Biochemical and Biophysical Research Communications 441 (2013) 463–468

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cloxyquin (5-chloroquinolin-8-ol) is an activator of the two-pore domain potassium channel TRESK

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ARTICLE INFO

Article history:
Received 11 October 2013
Available online 25 October 2013

Keywords:
TRESK
Migraine
Screening
Assay
Ion channel
Cloxyquin

ABSTRACT

TRESK is a two-pore domain potassium channel. Loss of function mutations have been linked to typical migraine with aura and due to TRESK's expression pattern and role in neuronal excitability it represents a promising therapeutic target. We developed a cell based assay using baculovirus transduced U20S cells to screen for activators of TRESK. Using a thallium flux system to measure TRESK channel activity we identified Cloxyquin as a novel activator. Cloxyquin was shown to have an EC50 of 3.8 μM in the thallium assay and displayed good selectivity against other potassium channels tested. Activity was confirmed using whole cell patch electrophysiology, with Cloxyquin causing a near two fold increase in outward current. The strategy presented here will be used to screen larger compound libraries with the aim of identifying novel chemical series which may be developed into new migraine prophylactics.

1. Introduction

Migraine is characterised as severe, recurring headache, often accompanied by nausea, vomiting and increased sensitivity to light and sound. It is the most common neurological disorder with a prevalence estimated at 18.2% in females and 6.5% in males [1]. Approximately one third of migraine attacks are preceded by visual disturbances, known as aura, that commonly appear as scintillating shapes, hallucinations, or black spots. Aura is believed to be linked to cortical spreading depression (CSD), a self-propagating wave of neuronal depolarization that slowly moves across the cerebral cortex. Evidence from rodent models suggests CSD is able to activate trigeminal nociceptors [2,3], leading to the release of pro-inflammatory peptides (such as CGRP and substance P) in the meninges that further activate trigeminal nerves. Migraine can therefore be thought of as a culmination of inappropriate neuronal hyperactivity, localized inflammation in the meninges and activation of trigeminal afferents and central pain pathways leading to the characteristic headache. Three susceptibility genes, *CACNA1A*, *ATP1A2* and *SCN1A* (reviewed [4]) have been described for the rare, autosomal dominant subtype of migraine with aura, familial hemiplegic migraine. Each of these encodes proteins involved in excitatory neurotransmission. Recently, mutations in the gene encoding TRESK, a two-pore domain potassium channel, have been described as segregating with typical migraine with aura in a large pedigree [5].

Two-pore domain potassium (K2P) channels act to establish background (or leak) potassium current. Functionally they maintain resting membrane potential and cell excitability. K2P channels are characterized by alpha subunits consisting of 4 transmembrane domains each containing two pore loops. Their activity is modulated by a number of physico-chemical parameters, such as pH and membrane stretch, and a variety of intracellular pathways e.g. calcium mobilization. The K2P family in humans includes 15 related channels [6], which are expressed widely throughout the central nervous system. They have been implicated in a variety of physiologies of particularly note, including nociception, and are a primary target for many neuroprotective agents and anesthetics.

TRESK, also known TWIK-related spinal cord potassium channel or $K_{2P}18.1$, is a 384 amino acid protein encoded by the *KCNK18* gene. The frameshift mutation F139WfsX24, was shown to be present only in individuals affected by migraine in a large proband [5]. This mutation leads to a prematurely truncated and non-functional channel and suggests loss of TRESK function as a rare cause of typical migraine with aura. Importantly, with relevance to its role in migraine and pain, TRESK is expressed at high levels in the dorsal root ganglion (DRG) and trigeminal ganglion (TGM) [7]. DRG neurons from TRESK KO mice display a lower threshold for activation, reduced action potential duration, and higher amplitudes of

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.10.090

after-hyperpolarization, indicating an *in vivo* role for TRESK in modulating neuronal excitability [8]. This is further supported by altered TRESK expression in a rodent nerve injury model [9]. Evidence that TRESK is activated by histamine [8] affords speculation that the physiological role of TRESK currents is to 'dampen' cellular excitability in inflammatory responses.

Based on the above evidence we sought to develop a cell based assay to identify TRESK activators. Using baculovirus gene delivery, a recombinant system was developed which employed thallium flux as a surrogate of K^+ conductance and thus TRESK channel function. This was used to identify compounds which increased channel activity. Inhibition of TRESK by immunosuppressants (cyclosporine) has been linked to increased frequency and severity of migraines [10] and volatile anesthetics such a halothane and isoflurane are potent activators of TRESK [11]. Collectively these demonstrate TRESK is susceptible to small molecule modulation and provide indirect pharmacological verification for a role in migraine. Moreover identification and optimization of selective and potent TRESK activators may provide novel therapeutic intervention in migraine.

2. Materials and methods

2.1. Cell culture

U2OS cells were maintained in McCoys 5A media, supplemented with 10% FBS and 1× Penicillin/Streptomycin (Gibco, USA). Baculovirus, expressing the *KCNK18* gene (NM_181840.1) was purchased from Life Technologies. Typically cells were plated at 2.5×10^6 cells in T-175 flasks and incubated overnight at 37 °C and 5% CO_2. The following day media was changed and supplemented with baculovirus solution, typically 10% v/v. Cells were incubated for a further 24 h in the presence of baculovirus. After this cells were plated into black sided clear bottom 384 well plates (Corning, USA) at a density of 5 K cells per well. Cells were incubated for a further 24 h at 37 °C and 5% CO_2.

2.2. Thallium flux assays

Thallium flux was measured using the FluxOR system (Life Technologies, USA), as per the manufacturer's guidelines. Media was removed by aspiration; cells were exposed to dye for 1 h at room temperature in the dark. Typically 15 μl of assay buffer was added to each well followed by 5 μl of compound prepared at 4×. Samples were analyzed using the FlexStation II (Molecular Devices, USA). Baseline was read for 20 s prior to the addition of 5 μl of thallium solution. This was added to give a final assay thallium concentration of 2 mM. Fluorescence was further measured for up to 180 s. In each case readings were taken every 3 s with excitation/emission at 490/525 nm. For each well, raw fluorescence counts were normalized to pre-thallium baseline, within that well.

2.3. Electrophysiology

Electrophysiological recordings were carried out at room temperature using a whole-cell patch clamp technique. U2OS cells were bathed in an external solution containing: 125 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM Glucose and 10 mM HEPES. Osmolarity was adjusted to 290 mOsm and pH adjusted to 7.3 with NaOH. Patch pipettes of 3–6 MΩ tip resistance were pulled from filamental borosilicate glass capillaries (1.2 mm outer diameter, 0.69 mm inner diameter; Harvard Apparatus, USA), using a horizontal puller (Sutter P-97) and filled with an internal solution containing: 140 mM KCl, 4 mM NaCl, 0.02 mM $CaCl_2$, 0.8 mM EGTA, 2 mM $MgCl_2$, 4 mM MgATP and 10 mM HEPES. Osmolarity was adjusted to 290 mOsm and pH adjusted to 7.3 with KOH. Recordings were made using an Axopatch 1D amplifier (Molecular Devices, USA) and data acquired using WinWCP Strathclyde Whole Cell Analysis software (V.3.9.7; University of Strathclyde, UK). K^+ currents were measured in voltage-clamp mode by applying a series of depolarizing currents from −80 mV to potentials between −70 and +50 mV. The digitized data were analyzed with pClamp9 (Axon Instruments, Inc., USA). *I/V* curves were generated by measuring the outward current at the end of each 1 s step.

3. Results

3.1. A cell based assay for TRESK activity

Using baculovirus a TRESK expressing U2OS cell line was generated. Expression was measured functionally using the FluxOR thallium flux assay (Life Technologies, USA). This assay uses fluorescence to report thallium (as a surrogate of potassium) flux through TRESK channels using a fluorescent dye. Fig. 1A shows an average of representative fluorescence traces recorded from individual wells of a 384-well plate containing U2OS cells expressing TRESK. No background TRESK signal was observed in U2OS cells and signal peaked at around 120 s, approximately 2.5-fold over baseline. The baculovirus system offers a number of advantages, including low cytotoxicity and portability. However, a primary reason for its selection in pharmacological studies is the ability to titrate target expression level and therefore channel function. Fig. 1A shows that by increasing the volume of baculovirus added we were able to increase the level of TRESK signal in a dose dependent fashion. To achieve maximum signal 10% v/v baculovirus was typically used in these studies. To confirm that the increase in fluorescence represented signal specifically related to ion movement through TRESK channels we sought to investigate the effects of the TRESK inhibitor tetrapentylammonium (TPA). Although pharmacological characterization was rudimentary, FluxOR signal was inhibited by TPA with an IC50 of 2.5 nM, largely in accordance with standard electrophysiology data. Similarly, we were also able to observe activation (Fig. 1B) using the previously described TRESK activator *phorbol*-12-myristate-13-acetate (PMA) [12]. PMA activates TRESK via protein kinase C activation and demonstrates that at least part of the native regulation of TRESK is preserved in the U20S system. To quantify the level of TRESK activation observed in our assay a number of parameters were assessed: peak fluorescence (with or without basal correction), area under the curve and rate of fluorescence increase. Each parameter was additionally analyzed at multiple time points. The largest activation was observed when comparing basal corrected maximum fluorescence at 30 s post addition. In the case of PMA this equated to 35% activation relative to DMSO containing controls. To assess the assays suitability for screening Z′ and co-efficient of variation (CV) parameters were calculated. In compound screening environments *Z′* is often used to describe assay sensitivity and reproducibility [13]. Using DMSO (high) and TPA (low) controls, *Z′* of 0.54–0.60 and CVs 0.2–4% were observed, indicating the assay would be suitable for high throughput screening.

3.2. Cloxyquin is an activator of TRESK

Using the baculovirus TRESK FluxOR assay we screened a 1 K library of diverse compounds. 12 compounds were shown to give robust activation. The most active of these compounds was Cloxyquin (5-chloroquinolin-8-ol). Representative traces (Fig. 2A) show the effect of Cloxyquin (50 and 5 μM) on TRESK expressing U20S cells. Concentration response curves showed Cloxyquin to