles) fitted to straight lines. $*P < 0.05$, paired Student's t test, $n = 5$.

Kv7 currents were recorded using a 5-second voltage step protocol from a -74 mV holding potential to test potentials ranging from -114 mV to -4 mV, followed by a 1-second step to -114 mV. To analyze the voltage dependence of channel activation, the instantaneous tail-current amplitude (estimated from the exponential fit of current deactivation measured at -114 mV) was converted to conductance according to the following equation: $G = I_{\text{tail}}/(-114 - E_K)$, where I_{tail} is the instantaneous tail current amplitude, -114 mV is the tail current step potential, and E_K is the reversal potential for potassium (-86 mV). Conductance plots in the absence (control) and in the presence of ML213 (100 nM, 300 nM, 1 μ M, 3 μ M, 10 μ M, and 30 μ M) or ICA-069673 (1 μ M, 3 μ M, 10 μ M, 30 μ M, and 100 μ M) for each experiment were fitted to a Boltzmann distribution: $G(V) = G_{\text{max}}/[1 + \exp(V_{0.5} - V)/s]$, where G is conductance, G_{max} is a maximal conductance, $V_{0.5}$ is the voltage of half-maximal activation, and s is the slope factor. To evaluate dose dependencies, the negative shift of the activation curve ($\Delta V_{0.5} = |V_{0.5, \text{drug}} - V_{0.5, \text{control}}|$), estimated by subtraction of $V_{0.5}$ in control from $V_{0.5}$ in the presence of the drug) and increase in conductance ($G/G_{\text{max, control}}$) for each drug concentration were plotted against concentration for each experiment, fitted with the Hill equation and averaged. Deactivation kinetics were analyzed by applying single exponential fits to the tail currents recorded using a 5-second voltage step protocol (from a -74 mV holding potential to 0 mV), followed by 1-second or 10-second repolarizations to voltages ranging from -130 mV to -90 mV.

Materials. Cell culture media were from Gibco-BRL (Gaithersburg, MD) or MediaTech (Herndon, VA). Lipofectamine reagent was from Invitrogen (Carlsbad, CA). Retigabine dihydrochloride was from Alomone Laboratories (Jerusalem, Israel). ML213 and ICA-069673 were from Tocris Bioscience (Bristol, UK). Amphotericin B was from Calbiochem (San Diego, CA). The vector pIRES2-EGFP was from Clontech (Mountain View, CA). The vector pShuttle-IRES-hrGFP-2 and the AdEasy Adenoviral Vector System were from Stratagene (La Jolla, CA). The human Kv7.4 cDNA (accession number: AF105202, originally in the mammalian expression vector pMT) was generously provided by Dr. Ian Wood at the University of Leeds (Leeds, UK). The human Kv7.5 cDNA (accession number: AF202977, originally in the mammalian expression vector pMT) was a generous gift from Prof. Thomas Jentsch at the Max-Delbrück-Centrum for Molecular Medicine (Berlin, Germany).

Statistics. SigmaStat (Systat Software, Inc., Point Richmond, CA) was used for all statistical analyses. Student's t test or Mann-Whitney

rank sum test was used for comparison between two groups as appropriate. Paired Student's t test was used for comparisons of parameters measured before and after treatments. Comparisons among multiple treatment groups were evaluated by analysis of variance (ANOVA) followed by a Holm-Sidak post hoc test. Cumulative concentration-response data were analyzed by repeated-measures ANOVA and post-hoc Holm-Sidak test. Differences associated with P values ≤ 0.05 were considered statistically significant.

Results

We used the A7r5 vascular smooth muscle cell line as an expression system for human Kv7.4, Kv7.5, and Kv7.4/7.5 to determine the efficacy of ML213 and ICA-069673 for activation of these smooth muscle-specific Kv7 channels. Exogenous channels introduced by adenoviral vectors produced currents with distinct biophysical properties and with more than 100-fold greater amplitudes compared with endogenous Kv7.5 currents in A7r5 cells (Brueggemann et al., 2011). Mean current densities measured at -4 mV were 0.38 ± 0.12 pA/pF for endogenous current, 68.3 ± 12.2 pA/pF for exogenously expressed Kv7.5, 84.3 ± 18.5 pA/pF for exogenously expressed Kv7.4, and 58.8 ± 15.7 pA/pF for exogenously expressed Kv7.4/7.5. We also have shown previously that, when both Kv7.4 and Kv7.5 channels are expressed together in A7r5 cells, functional channels predominantly form as Kv7.4/7.5 heteromers (Brueggemann et al., 2011).

ML213 was a potent activator of Kv7.4 channels, in agreement with a previous report (Yu et al., 2011). ML213, at concentrations between 100 nM and 30 μ M, increased maximal conductance to a peak at $212\% \pm 27\%$ of control, with an EC_{50} of 0.8 ± 0.3 μ M. A concentration-dependent negative shift of the activation curve of Kv7.4 channels was also observed, reaching a maximum of 25.0 ± 2.5 mV, with an EC_{50} of 1.6 ± 0.2 μ M (Fig. 1, A–D; Table 1). Application of ML213 (10 μ M) reduced the deactivation rates of Kv7.4 currents by 4.6-fold in the voltage range from -130 mV to -90 mV (Fig. 1, E and F), where near-complete current

Fig. 4. Summary of ML213-induced concentration-dependent negative shift of activation curves, increased maximal conductance, and decreased Kv7.4, Kv7.5, and Kv7.4/7.5 current deactivation rates. (A) Averaged ML213 dose dependence based on negative shift of $V_{0.5}$ estimated from Boltzmann fit of conductance plots for each experiment in A7r5 cells expressing Kv7.4 (black circles, $n = 5$), Kv7.5 (light gray circles, $n = 5$), and Kv7.4/7.5 (dark gray triangles, $n = 5$) fitted by the Hill equation. *Significant difference between Kv7.5 and Kv7.4 ($P < 0.05$, one-way ANOVA). (B) Averaged ML213 dose dependence based on increase in maximal conductance estimated from Boltzmann fit of conductance plots for each experiment in A7r5 cells expressing Kv7.4 (black circles, $n = 5$), Kv7.5 (light gray circles, $n = 5$) and Kv7.4/7.5 (dark gray triangles, $n = 5$) fitted by the Hill equation. (C) Time constant of current deactivation in the presence of 10 μ M ML213, normalized to the control time constant of deactivation, was plotted against voltages to illustrate the voltage-dependent change in deactivation kinetics in the presence of 10 μ M ML213 for Kv7.4 (black circles, $n = 5$), Kv7.5 (light gray circles, $n = 5$), and Kv7.4/7.5 (dark gray triangles, $n = 5$) fitted by straight lines. *Significant difference between Kv7.5 and Kv7.4 ($P < 0.05$, one-way ANOVA).

deactivation was achieved without a significant effect on activation rate (not shown).

Originally, ML213 was reported to be a selective activator of Kv7.2 and Kv7.4, being more than 80-fold more potent for activation of Kv7.2 and 12-fold more potent for activation of Kv7.4 than for Kv7.5 (Yu et al., 2011). However, we found that ML213 was a potent and effective activator of homomeric Kv7.5 channels overexpressed in A7r5 cells. Application of increasing concentrations of ML213 produced significant increases in the current amplitude, with marked negative shifts of the activation curves of Kv7.5 channels (Fig. 2, A–D). ML213 increased maximal conductance of Kv7.5 channels with an EC_{50} of $0.7 \pm 0.2 \mu\text{M}$, to a maximum of $181 \pm 17\%$ of control. The maximal negative shift of the activation curve of Kv7.5 channels reached $43.9 \pm 7.7 \text{ mV}$ (EC_{50} $4.6 \pm 2.0 \mu\text{M}$ (Table 1)). Application of ML213 ($10 \mu\text{M}$) also reduced deactivation rates of Kv7.5 currents by 5.9-fold on average (Fig. 2, E and F). The highest concentration of ML213 ($30 \mu\text{M}$) was also tested on endogenous Kv7.5 currents in A7r5 cells;

a similar increase in endogenous Kv7.5 current amplitude and negative shift of the current-voltage relationship (IV) curve were observed (Supplemental Fig. A).

ML213 produced similar effects on heteromeric Kv7.4/7.5 channels: $204\% \pm 11\%$ maximal increase in conductance with an EC_{50} of $1.1 \pm 0.6 \mu\text{M}$ and a $34.2 \pm 3.3 \text{ mV}$ maximal negative shift of the activation curve, with an EC_{50} of $3.8 \pm 1.2 \mu\text{M}$ (Fig. 3, A–D; Table 1). As found for homomeric Kv7.4 and Kv7.5 channels, ML213 ($10 \mu\text{M}$) reduced current deactivation rates through heteromeric Kv7.4/7.5 channels (Fig. 3, E and F).

Comparing potencies of ML213 based on negative shifts of activation curves or increases in maximal conductance, we found no significant differences among the three Kv7 channel types tested (Table 1). However, ML213 produced a stronger shift of the activation curve for Kv7.5 at the highest concentration (Fig. 4A; Table 1). ML213 induced a slowing of deactivation kinetics of currents through Kv7.5 homomeric channels that was significantly greater than for Kv7.4 channels only at -110 mV (Fig. 4C).

Fig. 5. Comparison of effects of $10 \mu\text{M}$ ML213 on wild-type Kv7.5 and retigabine-insensitive mutant Kv7.5 W235L. (A) IV curves of normalized steady-state Kv7.5 W235L currents recorded before (control, filled circles) and after 10-minute treatment with $10 \mu\text{M}$ of ML213 (open circles, $n = 6$), followed by 10-minute treatment with $10 \mu\text{M}$ retigabine (open triangles, $n = 5$). **Significant difference between all groups ($P < 0.01$, repeated measures ANOVA). (B) Averaged fractional conductance plots for Kv7.5 W235L normalized to maximal conductance in control for each experiment fitted to a Boltzmann distribution (treatments as in A). (C) The time constants (τ) of current deactivation of Kv7.5 W235L, calculated from the single exponential fits of tail currents measured in control (filled circles) and in the presence of $10 \mu\text{M}$ ML213 (open circles) and $10 \mu\text{M}$ retigabine (open triangles). (D) Averaged $V_{0.5}$ estimated from Boltzmann fit of conductance plots in control (black bar for Kv7.5 wild-type and gray bar for Kv7.5 W235L) and in the presence of $10 \mu\text{M}$ ML213 (open bar for Kv7.5 wild-type and striped bar for Kv7.5 W235L). ***Significant difference from all groups ($P < 0.001$, one-way ANOVA, $n = 5$ or 6). (E) Averaged maximal conductance estimated from Boltzmann fit of conductance plots in the presence of $10 \mu\text{M}$ of ML213 normalized to control maximal conductance for wild-type Kv7.5 and Kv7.5 W235L (treatment indicated as in D). ***Significant difference from all groups ($P < 0.001$, one-way ANOVA, $n = 5$ or 6). (F) Time constant of current deactivation in the presence of $10 \mu\text{M}$ ML213, normalized to the deactivation time constant in control for wild type Kv7.5 (black circles, $n = 5$) and Kv7.5 W235L (gray circles, $n = 5$). **Significant difference between wild-type Kv7.5 and Kv7.5 W235L; $P < 0.01$, Mann-Whitney rank sum test.

The apparent absence of ML213 selectivity among the tested smooth muscle Kv7 channel isoforms led us to test whether ML213 shares the essential tryptophan binding site of other nonselective Kv7.2–Kv7.5 channel activators, retigabine, S-1 ((S)-N-[1-(3-morpholin-4-yl-phenyl)-ethyl]-3-phenyl-acrylamide), and BMS-204352 ((3S)-(+)-(5-chloro-2-methoxyphenyl)-1,3-dihydro-3-fluoro-6-(trifluoromethyl)-2H-indol-2-one) (Bentzen et al., 2006). Mutation of the tryptophan residue at position 235 of Kv7.5 to leucine (W235L), previously shown to confer insensitivity to each of these Kv7 channel activators (Schenzer et al., 2005), also greatly reduced the increase in Kv7.5 current in response to application of ML213 (10 μ M, Fig. 5 A). The W235L mutation of Kv7.5 also prevented the shift of the activation curve in response to ML213 (10 μ M; Fig. 5 B). As expected, retigabine (10 μ M) also failed to enhance the current through mutant Kv7.5 W235L channels; a slight reduction of the current densities was observed in the voltage range from -19 mV to -4 mV (Fig. 5, A and B). Neither retigabine (10 μ M)

nor ML213 (10 μ M) slowed Kv7.5 W235L current deactivation (Fig. 5C). A side-by-side comparison of activation parameters of wild-type Kv7.5 and Kv7.5 W235L channels, shown in Fig. 5, D–F, clearly illustrates the loss of ML213 responsiveness with the tryptophan mutation.

Similar results were obtained with a Kv7.4 W242L retigabine-insensitive mutant. The W242L mutation of Kv7.4 abolished the increase in Kv7.4 current amplitude, negative shift of activation curve, and reduction in current deactivation rate observed on application of ML213 (10 μ M) for wild-type Kv7.4 currents (Fig. 6).

Since ML213 failed to be selective for Kv7.4 over Kv7.5, we turned our attention to a second commercially available candidate, ICA-069673. We found that ICA-069673 produced a slight concentration-dependent increase in Kv7.4 current amplitude, with a profound negative shift of its activation curve (-53.3 ± 2.0 mV at 100 μ M ICA-069673; Fig. 7, A–D). The kinetics of Kv7.4 current deactivation slowed dramatically in

Fig. 6. Comparison of the effects of 10 μ M ML213 on wild-type Kv7.4 and retigabine-insensitive mutant Kv7.4 W242L. (A) IV curves of normalized steady-state Kv7.4 W242L currents recorded before (control, filled circles) and after 10-minute treatment with 10 μ M of ML213 (open circles, $n = 4$), followed by 10-minute treatment with 10 μ M retigabine (open triangles, $n = 4$). (B) Averaged fractional conductance plots for Kv7.4 W242L normalized to maximal conductance in control for each experiment fitted to a Boltzmann distribution [treatments as in (A)]. (C) The time constants (τ) of current deactivation of Kv7.4 W242L, calculated from the single exponential fits of tail currents measured in control (filled circles) and in the presence of 10 μ M ML213 (open circles) and 10 μ M retigabine (open triangles). (D) Averaged $V_{0.5}$ estimated from Boltzmann fit of conductance plots in control (black bar) and in the presence of 10 μ M ML213 (open bar for Kv7.4 wild-type and striped bar for Kv7.4 W242L). **Significant difference from all groups ($P < 0.01$, one-way ANOVA, $n = 4$ or 5). (E) Averaged maximal conductance estimated from Boltzmann fit of conductance plots in the presence of 10 μ M of ML213 normalized to control maximal conductance for wild-type Kv7.4 and Kv7.4 W242L [treatment indicated as in (D)]. ***Significant difference from all groups ($P < 0.001$, one-way ANOVA, $n = 4$ to 5). (F) Time constant of current deactivation in the presence of 10 μ M ML213, normalized to the deactivation time constant in control for wild-type Kv7.4 (black circles, $n = 5$) and Kv7.4 W242L (gray circles, $n = 4$). ***Significant difference between wild-type Kv7.4 and Kv7.4 W242L, $P < 0.001$, Student's t test.

Fig. 7. ICA-069673 induced a profound negative shift of the Kv7.4 activation curve and drastically reduced Kv7.4 current deactivation rate. (A,B) Representative traces of Kv7.4 currents recorded with a voltage step protocol in the absence (A) and in the presence of 100 μ M ICA-069673 (B). (C) IV curves of normalized steady-state Kv7.4 currents recorded before (control, black circles) and after 10-minute treatment with increasing concentrations of ICA-069673 as indicated ($n = 4$ to 5). (D) Averaged fractional conductance plots normalized to maximal conductance in control for each experiment fitted to a Boltzmann distribution (at increasing concentrations of ICA-069673 as indicated in C). (E) Representative traces of tail currents recorded in the absence (black; current scale at left) and in the presence of 100 μ M ICA-069673 (red; current scale at right) scaled to size of currents recorded at 0 mV in the absence of ICA-069673 for visual comparison, 5 seconds of total 10-second tail duration shown for clarity. (F) The time constants (τ) of current deactivation, calculated from the single exponential fits of tail currents measured at voltages ranging from -130 mV to -90 mV in the absence (black circles) and in the presence of 100 μ M ICA-069673 (red circles) fitted to straight lines. $^{**}P < 0.01$, paired Student's t test, $n = 6$.

the presence of 100 μ M ICA-069673; a 1-second voltage step duration was not sufficient to achieve full current deactivation, so we increased voltage step duration to 10 seconds, revealing 74 ± 9 - to 93 ± 19 -fold increases in rate constants (τ) of single exponential fits between -130 and -100 mV (Fig. 7, E and F).

In contrast to its robust enhancement of Kv7.4 currents, ICA-069673, at concentrations of 10 μ M and 30 μ M, produced no significant increase in the Kv7.5 current amplitude. At 100 μ M, ICA-069673 induced a slight, reversible inhibition of Kv7.5 currents that reached significance only at voltages positive to -19 mV (Fig. 8, A–C). Despite this reduction in maximal conductance, the application of 100 μ M ICA-069673 induced a relatively modest (16.0 ± 3.2 mV) negative shift of the activation curve (Fig. 8D). Deactivation kinetics of Kv7.5 currents also significantly slowed in the presence of 100 μ M

Fig. 8. ICA-069673 induced slight inhibition of Kv7.5 currents at 100 μ M accompanied by negative shift of activation and reduction of current deactivation rate. (A,B) Representative traces of Kv7.5 currents recorded with voltage step protocol in the absence (A) and in the presence of 100 μ M ICA-069673 (B). (C) IV curves of normalized steady-state Kv7.5 currents recorded before (control, black circles) and after 10-minute treatment with increasing concentrations of ICA-069673 as indicated ($n = 4$ or 5). ** Significant difference from all groups ($P < 0.01$, repeated-measures ANOVA). (D) Averaged fractional conductance plots were normalized to maximal conductance in control for each experiment fitted to a Boltzmann distribution (at increasing concentrations of ICA-069673 as indicated in C). ** Significant difference from all groups ($P < 0.01$, repeated measures ANOVA). (E) Representative traces of tail currents recorded in the absence (black; current scale at left) and in the presence of 100 μ M ICA-069673 (red; current scale at right) scaled to size of currents recorded at 0 mV in the absence of ICA-069673 for visual comparison. (F) The time constants (τ) of current deactivation, calculated from the single exponential fits of tail currents measured at voltages ranging from -130 mV to -90 mV in the absence (black circles) and in the presence of 100 μ M ICA-069673 (red circles) fitted to straight lines. $^{*}P < 0.05$, paired Student's t test, $n = 5$.

ICA-069673 at all voltages tested (Fig. 8, E and F), although this effect was much less than that observed for Kv7.4 currents. ICA-069673 (100 μ M) was also tested on endogenous Kv7.5 currents in A7r5 cells; a slight reduction in endogenous Kv7.5 current amplitude was observed, along with a negative shift of the IV curve (Supplemental Fig. B).

The finding of promising selectivity of ICA-069673 for Kv7.4 versus Kv7.5 channels encouraged us to determine its efficacy as a Kv7.4/7.5 channel activator. ICA-069673, at concentrations ranging from 10 to 100 μ M, produced slight increases in the current amplitudes and significant negative shifts of the activation curves of Kv7.4/7.5 channels without a significant increase in maximal conductance (Fig. 9, A–D). Kinetics of

Fig. 9. ICA-069673 induces a negative shift of the Kv7.4/7.5 activation curve and reduced Kv7.4/7.5 current deactivation rate. (A,B) Representative traces of Kv7.4/7.5 currents recorded with voltage step protocol in the absence (A) and in the presence of 100 μ M ICA-069673 (B). (C) IV curves of normalized steady-state Kv7.4/7.5 currents recorded before (control, black circles) and after 10-minute treatment with increasing concentrations of ICA-069673 as indicated ($n = 4$ or 5). (D) Averaged fractional conductance plots normalized to maximal conductance in control for each experiment fitted to a Boltzmann distribution (at increasing concentrations of ICA-069673 as indicated in C). (E) Representative traces of tail currents recorded in the absence (black; current scale at left) and in the presence of 100 μ M ICA-069673 (red; current scale at right) scaled to size of currents recorded at 0 mV in the absence of ICA-069673 for visual comparison, 5 seconds of total 10-second tail duration shown for clarity. (F) The time constants (τ) of current deactivation, calculated from the single exponential fits of tail currents measured at voltages ranging from -130 mV to -90 mV in the absence (black circles) and in the presence of 100 μ M ICA-069673 (red circles) fitted to straight lines. $**P < 0.01$, paired Student's t test, $n = 5$.

deactivation of Kv7.4/7.5 current also drastically slowed in the presence of 100 μ M ICA-069673, with τ values intermediate between those for Kv7.4 current deactivation and Kv7.5 current deactivation (Fig. 9, E and F).

For a more direct comparison, we combined ICA-069673 dose dependencies for Kv7.4, Kv7.5, and Kv7.4/7.5 currents, as well as relative changes in deactivation kinetics of Kv7.4, Kv7.5, and Kv7.4/7.5 currents in the presence of 100 μ M ICA-069673 on separate graphs (Fig. 10). We could not adequately evaluate EC_{50} values of ICA-069673 because a maximal shift of activation curves was not achieved at 100 μ M ICA-069673 and higher concentrations of ICA-069673 were not practicable. Nevertheless, the extent of negative shift of the activation

curve at 30 μ M and 100 μ M ICA-069673 was significantly different among all three channels tested, with the greatest shift found for Kv7.4 channels and the least for Kv7.5 (Fig. 10A). The relative change in deactivation kinetics also fell into the same pattern: Kv7.4 > Kv7.4/7.5 > Kv7.5 (Fig. 10B).

We also made an attempt to identify the crucial binding site for ICA-069673. The retigabine-insensitive Kv7.4 W242L channels were sensitive to ICA-069673: currents were increased in association with a profound negative shift of the activation curve, from -34.2 ± 2.7 mV to -83.2 ± 1.6 mV, and a 57-fold reduction of current deactivation rate in the presence of 100 μ M ICA-069673 (Fig. 11, A–C). A conserved phenylalanine at position 137 in the middle of the S2 segment of Kv7.2 channels was previously identified as a critical binding site for a structurally related Kv7 channel activator, ztz240 (Li et al., 2013). We therefore mutated the corresponding phenylalanine at position 143 in the Kv7.4 channel to alanine (F143A). Currents through the mutant Kv7.4 F143A channels were increased in the presence of ICA-069673 (100 μ M) but to a much lesser extent and with a reduced negative shift of the activation curve (from -43.1 ± 1.6 mV to -54.8 ± 0.7 mV) and significantly attenuated decrease in current deactivation rate compared with wild-type Kv7.4 (Fig. 11, D–F). A side-by-side comparison of half-activation voltage of wild-type Kv7.4, Kv7.4 W242L, and Kv7.4 F143A channels, shown in Fig. 11G, illustrates that activation parameters in control and in the presence of 100 μ M ICA-069673 were not different in wild-type Kv7.4 and retigabine-insensitive Kv7.4 W242L mutant but were significantly attenuated by the F143A mutation. Not only was the negative shift of the activation curve in the presence of ICA-069673 reduced (from -52 mV to -12 mV), but the $V_{0.5}$ of the Kv7.4 F143A channel activation curve was also significantly more negative than for wild-type Kv7.4 in the absence of any drugs (-43.1 ± 1.6 mV vs. -34.3 ± 1.9 mV). The relative decrease in deactivation rate was also significantly reduced by the F143A mutation (from ~ 85 -fold for Kv7.4 to only 4-fold for Kv7.4 F143A, in the presence of 100 μ M ICA-069673) (Fig. 11H). A slight reduction in the relative decrease in the deactivation rate for the retigabine-insensitive Kv7.4 W242L mutant in the presence of 100 μ M ICA-069673 did not reach statistical significance.

Discussion

There is considerable interest in Kv7 channels as drug targets for treatment of a wide range of human diseases. The channels form as homomeric or heteromeric tetramers of the five α -subunits (Kv7.1–Kv7.5) encoded by KCNQ 1–5 genes (Schwake et al., 2003). It is increasingly apparent that different tetrameric subunit combinations exert specific functions in different cell types in tissues throughout the body. Drugs such as retigabine, a Kv7.2–Kv7.5 channel activator that has been approved for use as an antiepileptic agent, and flupirtine, an analgesic agent, have been recently demonstrated to act as vasodilators in mouse, rat, and human arteries and as bronchorelaxants in mouse, rat, and human lungs (Yeung et al., 2007; Joshi et al., 2009; Ng et al., 2011; Brueggemann et al., 2012, 2014a; Evseev et al., 2013). These findings highlight the potential for off-target effects of drugs that do not discriminate among the different tissue-specific Kv7 channel subtypes. These selectivity issues are further complicated by the demonstrated presence of heteromeric

Fig. 10. ICA-069673 induced a concentration-dependent negative shift of activation curves and decreased Kv7.4, Kv7.5 and Kv7.4/7.5 current deactivation rates. (A) Averaged dose dependence of negative shift of $V_{0.5}$ estimated from Boltzmann fit of conductance plots for each experiment in A7r5 cells expressing Kv7.4 (black circles, $n = 5$), Kv7.5 (light gray circles, $n = 5$) and Kv7.4/7.5 (dark gray triangles, $n = 5$). ***Significant difference between all groups ($P < 0.001$ one-way ANOVA), ###Significant difference of Kv7.5 from Kv7.4 and Kv7.4/7.5 ($P < 0.001$ one-way ANOVA). (B) Time constant of current deactivation in the presence of 100 μM ICA069673, normalized to the control time constant of deactivation, was plotted against voltages to illustrate the voltage-dependent change in deactivation kinetics in the presence of 100 μM ICA069673 for Kv7.4 (black circles, $n = 5$), Kv7.5 (light gray circles, $n = 3$) and Kv7.4/7.5 (dark gray triangles, $n = 4$). **Significant difference between all groups ($P < 0.01$ one-way ANOVA).

channels that have distinct electrophysiologic and pharmacologic characteristics compared with homomeric tetramers of Kv7 α -subunits.

Recent studies suggest that vascular smooth muscle Kv7 channels form predominantly as heteromeric Kv7.4/Kv7.5 channels (Brueggemann et al., 2011, 2014b; Chadha et al., 2014); unfortunately, few drugs have been evaluated for their effects on this combination of Kv7 subunits. Our rigorous electrophysiologic evaluation of the efficacy of ML213 on homomeric Kv7.4, Kv7.5, and heteromeric Kv7.4/7.5 channels expressed in vascular smooth muscle cells revealed that ML213 lacks the previously reported selectivity for Kv7.4. ML213 was previously reported to be 80-fold more selective for Kv7.4 channels than for Kv7.5 channels (Yu et al., 2011). These determinations were based on Ti^+ flux assays with Chinese hamster ovary cells expressing various Kv7 channels; a subset of the flux assay results were verified using an IonWorks (Sunnyvale, CA) automated electrophysiologic assay (Yu et al., 2011). It is not clear why the more high-throughput approaches yielded different results, but our findings suggest a less selective profile of ML213. This is important considering that this commercially available drug has already been used as a selective activator of Kv7.4 channels in functional assays in two recent studies (Svalø et al., 2013; Chadha et al., 2014). For example, Svalø et al. (2013) used ML213 to support the conclusion that Kv7 channels in bladder detrusor smooth muscle are predominantly Kv7.4, based on the functional responses to ML213 treatment and its supposed selectivity for Kv7.4 over Kv7.5.

The effects of ML213 on Kv7 smooth muscle isoforms include an overall increase in current amplitude, a negative shift of activation curves, and an increase in the time constant of current deactivation. Other known activators of Kv7.2-7.5 channels, such as retigabine, S-1, and BMS-204352, have been reported to exert similar effects on Kv7.4 and Kv7.5 channels (Schröder et al., 2001; Tatulian et al., 2001; Dupuis et al., 2002; Schenzer et al., 2005; Bentzen et al., 2006). All these drugs share an essential tryptophan within the S5 transmembrane domain as their main binding site (Schröder et al., 2001; Tatulian et al., 2001; Schenzer et al., 2005; Bentzen et al., 2006). We found that an equivalent tryptophan residue was essential for the Kv7 current-enhancing effects of ML213 since mutations W235L for Kv7.5 and W242L for Kv7.4 abolished all effects of ML213. It is worth noting that Kv7.2-7.5 enhancers sharing the tryptophan binding site

induced an increase in maximal conductance in addition to a negative shift of Kv7.4 and Kv7.5 activation curves, whereas a negative shift of the activation curves of Kv7.2 and Kv7.2/7.3 channels was not accompanied by an increase in maximal conductance (Schröder et al., 2001; Tatulian et al., 2001; Schenzer et al., 2005; Bentzen et al., 2006); this difference might be used as a diagnostic test to distinguish between Kv7.2/7.3 channels and Kv7.4/7.5 channels in cells that express an undetermined complement of Kv7 channels.

Another class of Kv7 channel activators has been named *gating modifiers* based on their binding to the S1-S4 voltage-sensing domain rather than to the S5-S6 pore domain segment (Peretz et al., 2010). Gating modifiers of Kv7 channels include two groups of structurally unrelated compounds: *N*-pyridyl and pyrimidine benzamides (ICA-27243, ICA-069673, and ztz240) and *N*-phenylanthranilic acid derivatives (diclofenac, meclofenamic acid (2-[(2,6-dichloro-3-methylphenyl) amino]benzoic acid), and NH29 (2-[(2,6-dichloro-4-nitrophenyl) amino]-*N*-(hydroxymethyl)-3,5-dinitrobenzamide) (Peretz et al., 2007; Wickenden et al., 2008; Padilla et al., 2009; Peretz et al., 2010; Gao et al., 2010; Amato et al., 2011; Li et al., 2013). ICA-069673 and ICA-27243 have similar structures, sharing the same two fluorines in a phenyl ring and an amide linker between aromatic rings, but ICA-27243 has a 6-chloro-pyridine ring, whereas ICA-069673 has a 2-chloro-5-aminopyrimidine ring (Amato et al., 2011). To date, no detailed examination of the selectivity profile or differences in the biophysical effects of ICA-069673 on the different Kv7 isoforms and no identification of the ICA-069673 binding site have been done. The only available information regarding ICA-069673 selectivity is a comparison of EC_{50} values, based on Rb^+ efflux assays, for Kv7.2/7.3, Kv7.3/7.5, and Kv7.1, which indicated that ICA-069673 is 20-fold more selective for Kv7.2/7.3 over Kv7.3/7.5 and ineffective against Kv7.1 (Amato et al., 2011). We compared the efficacy of ICA-069673 on vascular smooth muscle Kv7 isoforms and found that ICA-069673 effectively activated homomeric Kv7.4 by producing a strong negative shift of its activation curve without significantly increasing maximal conductance. ICA-069673 also drastically decreased Kv7.4 current deactivation. These effects differ qualitatively from the effects of ICA-27243 and ztz240 on homomeric Kv7.4: both drugs increased maximal Kv7.4 current amplitude in addition to negatively shifting its activation curve and only modestly decreasing its deactivation rate (Blom et al., 2010;

Fig. 11. Comparison of effects of 100 μ M ICA-069673 on wild-type Kv7.4, retigabine-insensitive mutant Kv7.4 W242L, and zt240-insensitive mutant Kv7.4 F143A. (A) IV curves of normalized steady-state Kv7.4 W242L currents recorded before (control, filled circles) and after 10-minute treatment with 100 μ M of ICA-069673 (open circles, $n = 8$). (B) Averaged fractional conductance plots of Kv7.4 W242L channels normalized to maximal conductance in control for each experiment fitted to a Boltzmann distribution [treatments as in (A)]. (C) The time constants (τ) of Kv7.4 W242L current deactivation, calculated from the single exponential fits of tail currents measured in control (filled circles) and in the presence of 100 μ M ICA-069673 (open circles) fitted to straight lines. $**P < 0.01$, paired Student's t test, $n = 8$. (D) IV curves of normalized steady-state Kv7.4 F143A currents recorded before (control, filled circles) and after 10-minute treatment with 100 μ M of ICA-069673 (open circles, $n = 5$). (E) Averaged fractional conductance plots of Kv7.4 F143A channels normalized to maximal conductance in control for each experiment fitted to a Boltzmann distribution [treatments as in (A)]. (F) The time constants (τ) of Kv7.4 F143A current deactivation, calculated from the single exponential fits of tail currents measured in control (filled circles) and in the presence of 100 μ M ICA-069673 (open circles) fitted to straight lines. $**P < 0.01$, paired Student's t test, $n = 5$. (G) Averaged $V_{0.5}$ estimated from Boltzmann fit of conductance plots in control (black bar for Kv7.4 wild-type, light gray bar for Kv7.4 W242L and dark gray bar for Kv7.4 F143A) and in the presence of 100 μ M ICA-069673 (open bar for Kv7.4 wild type, striped bar for Kv7.4 W242L and dotted bar for Kv7.4 F143A). $***$ Significant difference from Kv7.4 wild type, and Kv7.4 W242L, Kv7.4F143A in control, and Kv7.4 F143A in the presence of 100 μ M ICA-069673, ($P < 0.001$, one-way ANOVA, $n = 5-8$), $###$ Significant difference from Kv7.4 wild-type and Kv7.4 W242L in control ($P < 0.001$, one-way ANOVA, $n = 5-8$), $^^^$ Significant difference from all groups ($P < 0.001$, one-way ANOVA, $n = 5-8$). H. Time constant of current deactivation in the presence of 100 μ M ICA-069673, normalized to the deactivation time constant in control for wild-type Kv7.4 (open circles, $n = 6$), hKv7.4 W242L (light gray circles, $n = 8$), and Kv7.4 F143A (dark gray circles, $n = 5$). Dotted line (at y-axis value = 1) represents the level of no changes in deactivation kinetic. $**$ Significant difference from wild-type Kv7.4 and Kv7.4 W242L, $P < 0.01$, ANOVA on rank sum test).

Gao et al., 2010). Zt240 activated homomeric Kv7.4 and Kv7.5 channels with similar potencies (Gao et al., 2010), whereas we found that ICA-069673 had no appreciable effect on homomeric Kv7.5 at concentrations up to 100 μ M. At that concentration, a slight negative shift of the activation curve was accompanied by a reduction of maximal conductance, reminiscent of effects of diclofenac on homomeric Kv7.5 (Brueggemann et al., 2011) but not as robust.

Based on mutagenesis and molecular simulation, the zt240 binding pocket in Kv7.2 was found to colocalize with the external part of the gating charge pathway formed between transmembrane segments S1–S4 and a conserved phenylalanine at position 137 in the middle of the S2 segment (Li et al., 2013). The Kv7.2 F137A mutation abolished both the zt240-induced negative shift of the Kv7.2 activation curve and the decrease of current deactivation; it was also

reported to reduce the efficacy of zt240 to increase Kv7.2 current amplitude by approximately 100-fold (Li et al., 2013). Mutation of the corresponding phenylalanine at position 143 in Kv7.4 channels also significantly reduced the effects of ICA-069673. Some remaining effects of ICA-069673 on the Kv7.4 F143A mutant suggest that, despite involvement of F143 in binding of ICA-069673, additional essential residues are involved. Precision mapping of the ICA-069673 binding site requires additional work involving mutagenesis and docking simulations. It is worth noting that phenylalanine at position 143 seems to be involved in voltage sensing of Kv7.4 since mutation of phenylalanine at that position to alanine caused a 9-mV negative shift of the Kv7.4 channel activation curve; surprisingly, the corresponding F137A mutation in Kv7.2 channel had no effect on Kv7.2 voltage sensitivity (Li et al., 2013).

Based on currently available information, a promising direction for development of isoform-specific Kv7 channel activators would be toward the design of new gating modifiers. Isoform-specific drugs that exclusively target Kv7.2/Kv7.3 channels might be good neuronal Kv7 channel activators with reduced smooth muscle side effects. Smooth muscle-selective Kv7 channel activators, targeting predominantly Kv7.4/Kv7.5 channels, might also be developed to treat disorders associated with smooth muscle hyperresponsiveness (Jepps et al., 2013).

In summary, we evaluated the selectivities of two new commercially available Kv7 channel activators, ML213 and ICA-069673, on the smooth muscle isoforms of Kv7 channels: homomeric Kv7.4 and Kv7.5 and heteromeric Kv7.4/7.5. We found that, in disagreement with a previous report, ML213 was an effective activator of Kv7.5 and cannot distinguish among Kv7.4, Kv7.5, or Kv7.4/Kv7.5 channels. On the other hand, ICA-069673 was more selective for Kv7.4 over Kv7.5 but only at the highest concentrations tested could it distinguish between homomeric Kv7.4 and heteromeric Kv7.4/7.5. We also found that a tryptophan residue essential for retigabine binding is also essential for ML213 binding and that the ICA-069673 binding pocket in Kv7.4 channels involves phenylalanine at position 143.

Authorship Contributions

Participated in research design: Brueggemann, Byron.

Conducted experiments: Brueggemann.

Contributed new reagents or analytic tools: Cribbs, Haick.

Performed data analysis: Brueggemann.

Wrote or contributed to the writing of the manuscript: Brueggemann, Haick, Cribbs, Byron.

References

- Afeli SA, Malysz J, and Petkov GV (2013) Molecular expression and pharmacological evidence for a functional role of kv7 channel subtypes in Guinea pig urinary bladder smooth muscle. *PLoS ONE* **8**:e75875.
- Amato G, Roeloffs R, Rigdon GC, Antonio B, Mersch T, McNaughton-Smith G, Wickenden AD, Fritch P, and Suto MJ (2011) N-pyridyl and pyrimidine benzamides as KCNQ2/Q3 potassium channel openers for the treatment of epilepsy. *ACS Med Chem Lett* **2**:481–484.
- Barhanin J, Lesage F, Guillemare E, Fink M, Lazdunski M, and Romey G (1996) K(V) LQT1 and IsK (minK) proteins associate to form the I(Ks) cardiac potassium current. *Nature* **384**:78–80.
- Bentzen BH, Schmitt N, Calloe K, Dalby Brown W, Grunnet M, and Olesen S-P (2006) The acrylamide (S)-1 differentially affects Kv7 (KCNQ) potassium channels. *Neuropharmacology* **51**:1068–1077.
- Biervert C, Schroeder BC, Kubisch C, Berkovic SF, Propping P, Jentsch TJ, and Steinlein OK (1998) A potassium channel mutation in neonatal human epilepsy. *Science* **279**:403–406.
- Blom SM, Schmitt N, and Jensen HS (2010) Differential effects of ICA-27243 on cloned K(V)7 channels. *Pharmacology* **86**:174–181.
- Brickel N, Gandhi P, VanLandingham K, Hammond J, and DeRossett S (2012) The urinary safety profile and secondary renal effects of retigabine (ezogabine): a first-in-class antiepileptic drug that targets KCNQ (Kv7) potassium channels. *Epilepsia* **53**:606–612.
- Brueggemann LI, Haick JM, Neuburg S, Tate S, Randhawa D, Cribbs LL, and Byron KL (2014a) KCNQ (Kv7) potassium channel activators as bronchodilators: combination with a β 2-adrenergic agonist enhances relaxation of rat airways. *Am J Physiol Lung Cell Mol Physiol* **306**:L476–L486.
- Brueggemann LI, Kakad PP, Love RB, Solway J, Dowell ML, Cribbs LL, and Byron KL (2012) Kv7 potassium channels in airway smooth muscle cells: signal transduction intermediates and pharmacological targets for bronchodilator therapy. *Am J Physiol Lung Cell Mol Physiol* **302**:L120–L132.
- Brueggemann LI, Mackie AR, Cribbs LL, Freda J, Tripathi A, Majetschak M, and Byron KL (2014b) Differential protein kinase C-dependent modulation of Kv7.4 and Kv7.5 subunits of vascular Kv7 channels. *J Biol Chem* **289**:2099–2111.
- Brueggemann LI, Mackie AR, Mani BK, Cribbs LL, and Byron KL (2009) Differential effects of selective cyclooxygenase-2 inhibitors on vascular smooth muscle ion channels may account for differences in cardiovascular risk profiles. *Mol Pharmacol* **76**:1053–1061.
- Brueggemann LI, Mackie AR, Martin JL, Cribbs LL, and Byron KL (2011) Diclofenac distinguishes among homomeric and heteromeric potassium channels composed of KCNQ4 and KCNQ5 subunits. *Mol Pharmacol* **79**:10–23.
- Brueggemann LI, Moran CJ, Barakat JA, Yeh JZ, Cribbs LL, and Byron KL (2007) Vasopressin stimulates action potential firing by protein kinase C-dependent inhibition of KCNQ5 in ATR5 rat aortic smooth muscle cells. *Am J Physiol Heart Circ Physiol* **292**:H1352–H1363.
- Byron KL and Taylor CW (1993) Spontaneous Ca²⁺ spiking in a vascular smooth muscle cell line is independent of the release of intracellular Ca²⁺ stores. *J Biol Chem* **268**:6945–6952.
- Chadha PS, Jepps TA, Carr G, Stott JB, Zhu HL, Cole WC and Greenwood IA (2014) Contribution of Kv7.4/Kv7.5 heteromers to intrinsic and calcitonin gene-related peptide-induced cerebral reactivity. *Arterioscler Thromb Vasc Biol*.
- Chadha PS, Zunko F, Zhu H-L, Davis AJ, Jepps TA, Olesen SP, Cole WC, Moffatt JD, and Greenwood IA (2012) Reduced KCNQ4-encoded voltage-dependent potassium channel activity underlies impaired β -adrenoceptor-mediated relaxation of renal arteries in hypertension. *Hypertension* **59**:877–884.
- Charlier C, Singh NA, Ryan SG, Lewis TB, Reus BE, Leach RJ, and Leppert M (1998) A pore mutation in a novel KQT-like potassium channel gene in an idiopathic epilepsy family. *Nat Genet* **18**:53–55.
- Dupuis DS, Schröder RL, Jespersen T, Christensen JK, Christophersen P, Jensen BS, and Olesen SP (2002) Activation of KCNQ5 channels stably expressed in HEK293 cells by BMS-204352. *Eur J Pharmacol* **437**:129–137.
- Evshev AI, Semenov I, Archer CR, Medina JL, Dube PH, Shapiro MS, and Brenner R (2013) Functional effects of KCNQ K(+) channels in airway smooth muscle. *Front Physiol* **4**:277.
- French JA, Abou-Khalil BW, Leroy RF, Yacubian EM, Shin P, Hall S, Mansbach H, and Nohria V; RESTORE 1/Study 301 Investigators (2011) Randomized, double-blind, placebo-controlled trial of ezogabine (retigabine) in partial epilepsy. *Neurology* **76**:1555–1563.
- Gao Z, Zhang T, Wu M, Xiong Q, Sun H, Zhang Y, Zu L, Wang W, and Li M (2010) Isoform-specific prolongation of Kv7 (KCNQ) potassium channel opening mediated by new molecular determinants for drug-channel interactions. *J Biol Chem* **285**:28322–28332.
- Grunnet M, Strøbæk D, Hougaard C, and Christophersen P (2014) Kv7 channels as targets for anti-epileptic and psychiatric drug-development. *Eur J Pharmacol* **726**:133–137.
- Ipavec V, Martire M, Barrese V, Tagliatalata M, and Curro D (2011) KV7 channels regulate muscle tone and nonadrenergic noncholinergic relaxation of the rat gastric fundus. *Pharmacol Res* **64**:397–409.
- Jepps TA, Greenwood IA, Moffatt JD, Sanders KM, and Ohya S (2009) Molecular and functional characterization of Kv7 K⁺ channel in murine gastrointestinal smooth muscles. *Am J Physiol Gastrointest Liver Physiol* **297**:G107–G115.
- Jepps TA, Olesen SP, and Greenwood IA (2013) One man's side effect is another man's therapeutic opportunity: targeting Kv7 channels in smooth muscle disorders. *Br J Pharmacol* **168**:19–27.
- Joshi S, Sedivy V, Hodyc D, Herget J, and Gurney AM (2009) KCNQ modulators reveal a key role for KCNQ potassium channels in regulating the tone of rat pulmonary artery smooth muscle. *J Pharmacol Exp Ther* **329**:368–376.
- Kubisch C, Schroeder BC, Friedrich T, Lütjohann B, El-Amraoui A, Marlin S, Petit C, and Jentsch TJ (1999) KCNQ4, a novel potassium channel expressed in sensory outer hair cells, is mutated in dominant deafness. *Cell* **96**:437–446.
- Lerche C, Scherer CR, Seeböhm G, Derst C, Wei AD, Busch AE, and Steinmeyer K (2000) Molecular cloning and functional expression of KCNQ5, a potassium channel subunit that may contribute to neuronal M-current diversity. *J Biol Chem* **275**:22395–22400.
- Li P, Chen Z, Xu H, Sun H, Li H, Liu H, Yang H, Gao Z, Jiang H, and Li M (2013) The gating charge pathway of an epilepsy-associated potassium channel accommodates chemical ligands. *Cell Res* **23**:1106–1118.
- Mackie AR, Brueggemann LI, Henderson KK, Shiels AJ, Cribbs LL, Srogin KE, and Byron KL (2008) Vascular KCNQ potassium channels as novel targets for the control of mesenteric artery constriction by vasopressin, based on studies in single cells, pressurized arteries, and in vivo measurements of mesenteric vascular resistance. *J Pharmacol Exp Ther* **325**:475–483.
- McCallum LA, Greenwood IA, and Tribe RM (2009) Expression and function of K(v)7 channels in murine myometrium throughout oestrous cycle. *Pflugers Arch* **457**:1111–1120.
- McCallum LA, Pierce SL, England SK, Greenwood IA, and Tribe RM (2011) The contribution of Kv7 channels to pregnant mouse and human myometrial contractility. *J Cell Mol Med* **15**:577–586.
- Ng FL, Davis AJ, Jepps TA, Harhun MI, Yeung SY, Wan A, Reddy M, Melville D, Nardi A, and Khong TK et al. (2011) Expression and function of the K⁺ channel KCNQ genes in human arteries. *Br J Pharmacol* **162**:42–53.

- Ohya S, Asakura K, Muraki K, Watanabe M, and Imaizumi Y (2002) Molecular and functional characterization of ERG, KCNQ, and KCNE subtypes in rat stomach smooth muscle. *Am J Physiol Gastrointest Liver Physiol* **282**:G277–G287.
- Padilla K, Wickenden AD, Gerlach AC, and McCormack K (2009) The KCNQ2/3 selective channel opener ICA-27243 binds to a novel voltage-sensor domain site. *Neurosci Lett* **465**:138–142.
- Peretz A, Degani-Katzav N, Talmon M, Danieli E, Gopin A, Malka E, Nachman R, Raz A, Shabat D, and Attali B (2007) A tale of switched functions: from cyclooxygenase inhibition to M-channel modulation in new diphenylamine derivatives. *PLoS ONE* **2**:e1332.
- Peretz A, Pell L, Gofman Y, Haitin Y, Shamgar L, Patrich E, Kornilov P, Gourgy-Hacohen O, Ben-Tal N, and Attali B (2010) Targeting the voltage sensor of Kv7.2 voltage-gated K⁺ channels with a new gating-modifier. *Proc Natl Acad Sci USA* **107**:15637–15642.
- Schenzer A, Friedrich T, Pusch M, Saftig P, Jentsch TJ, Grützinger J, and Schwake M (2005) Molecular determinants of KCNQ (K_v7) K⁺ channel sensitivity to the anticonvulsant retigabine. *J Neurosci* **25**:5051–5060.
- Schröder RL, Jespersen T, Christophersen P, Strøbaek D, Jensen BS, and Olesen S-P (2001) KCNQ4 channel activation by BMS-204352 and retigabine. *Neuropharmacology* **40**:888–898.
- Schroeder BC, Hechenberger M, Weinreich F, Kubisch C, and Jentsch TJ (2000) KCNQ5, a novel potassium channel broadly expressed in brain, mediates M-type currents. *J Biol Chem* **275**:24089–24095.
- Schroeder BC, Kubisch C, Stein V, and Jentsch TJ (1998) Moderate loss of function of cyclic-AMP-modulated KCNQ2/KCNQ3 K⁺ channels causes epilepsy. *Nature* **396**:687–690.
- Schwake M, Jentsch TJ, and Friedrich T (2003) A carboxy-terminal domain determines the subunit specificity of KCNQ K⁺ channel assembly. *EMBO Rep* **4**:76–81.
- Sims SM, Singer JJ, and Walsh JV, Jr (1985) Cholinergic agonists suppress a potassium current in freshly dissociated smooth muscle cells of the toad. *J Physiol* **367**:503–529.
- Singh NA, Charlier C, Stauffer D, DuPont BR, Leach RJ, Melis R, Ronen GM, Bjerre I, Quattlebaum T, and Murphy JV et al. (1998) A novel potassium channel gene, KCNQ2, is mutated in an inherited epilepsy of newborns. *Nat Genet* **18**:25–29.
- Svalø J, Bille M, Parameswaran Theepakaran N, Sheykhzade M, Nordling J, and Bouchelouche P (2013) Bladder contractility is modulated by Kv7 channels in pig detrusor. *Eur J Pharmacol* **715**:312–320.
- Tatulian L, Delmas P, Abogadie FC, and Brown DA (2001) Activation of expressed KCNQ potassium currents and native neuronal M-type potassium currents by the anti-convulsant drug retigabine. *J Neurosci* **21**:5535–5545.
- Wang HS, Pan Z, Shi W, Brown BS, Wymore RS, Cohen IS, Dixon JE, and McKinnon D (1998) KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel. *Science* **282**:1890–1893.
- Wickenden AD, Krajewski JL, London B, Wagoner PK, Wilson WA, Clark S, Roeloffs R, McNaughton-Smith G, and Rigdon GC (2008) N-(6-chloro-pyridin-3-yl)-3,4-difluoro-benzamide (ICA-27243): a novel, selective KCNQ2/Q3 potassium channel activator. *Mol Pharmacol* **73**:977–986.
- Wickenden AD and McNaughton-Smith G (2009) Kv7 channels as targets for the treatment of pain. *Curr Pharm Des* **15**:1773–1798.
- Yeung SY, Pucovsky V, Moffatt JD, Saldanha L, Schwake M, Ohya S, and Greenwood IA (2007) Molecular expression and pharmacological identification of a role for K(v)7 channels in murine vascular reactivity. *Br J Pharmacol* **151**:758–770.
- Yu H, Wu M, Townsend SD, Zou B, Long S, Daniels JS, McManus OB, Li M, Lindsley CW, and Hopkins CR (2011) Discovery, synthesis, and structure activity relationship of a series of N-aryl-bicyclo[2.2.1]heptane-2-carboxamides: characterization of ML213 as a Novel KCNQ2 and KCNQ4 potassium channel opener. *ACS Chem Neurosci* **2**:572–577.

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