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The KCNQ2/3 selective channel opener ICA-27243 binds to a novel voltage-sensor domain site

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ABSTRACT

The mammalian KCNQ (Kv7) gene family is composed of five members (KCNQ1–5). KCNQ2, Q4 and Q5 (KCNQ2–5) channels co-express with KCNQ3 to form heterotetrameric voltage-gated K^+ (KCNQ2–5/3) channels that underlie the endogenous M-current and regulate neuronal excitability in CNS and PNS neurons. Openers of one or a mixture of these channels may be an attractive therapeutic agent for epilepsy and pain. Non-selective KCNQ2–5/3 activators have shown efficacy in pre-clinical and clinical studies. However, more selective pharmacological profiles, including greater KCNQ sub-type-selective activation, could provide efficacy with fewer side effects. One such compound, ICA-27243, sub-type selectively enhances the activation of KCNQ2/3 channels and also exhibits efficacy in pre-clinical anticonvulsant models; Roeloffs et al. (2008) [15]; Wickenden et al. (2008) [27]. The binding site of non-selective KCNQ2–5/3 openers maps to the S5–S6 pore domain and is altered by mutation of a tryptophan residue (Trp236 in KCNQ2, Trp265 in KCNQ3) conserved among KCNQ2–5 channels; Schenzer et al. (2005) [19]; Wuttke et al. (2005) [30]. Here we report that the activity of the KCNQ2/3 selective opener ICA-27243 is not affected by these Trp mutations and does not map to the S5–S6 domain. Rather, the selective activity of ICA-27243 is determined by a novel site within the S1–S4 voltage-sensor domain (VSD) of KCNQ channels. The sub-type-selective activity of ICA-27243 may arise from greater sequence diversity of KCNQ family members within the ICA-27243 binding pocket, allowing for more selective small molecule–protein interactions.

Voltage-gated K^+ channels play a significant role in regulating membrane excitability properties [7]. In the KCNQ (Kv7) family, mutations in four of the five human family members (KCNQ1–4) underlie excitability disorders consistent with their cellular expression patterns. Mutations in KCNQ1 are associated with cardiac arrhythmias and deafness [12,25] while mutations in KCNQ4 are associated with progressive hearing loss [5]. KCNQ2, Q4 and Q5 channels found in CNS and PNS tissues express as functional heterotetrameric channels with KCNQ3 and underlie the neuronal M-current [8,21,26]. Mutations in the CNS expressing KCNQ channels KCNQ2 and KCNQ3 are associated with forms of neonatal epilepsy [1,23]. Openers of KCNQ2–5/3 channels are currently being pursued as therapeutic agents for CNS indications including epilepsy and pain.

Retigabine (N-(2-Amino-4-[fluorobenzylamino]-phenyl) carbamic acid) enhances KCNQ channel activation by inducing a hyperpolarizing shift in the voltage-dependence of activation [11,17,29]. While retigabine has shown promising anticonvulsant properties in both pre-clinical and clinical studies [14,16], it exhibits little selectivity between KCNQ2/3 and other KCNQ2–5 homo- and heteromeric channels [20,24,28]. In addition, it has potential effects on GABAergic transmission and other ion channels [4,18]. Agents capable of selectively enhancing the activation of distinct neuronal KCNQ heteromers such as KCNQ2/3 may represent particularly attractive anticonvulsant and/or pain therapeutics [3,15,31].

Here we explore the location of the binding site for the KCNQ2/3 selective opener ICA-27243 (N-(6-chloro-pyridin-3-yl)-3,4-difluoro-benzamide). Chimeric constructs were generated utilizing PCR and restriction sites endogenous to either human KCNQ2 or Q5 as detailed. Mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene). KCNQ channel constructs were co-expressed with KCNQ3 to increase current expression and facilitate analysis in native heteromers. Chinese hamster ovary (CHO) cells were transiently transfected using Fugene (Roche, Applied Science) with 1 μg of chimera and 1 μg of KCNQ3 DNA, and 0.2 μg of hCD4 DNA (all in pCDNA3.1, Invitrogen). After 24 h, cells were trypsinized and plated on coverslips for visualization with anti-CD4 beads (Dynal). K^+ currents recorded in whole cell configuration patch clamp at 2–10 kHz and

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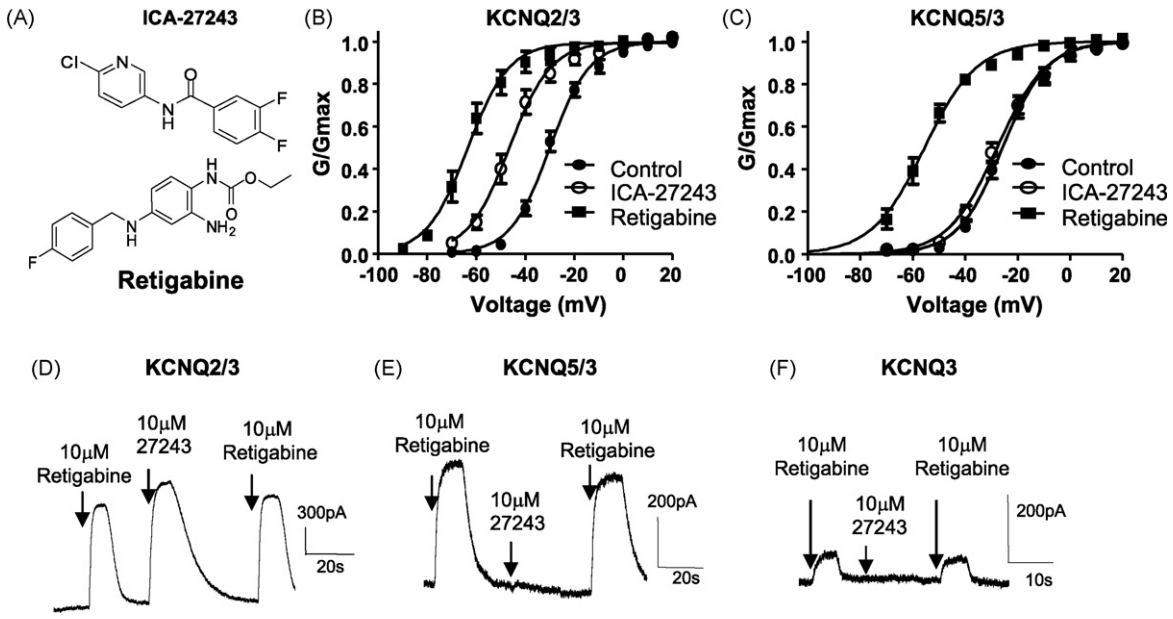

Fig. 1. ICA-27243 selectively activates KCNQ2/3 heteromeric channels and not KCNQ5/3 or KCNQ3 channels. (A) Structures of retigabine and ICA-27243. Normalized conductance–voltage relations for KCNQ2/3 (B) and KCNQ5/3 (C) channels in the presence (10 μM) or absence of indicated compounds. KCNQ2/3 $V_{1/2}$ values were control = −29 ± 2 mV, ICA-27243 = −46 ± 3 mV, retigabine = −62 ± 3 mV (n = 3 each). KCNQ5/3 $V_{1/2}$ values were control = −26 ± 2 mV, ICA-27243 = −27 ± 2 mV, retigabine = −56 ± 2 mV (n = 4 each) Representative (D) KCNQ2/3, (E) KCNQ5/3 and (F) KCNQ3 channel currents during prolonged −40 mV depolarizing pulses in the presence of the indicated concentrations of compound.

filtered at 1–2 kHz using an Axon 200B amplifier and pCLAMP software (Molecular Devices, Sunnyvale, CA) with bathing and pipette solutions described previously [27–29] at 22–24 °C. Drug-induced currents were measured as increases in outward current at sub-maximal holding potentials (−40 mV), thereby providing a window for drug-induced current. To determine the voltage for half-maximal activation ($V_{1/2}$), current amplitude was measured at the end of three second depolarizing steps (−100 to +30 mV in 10 mV increments from a holding potential of −80 mV). Whole cell conductance (G) was calculated according to the equation $G = I/(V - E_K)$, where I is the steady-state current, V is the step potential, and E_K is the reversal potential for potassium (−82.9 mV). Normalized conductance was plotted against the step potential and fitted to a Boltzman equation to derive $V_{1/2}$ values. ICA-27243 and retigabine were synthesized at Icagen. Data are represented as mean ± SEM. Data were analyzed using one way analysis of variance with post hoc Tukey t-test and significance reported at values of $p < 0.05$ where indicated.

Previous reports have shown that ICA-27243 exhibits little activity against either KCNQ4 or KCNQ1 channels [27]. The effects of retigabine and ICA-27243 on KCNQ2/3, and KCNQ5/3 hetero- and KCNQ3 homomeric channels are illustrated in Fig. 1. Retigabine and ICA-27243 enhanced KCNQ channel activation by shifting channel opening to more hyperpolarized potentials. Retigabine non-selectively enhanced all channel currents while ICA-27243 enhanced activation of KCNQ2/3 channels but had little effect on KCNQ5/3 or KCNQ3. The data suggest that the selective activation of KCNQ2/3 vs KCNQ5/3 channels is driven by molecular differences between the KCNQ2 and KCNQ5 channel proteins and that ICA-27243 exhibits significant selectivity for KCNQ2/3 over other (non-KCNQ2 containing) homo- and heteromeric KCNQ channels. Chimeric studies have previously mapped the retigabine binding site to the S5–S6 pore domain segments [19,30]. Recent studies have further proposed interactions of retigabine with four specific amino acid residues within the S5, pore loop and S6 regions [6] to stabilize inter-subunit contacts and the open conformation of the tetrameric pore domain. To date, the binding sites of all KCNQ openers have mapped to the S5–S6 pore domain; the activity of the KCNQ2–5 openers (S)-1 and BMS-204352 are also dependent on Trp236 while ZnPy (activates KCNQ1 but not KCNQ3) and RL3 (activates KCNQ1 but not other KCNQ channels) map to other S5–S6 pore domain sites [2,3,22,31]. To explore the ICA-27243 binding site, we co-expressed KCNQ2/3 channels containing the W236L/W265L pore domain mutations and tested the effect of ICA-27243 on these channels. While abrogating the activity of retigabine [19,30], the mutations did not inhibit the activity of ICA-27243 (Fig. 2). The EC_{50} value for ICA-27243 on KCNQ2/3 W236L/W265L channels was 0.28 ± 0.12 μM (n = 4) in comparison to 0.44 ± 0.07 μM (n = 5) for wild-type (WT) KCNQ2/3 channels. Although retigabine and ICA-27243 show qualitatively similar effects on KCNQ2/3 heteromeric channels, our results indicate that ICA-27243 may act through a novel site and therefore by a distinct mechanism.

To map the binding site for ICA-27243, we generated chimeric constructs utilizing human KCNQ2 and KCNQ5 channel cDNAs and co-expressed them with KCNQ3 [28,29]. The cDNAs were ini-

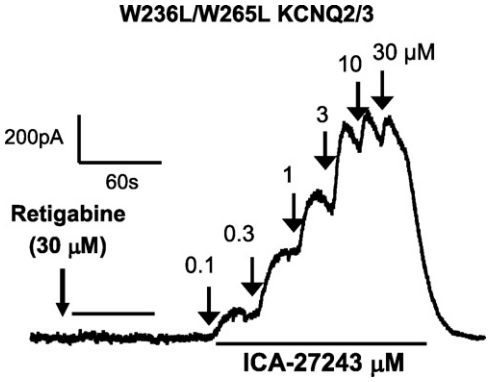

Fig. 2. ICA-27243 potently activates W236L/W265L KCNQ2/3 mutant channels. Representative −40 mV current trace of W236L/W265L KCNQ2/3 in response to retigabine and ICA-27243. The mutations render the currents insensitive to retigabine but have no comparable effects on ICA-27243 activity.