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

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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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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

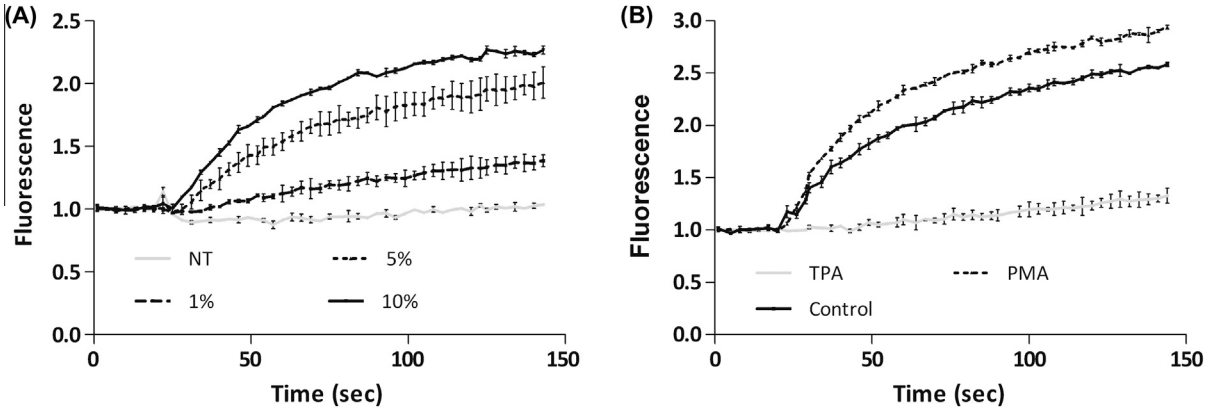

Fig. 1. FluxOR analysis of U2OS cells expressing TRESK. (A) TRESK function is titratable. U20S cells transduced with 1%, 5% or 10% (v/v) TRESK baculovirus show a dose dependent increase in function. TRESK channel function is measured by thallium flux which causes an increase in intracellular fluorescence. (B) TRESK channel function is enhanced by the addition of 1 μM *phorbol*-12-myristate-13-acetate (PMA) and inhibited by tetrapentylammonium (TPA). In both graphs thallium is added at 20 s and all fluorescence is relative to pre-addition baseline. Errors bars represent standard deviation.

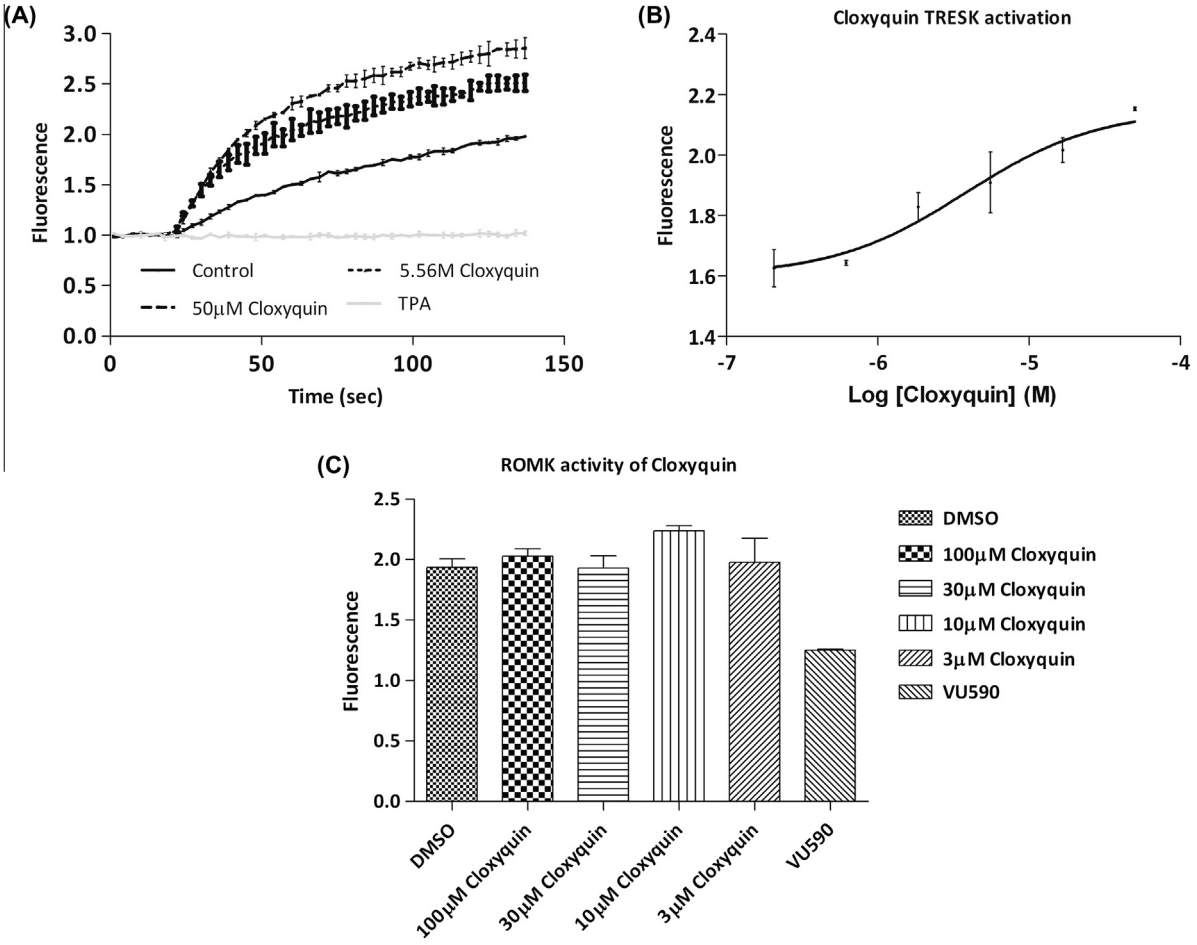

Fig. 2. Cloxyquin in an activator of TRESK. (A) FluxOR analysis of transduced U20S cells shows an increase in TRESK activity after pre-incubation with Cloxyquin. Traces shows fluorescence increases after the addition of thallium. Cloxyquin causes a significant increase in channel function relative to DMSO containing controls. Tetrapentylammonium (TPA) inhibits the TRESK signal. Thallium is added at 20 s and all measurements are relative to pre-addition baseline. Errors bars represent standard deviation. (B) Compound response curve for Cloxyquin activation. Graph shows fluorescence at 50 s. Cloxyquin has an EC50 of 3.8 μM. Error bars represent standard deviation. (C) Cloxyquin shows no significant activation at ROMK. U20S cells transduced with ROMK were analysed using FluxOR assay. Bars represent fluorescence, relative to baseline, at 180 s. Error bars show standard deviation.

have a saturable response at around 30 μM, with an EC50 of 3.8 μM (Fig. 2B). The maximal effect equated to a near 100% increase in TRESK channel activity. To assess the specificity of Cloxyquin activation it was 'counter-screened' against other potassium channels, ROMK (Kir1.1) and hERG (human *Ether-à-go-go*-Related Gene). ROMK, an inwardly rectifying potassium channel, was expressed in U2OS using baculovirus, and analyzed using FluxOR. This additionally allowed us to rule out the possibility Cloxyquin

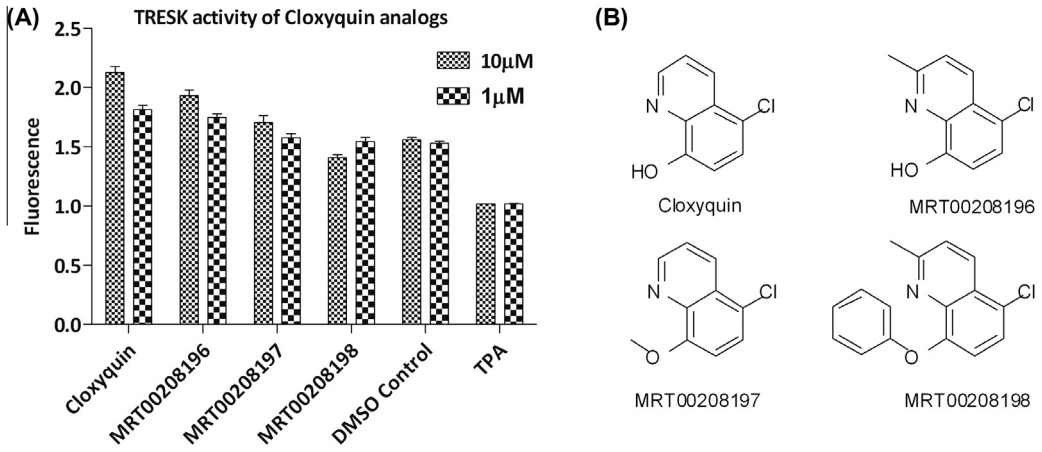

Fig. 3. (A) Screening for TRESK activation by Cloxyquin analogs. Graph shows activity of TRESK in U20S cells in FluxOR thallium flux assay. Cells treated with 10 or 1 μM of Cloxyquin or the Cloxyquin analogs MRT00208196, MRT00208197 or MRT00208198. Bars represent fluorescence relative to baseline at 50 s. Baseline read for 20 s before thallium addition. Error bars show standard deviation. (B) Structures of Cloxyquin and analogs. Cloxyquin (top left), MRT00208196 (top right), MRT00208197 (bottom left), MRT00208198 (bottom right).

was having a non-specific effect on U2OS cells or the thallium flux *per se*. No significant activation was observed at concentrations between 100 and 0.1 μM (Fig. 2C). Additionally, Cloxyquin showed no inhibition of hERG in a competition binding fluorescence polarization assay (Life Technologies, USA, data not shown). hERG is an important target for cardiac safety and due its structure is known to have a high propensity for small molecule interaction.

Initial structure–activity relationship (SAR) analysis of Cloxyquin was also carried out (Fig. 3) and three analogs were generated. The closest related structure, MRT00208196, which has an extra methyl group, retained the ability to activate TRESK. MRT00208198, which has more significant modifications, specifically an extra phenyl ring, showed a marked decrease in its ability to activate at TRESK, the change in fluorescence being comparable

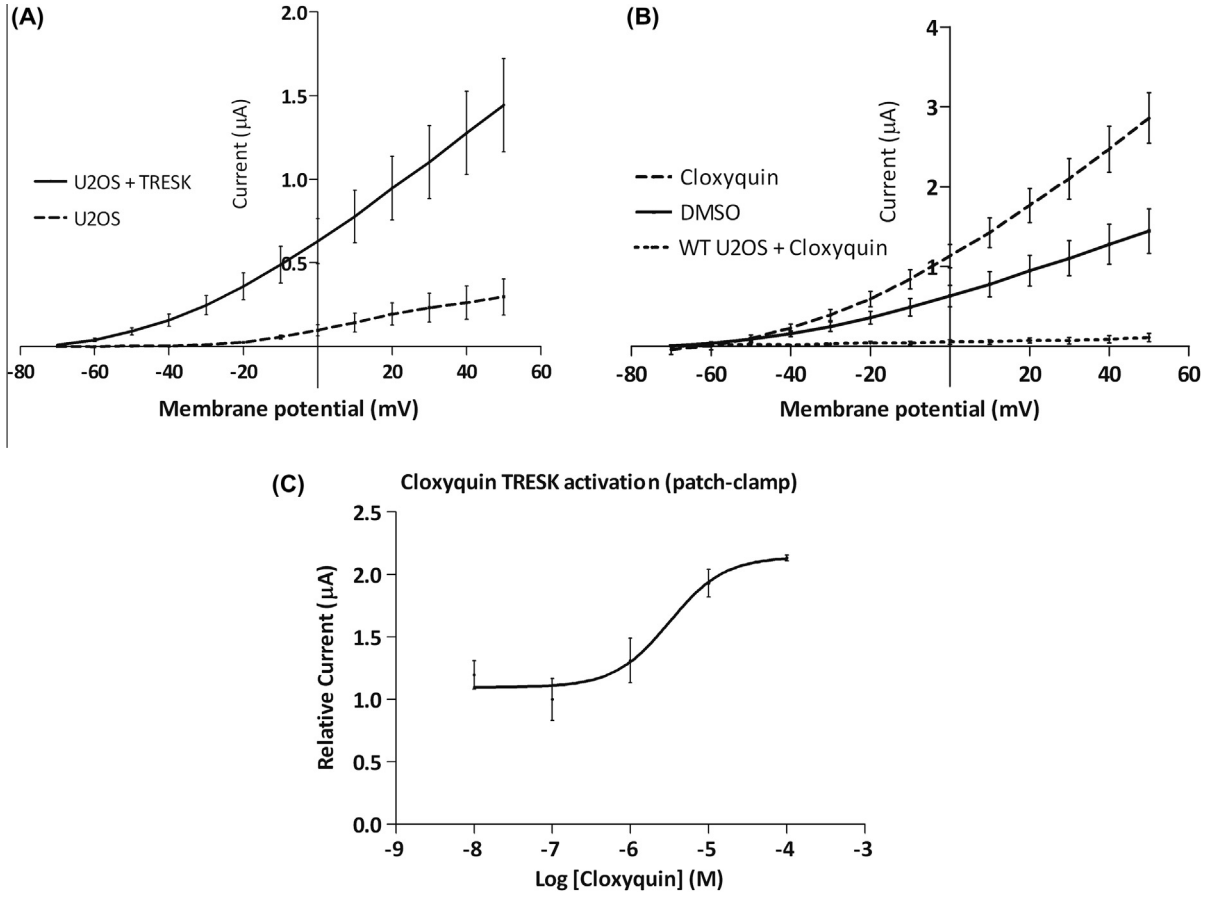

Fig. 4. Whole cell patch clamp analysis confirms TRESK activity and activation by Cloxyquin. (A) U20S cells transduced with TRESK baculovirus (U20S + TRESK) show an outward current. (B) Outward current of U2OS cells transduced with TRESK is enhanced by the application of 10 μM Cloxyquin. WT U2OS cells (non-transduced) show no increase in outward current when treated with Cloxyquin. Cells held in voltage clamp and stepped to depolarizing voltages. (C) Compound response curve for Cloxyquin activation using whole cell patch clamp electrophysiology. Graph shows current (at 50 mV) relative to vehicle control. Cloxyquin has an EC50 of 3.2 μM, when analysed using whole cell patch clamp. Error bars show SEM.

to that observed in the DMSO controls. Analysis of less potent hits from our initial screen revealed diversity in structures, although it is noteworthy that chloro-acetoxy-quinoline, another structurally related compound, was also identified as an activator of TRESK.

3.3. Cloxyquin mediated TRESK activation is observed in whole cell patch clamp electrophysiology

Although the FluxOR system is robust and largely predictive, the use of thallium and fluorescence as a surrogate necessitated confirmation of TRESK activity through analogous means. Patch clamp recordings in the whole-cell voltage clamp mode were used to directly analyze electrophysiological properties of transduced lines. The cells were clamped at a holding potential of −80 mV and then depolarised, in 10 mV increments, to a positive potential of 50 mV. A large, outwardly rectifying current was observed in TRESK expressing cells, which was largely absent in non-transduced U20S cells. Transduced and non-transduced cells showed maximal outward currents of 1.44 ± 0.28 μA and 0.3 ± 0.11 μA respectfully (Fig. 4A). Administration of 500 nM TPA caused near total inhibition, indicating the current observed was predominantly TRESK mediated. Addition of 10 μM Cloxyquin caused a near twofold increase in TRESK current (Fig. 4B), with treated cells exhibiting a peak outward current of 2.86 ± 0.32 μA. Consistent with activation of TRESK and not native currents, Cloxyquin had no effect on non-transduced cells (Fig. 4B). Compound response curves performed using whole cell patch clamp electrophysiology gave rise to an EC50 of 3.2 μM (Fig. 4C), which is in accordance with that observed in the FluxOR assay. Although limited pharmacological characterization has been performed to date, these data are compelling evidence that Cloxyquin is able to increase TRESK activity.

4. Discussion

The aim of this work was to identify activators of the two-pore domain potassium channel TRESK, which may be of therapeutic relevance in the treatment of migraine. Baculovirus was used to generate U20S cells expressing TRESK and channel function was measured using the FluxOR thallium flux system. Compound screening identified Cloxyquin as a novel activator of TRESK, and its activity was confirmed using whole cell patch electrophysiology. Cloxyquin was shown not to activate at a subset of alternative potassium channels and preliminary SAR was confirmed using a subset of structurally related compounds.

TRESK is known to be activated by increases in intracellular calcium [14]. This activation does not involve the direct biding of calcium to TRESK but is modulated by calcineurin, which acts to dephosphorylate two major regulatory regions, S264 and the S276 cluster. Independent of the calcineurin pathway and independent of protein kinase A (which phosphorylates TRESK and restores baseline) is the PMA induced activation of TRESK, which occurs via protein kinase C [12]. Also of note are the observations that membrane stretch [15], induced by cell swelling, elevates TRESK currents (whereas cell shrinkage acts to inhibit TRESK current) and volatile anesthetics are believed to increase TRESK current by enhancing channel gating to increase open probability [11]. These varied mechanisms of TRESK activation each provide novel opportunities for small molecule intervention.

Cloxyquin is a member of the quinolin-8-ol family, compounds with anti-amoebic properties used to treat intestinal infection. It is also described as having anti-bacterial and anti-fungal properties. The antimicrobial action is likely to relate to its chelating activities, the hypothesis being that iron chelation removes an essential nutrient or may involve the inhibition of transcription [16]. We may therefore speculate that perturbations in ion levels are leading to activation of TRESK via intracellular mechanisms such as those observed with calcium. Alternatively Cloxyquin may be acting via protein kinase C activation, as PMA does. Whilst we cannot rule out Cloxyquin is binding directly to TRESK it is likely given Cloxyquin's chemical properties it would cross the cell membrane. Importantly, we did not observe an increase in intracellular calcium (data not shown) after administration of Cloxyquin, thus the mechanism of activation is not likely to be similar in nature to the Gq receptor mediated activation observed with the muscarinic agonist carbachol [14] and is probably not related to the calcium/calcineurin pathway. Further investigations on the effect of Cloxyquin on each of the known mechanisms of TRESK activation, and even the interactions between the distinct pathways, may provide greater insight into the mechanisms of TRESK regulation.

Based on well documented target validation it is tempting to speculate TRESK activation would be of potential benefit in migraine by acting to make cells less excitable, specifically those in the trigeminal nerve. A number of treatments are available for acute symptoms such as triptans and for migraine prophylaxis, such as topiramate. Experimental therapies, such as CGRP antagonists, are also currently in trial. However, current therapies do not provide relief for all patients and triptans can lead to medication over-use headache. Although speculative a TRESK activator given prophylactically might be an effective treatment without the troublesome side effects of existing migraine therapies. Moreover, by virtue of an alternative mechanism of action combined with TRESK's restricted expression patterns, currently intractable patients may be treated with an improved therapeutic index. Indeed, due to its expression in the dorsal root ganglion a novel TRESK activator could also be of major therapeutic interest in the treatment of neuropathic pain and other nociceptive disorders.

In conclusion, Cloxyquin is a novel activator of the two-pore domain potassium channel TRESK. This identification may enable further elucidation of TRESK's role within neuronal excitability and migraine or pain. The strategy presented here describes an effective way to identify new molecules capable of activating TRESK, affording the screening of large compound libraries and thus potentially the development of new therapies.

References

[1] R.B. Lipton, W.F. Stewart, S. Diamond, M.L. Diamond, M. Reed, Prevalence and burden of migraine in the United States: data from the American Migraine Study II, Headache 41 (2001) 646–657.
[2] X. Zhang, D. Levy, R. Noseda, V. Kainz, M. Jakubowski, R. Burstein, Activation of meningeal nociceptors by cortical spreading depression: implications for migraine with aura, The Journal of Neuroscience 30 (2010) 8807–8814.
[3] X. Zhang, D. Levy, V. Kainz, R. Noseda, M. Jakubowski, R. Burstein, Activation of central trigeminovascular neurons by cortical spreading depression, Annals of Neurology 69 (2011) 855–865.
[4] B. de Vries, R.R. Frants, M.D. Ferrari, A.M.J.M. van den Maagdenberg, Molecular genetics of migraine, Human Genetics 126 (2009) 115–132.
[5] R.G. Lafrenière, M.Z. Cader, J.-F. Poulin, I. Andres-Enguix, M. Simoneau, N. Gupta, et al., A dominant-negative mutation in the TRESK potassium channel is linked to familial migraine with aura, Nature Medicine 16 (2010) 1157–1160.
[6] P. Enyedi, G. Czirják, Molecular background of leak K^+ currents: two-pore domain potassium channels, Physiological Reviews 90 (2010) 559–605.
[7] R.G. Lafrenière, G.A. Rouleau, Migraine: role of the TRESK two-pore potassium channel, The International Journal of Biochemistry & Cell Biology 43 (2011) 1533–1536.
[8] T. Dobler, A. Springauf, S. Tovornik, M. Weber, A. Schmitt, R. Sedlmeier, et al., TRESK two-pore-domain K^+ channels constitute a significant component of background potassium currents in murine dorsal root ganglion neurones, The Journal of Physiology 585 (2007) 867–879.
[9] A. Tulleuda, B. Cokic, G. Callejo, B. Saiani, J. Serra, X. Gasull, TRESK channel contribution to nociceptive sensory neurons excitability: modulation by nerve injury, Molecular Pain 7 (2011) 30.
[10] M.J. Steiger, T. Farrah, K. Rolles, P. Harvey, A.K. Burroughs, Cyclosporin associated headache, Journal of Neurology, Neurosurgery, and Psychiatry 57 (1994) 1258–1259.
[11] C. Liu, J.D. Au, H.L. Zou, J.F. Cotten, C.S. Yost, Potent activation of the human tandem pore domain K channel TRESK with clinical concentrations of volatile anesthetics, Anesthesia and Analgesia 99 (2004) 1715–1722.

[12] A.-K. Rahm, J. Gierten, J. Kisselbach, I. Staudacher, K. Staudacher, P.A. Schweizer, et al., PKC-dependent activation of human K(2P) 18.1 K(+) channels, British Journal of Pharmacology 166 (2012) 764–773.
[13] J. Zhang, T. Chung, K. Oldenburg, A simple statistical parameter for use in evaluation and validation of high throughput screening assays, Journal of Biomolecular Screening 4 (1999) 67–73.
[14] G. Czirják, Z.E. Tóth, P. Enyedi, The two-pore domain K^+ channel, TRESK, is activated by the cytoplasmic calcium signal through calcineurin, The Journal of Biological Chemistry 279 (2004) 18550–18558.
[15] G. Callejo, J.P. Giblin, X. Gasull, Modulation of TRESK background K^+ channel by membrane stretch, PLoS One 8 (2013) e64471.
[16] W. Rohde, B. Cordell, R. Webster, W. Levinson, Inhibition of amino acyl tRNA synthetase activity by copper complexes of two metal binding ligands. *N*-Methyl isatin beta-thiosemicarbazone and 8-hydroxyquinoline, Biochimica Et Biophysica Acta 477 (1977) 102–111.