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KCNQ2/3 openers show differential selectivity and site of action across multiple KCNQ channels

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ABSTRACT

KCNQ2/3 voltage-gated potassium channels conduct low-threshold, slowly activating and non-inactivating currents to repolarize the neuronal resting membrane potential. The channels negatively regulate neuronal excitability and KCNQ2/3 openers are efficacious in hyperexcited states such as epilepsy and pain. We developed and utilized thallium influx assays to profile novel KCNQ2/3 channel openers with respect to selectivity across KCNQ subtypes and on requirement for tryptophan 236 of KCNQ2, a critical residue for activity of the KCNQ opener retigabine. Using distinct chemical series of openers, a quinazolinone series showed relatively poor selectivity across multiple KCNQ channels and lacked activity at the KCNQ2(W236L) mutant channel. In contrast, several novel benzimidazole openers showed selectivity for KCNQ2/3 and KCNQ2 and retain activity at KCNQ2(W236L). Profiling of several hundred KCNQ2/3 openers across multiple diverse chemical series revealed that openers show differential degrees of selectivity across subtypes, with selectivity most difficult to achieve against KCNQ2. In addition, we report the significant finding that KCNQ openers can pharmacologically differentiate between homomeric and heteromeric channels containing subtypes in common. Moreover, most openers assayed were dependent on the W236 for activity, whereas only a small number appear to use a distinct mechanism. Collectively, we provide novel insights into the molecular pharmacology of KCNQ channels by demonstrating differential selectivity and site of action for KCNQ2/3 openers. The high-throughput thallium influx assays should prove useful for rapid characterization of KCNQ openers and in guiding efforts to identify selective compounds for advancement towards the clinic.

1. Introduction

KCNQ (Kv7) channels are voltage-activated potassium channels with important roles in many excitable cells, such as neurons, cardiac myocytes and vascular smooth muscle cells (Brown and Passmore, 2009; Mackie and Byron, 2008; Robbins, 2001). Unlike most other voltage-gated potassium channels, these channels are activated at a low threshold that is close to the resting membrane potential and mediate the so called "M current", a slowly activating and deactivating, non-inactivating outward current (Wang et al., 1998). As such, the activation of KCNQ channels results in repolarization of the resting membrane potential and hence has long-lasting effects on dampening cell excitability.

The KCNQ family is composed of five subtypes (KCNQ1–KCNQ5) and functional potassium channels are formed as tetramers of these subtypes, either as homomers or heteromers (Delmas and Brown, 2005). Of these five subtypes, KCNQ2–5 have higher homology to each other while KCNQ1 is the most divergent in sequence (Wei et al., 2005). KCNQ2–5 subtypes are predominantly found in various central and peripheral neurons and the resulting M-current hyperpolarizes the resting membrane potential and functions as a "brake" on repetitive action potential discharges, thus exerting an inhibitory control over neuronal excitability (Brown and Adams, 1980; Jentsch, 2000; Passmore et al., 2003; Selyanko and Sim, 1998). Of these, KCNQ2, KCNQ3 and KCNQ5 have been shown to have a relatively broad expression profile in the nervous system (Jentsch, 2000). In contrast, KCNQ4 has restricted expression in the auditory system and a specific mutation is linked to deafness (Kharkovets et al., 2000). It is also expressed in discrete dopaminergic areas of the brain (Hansen et al., 2006, 2008). Recently, KCNQ4 and KCNQ5 have also been found to be expressed in vascular smooth muscle cells and implicated in the regulation of vascular tone (Mackie and Byron, 2008).

Abbreviations: FLIPR, fluorometric imaging plate reader; Tl, thallium.

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Among the neuronal KCNQ channels, the heterotetrameric channels co-assembled by KCNQ2 and KCNQ3 are believed to be the major mediator of the M current in neurons, where they are involved in stabilizing negative resting membrane potential (Hadley et al., 2003; Wang et al., 1998). Mutations in KCNQ2 and KCNQ3 have been implicated in a form of neonatal epilepsy known as benign familial neonatal convulsions (Biervert et al., 1998; Charlier et al., 1998; Singh et al., 1998). In addition, deletion of one KCNQ2 allele in mice enhances sensitivity to epileptogenic agents (Watanabe et al., 2000). This suggests that KCNQ2/3 acts as a critical negative regulator of neuronal excitability and openers of the KCNQ2/3 channel can enhance this action. Such modulators have shown efficacy in pathological conditions with abnormal neuronal excitability such as epilepsy and neuropathic pain. For example, retigabine (*N*-(2-amino-4-(4-fluorobenzylamino)-phenyl) carbamic acid ethyl ester) and ICA-27243 (*N*-(6-chloropyridin-3-yl)-3,4-difluorobenzamide) exert anticonvulsant and analgesic activities in a broad range of animal models of seizure and pain (Blackburn-Munro et al., 2005; Blackburn-Munro and Jensen, 2003; Passmore et al., 2003; Rostock et al., 1996; Wickenden et al., 2008) and show efficacy in patients with seizures in clinical trails (Porter et al., 2007). Flupirtine, a KCNQ channel opener and close structural analog of retigabine, has proven to be an effective analgesic drug (Friedel and Fitton, 1993; Klawe and Maschke, 2009). These observations reveal the therapeutic potential of KCNQ2/3 channel openers in pain and epilepsy.

Non-selective KCNQ modulators may have side effect liabilities in the clinic, most likely as a result of effects in the neuronal and/or cardiovascular systems (Dalby-Brown et al., 2006; Mackie and Byron, 2008). Thus, KCNQ2/3 channel openers that are selective over multiple other KCNQ channels with diverse physiological roles could offer a significant advantage with respect to therapeutic index. In this study, we report on the evaluation of the selectivity and site of action of KCNQ2/3 openers using high-throughput Tl^+ influx assays and on novel insights into the molecular pharmacology of KCNQ channel openers.

2. Methods

2.1. Reagents

Retigabine, compound A1 ((±)-*exo*-2-bicyclo[2.2.1]hept-2-yl-*N*-(2-isopropyl-4-oxoquinazolin-3(4H)-yl)acetamide), compound A2 (*endo* 2-[bicyclo[3.2.1]oct-3-yl]-*N*-(2-ethyl-4-oxoquinazolin-3(4H)-yl)acetamide), and compound A3 (2-(1-adamantyl)-*N*-(2,6-dimethyl-4-oxo-5-phenylthieno[2,3-d]pyrimidin-3(4H)-yl)acetamide) were synthesized according to published methods (Blackburn-Munro et al., 2005; Scanio et al., 2010). Compound B1 (5-chloro-2-(6-chloro-pyridin-3-yl)-1H-benzoimidazole) and compound B2 (2-(6-chloro-pyridin-3-yl)-5,6-dimethyl-1H-benzoimidazole) were obtained from Key Organics (UK). Compound B3 (6-chloro-2-(6-chloro-pyridin-3-yl)-5-fluoro-1H-benzoimidazole) was made in house: In brief, 6-chloronicotinic acid and CDI in a 1:1 pyridine and DMF solution reacted with 4-chloro-5-fluorobenzene-1,2-diamine. The mixture was then heated in acetic acid in a microwave at 110 °C for 10 min. BTC-AM dye was obtained from Invitrogen (Carlsbad, CA). Thallium nitrate and ouabain octahydrate were purchased from Sigma (St. Louis, MO).

2.2. Cloning of human KCNQ channel cDNA

The coding sequences of human KCNQ2 (GenBank™ accession number: NM_172107), KCNQ3 (NM_004519), KCNQ4 (NM_004700), KCNQ5 (NM_019842) genes were derived either by PCR from human full length cDNA clones obtained from OriGene (Rockville, MD) or by RT-PCR from human brain RNA. The coding sequence of KCNQ3 was cloned into the pcDNA3.1/Zeo(+) expression vector (Invitrogen) whereas all other KCNQ subunits were cloned into the pcDNA3.1/V5-His TOPO vector (Invitrogen). The KCNQ2 expression construct with a W236L mutation was obtained by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer's instructions.

2.3. Transfection and cell culture

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin–streptomycin (Invitrogen) in a humidified 5% CO_2, 95% air incubator at 37 °C. Cells were transfected with the KCNQ expression constructs using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

To generate HEK293 cell lines stably expressing KCNQ channels composed of KCNQ2–5, the expression constructs were transfected into HEK293 cells. For the heteromeric channels KCNQ2/3 and KCNQ5/3, an equal amount of the expression construct for each subtype was transfected. Stable cell lines were then generated by antibiotic selection (0.2 mg/ml zeocin (Invitrogen) for KCNQ3 and 0.5 mg/ml geneticin (Invitrogen) for KCNQ2, 4, 5). The expression of KCNQ channel subtypes in the resultant cell lines was assessed and confirmed by Western blots, immunostaining and Tl^+ influx assays.

2.4. Tl^+ influx assay

The assay was performed using a fluorometric imaging plate reader (FLIPR^{TETRA}) system (Molecular Devices, Sunnyvale, CA), a high throughput and real time cellular assay screening system, in 384-well format similar to the method previously described (Weaver et al., 2004), with some modifications. Cells were seeded in 384-well, black-walled, clear-bottomed, poly-D-lysine coated plates (Greiner Bio-One, Germany) at a density of 1.5×10^4 cells per well 24 h before the assay. On the assay day, BTC-AM dye (Invitrogen) was loaded into the cells by replacing the cell culture medium with 30 μl/well of 2.8 μM dye in Dulbecco's Phosphate Buffered Saline (Invitrogen). Dye loading was allowed to proceed for 2 h at room temperature and then cells were washed once in 30 μl/well of assay buffer (in mM: 10 HEPES pH 7.3, 5 glucose, 140 Na-gluconate, 2.5 K-gluconate, 3.6 Ca-gluconate, 2 $MgSO_4$, 0.1 ouabain octahydrate) to remove unloaded dye. Cells were incubated in 30 μl of assay buffer before loading onto a FLIPR^{TETRA} system. Compounds to be assayed were added to the cells as 4× of final concentration in 15 μl of assay buffer and incubated for 3 min at room temperature. The influx signal was initiated by adding 15 μl of assay buffer containing 4.2 mM $TlNO_3$ and 7 mM K_2SO_4. Final concentrations of externally added Tl^+ and K^+ employed to initiate the influx signal are 1.05 mM and 3.5 mM, respectively. Fluors on BTC-AM dye were excited using LED lights with 470–495 nm excitation wavelength and emission was filtered using a 545 ± 30 nm bandpass filter. Fluorescent signals were detected by camera and recorded for 3 min.

Tl^+ influx signal was quantified as light unit changes after Tl^+ addition using the "SUM" statistic from the FLIPR^{TETRA} software. Fold of activation was calculated as fold of signal change over the basal signal level. For compound potency determination, fold of activation at a given dose was plotted against compound concentration and fit with a nonlinear regression curve using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). EC_{50} val-