Development and Validation of Fluorescence-Based and Automated Patch Clamp–Based Functional Assays for the Inward Rectifier Potassium Channel Kir4.1

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ABSTRACT

The inward rectifier potassium (Kir) channel Kir4.1 plays essential roles in modulation of neurotransmission and renal sodium transport and may represent a novel drug target for temporal lobe epilepsy and hypertension. The molecular pharmacology of Kir4.1 is limited to neurological drugs, such as fluoxetine (Prozac®), exhibiting weak and nonspecific activity toward the channel. The development of potent and selective small-molecule probes would provide critically needed tools for exploring the integrative physiology and therapeutic potential of Kir4.1. A fluorescence-based thallium (Tl⁺) flux assay that utilizes a tetracycline-inducible T-Rex-HEK293-Kir4.1 cell line to enable high-throughput screening (HTS) of small-molecule libraries was developed. The assay is dimethyl sulfoxide tolerant and exhibits robust screening statistics ($Z' = 0.75 \pm 0.06$). A pilot screen of 3,655 small molecules and lipids revealed 16 Kir4.1 inhibitors (0.4% hit rate). 3,3-Diphenyl-N-(1-phenylethyl)propan-1-amine, termed VU717, inhibits Kir4.1-mediated thallium flux with an IC_{50} of $\sim 6 \mu M$. An automated patch clamp assay using the IonFlux HT workbench was developed to facilitate compound characterization. Leak-subtracted ensemble “loose patch” recordings revealed robust tetracycline-inducible and Kir4.1 currents that were inhibited by fluoxetine ($IC_{50} = 10 \mu M$), VU717 ($IC_{50} = 6 \mu M$), and structurally related calcium channel blocker prenylamine ($IC_{50} = 6 \mu M$). Finally, we demonstrate that VU717 inhibits Kir4.1 channel activity in cultured rat astrocytes, providing proof-of-concept that the Tl⁺ flux and Ion-Flux HT assays can enable the discovery of antagonists that are active against native Kir4.1 channels.

INTRODUCTION

Inward rectifier potassium (Kir) channels are broadly expressed in excitable and nonexcitable tissues where they regulate numerous physiological processes, including nerve and muscle cell excitability, hormone secretion, and epithelial ion transport.¹ The Kir channel superfamily is comprised of 16 known genes (*KCNJ1x*) in humans, which are separated into seven subgroups (Kir1.x–Kir7.x) based on amino acid identity. Kir4.1 (*KCNJ10*) is expressed predominantly in brain and spinal cord astrocytes, retinal Muller glia, stria vascularis and glia of the inner ear, and renal tubule epithelial cells. Consistent with its localization, targeted deletion of *KCNJ10* in mice produces severe motor impairment, deafness, and premature death.^{2–6} The recent discovery^{7,8} of loss-of-function mutations in *KCNJ10* in patients with SeSAME (seizures, sensorineural deafness, ataxia, mental impairment, electrolyte imbalance) or EAST (epilepsy, ataxia, sensorineural deafness, salt-wasting tubulopathy) syndrome confirmed that Kir4.1 plays important roles in humans and may represent a druggable target for epilepsy and hypertension.

Kir4.1 constitutes the major K⁺ conductance in brain and spinal cord astrocytes and contributes to a large negative membrane potential in these cells. It is generally believed that K⁺ released into the extracellular space during trains of action potentials moves down its electrochemical gradient and into astrocytes via Kir4.1. The large negative membrane potential generated by Kir4.1 also contributes to glutamate uptake by astrocytes. Accordingly, knockout of *KCNJ10* depolarizes the astrocyte membrane potential and slows the rate of K⁺ and glutamate uptake.^{5,6} The loss of spatial buffering likely accounts, at least in part, for the reduced seizure threshold in SeSAME/EAST syndrome.^{7,8} However, the severity of the SeSAME/EAST syndrome may be due in part to gliosis, aberrant myelination, and neuronal death during embryological development.^{3,6} The development of selective small-molecule antagonists that are active *in vivo* would provide important tools for exploring the “druggability” of Kir4.1 and dissecting the relative contributions of acute versus chronic Kir4.1 loss-of-function in SeSAME/EAST syndrome. Furthermore, small-molecule activators of Kir4.1 may facilitate spatial buffering and lower the seizure threshold in epilepsy patients.

The renal consequences of SeSAME/EAST syndrome include polyuria, hypokalemia, and metabolic alkalosis, and are consistent with impaired NaCl reabsorption in the distal convoluted tubule

(DCT). In the DCT, NaCl absorption is mediated by the thiazide diuretic-sensitive NaCl cotransporter (NCC), which is located in the apical membrane of this nephron segment. Heteromeric Kir4.1/5.1 channels expressed in the basolateral membrane of the DCT (1) recycle K^+ across the basolateral membrane to help maintain the activity of the $Na^+-K^+-ATPase$, and (2) hyperpolarize the basolateral membrane potential to facilitate the electrogenic exit of Cl^- ions. Knockout of *KCNJ10* in mice recapitulates the salt-wasting phenotype of subjects with SeSAME/EAST syndrome.⁸ However, deletion of the Kir5.1-encoding gene *KCNJ16* paradoxically increases renal NaCl reabsorption.⁹ As alluded to earlier, unlike homomeric Kir4.1 channels, Kir4.1/5.1 is critically regulated by intracellular pH (pHi) and is partially inhibited at physiological pHi. A loss of this negative regulation in *KCNJ16*-knockout mouse leads to an increase in Kir4.1 activity and NaCl reabsorption in the DCT.⁷ Kir4.1/5.1 antagonists would be expected to mimic thiazide diuretics by indirectly inhibiting NCC-mediated NaCl reabsorption. However, they could offer some important advantages over conventional diuretics due to the localization of the channel on the basolateral membrane.^{10–12} Loop and thiazide diuretics must first be secreted by renal proximal tubule cells before reaching their sites of action on the apical membrane.^{13–16} Interactions between diuretics and other secreted drugs, including antibiotics, NSAIDs, antivirals, as well as organic acid in the setting of renal failure, can limit their secretion and natriuretic effects. Kir4.1/5.1 inhibitors that act directly on the basolateral membrane should circumvent this limitation. Furthermore, Zaika *et al.* (2013) reported recently that dopamine inhibits Na^+ reabsorption in the cortical collecting duct (CCD) through inhibition of Kir4.1 homomeric and Kir4.1/5.1 heteromeric channels.¹⁷ Conceivably, Kir4.1/5.1 antagonists would exhibit greater clinical efficacy than thiazide diuretics due to inhibition of sodium reabsorption in multiple nephron segments, unlike conventional diuretics that tend to work on single segments. Identifying subtype-selective modulators active against Kir4.1 or Kir4.1/5.1 channels will be essential for investigating the druggability of Kir4.1 as an antihypertensive target.

Loss-of-function mutations in the methyl CpG binding protein 2 (*MECP2*) gene give rise to Rett syndrome, a neurological disorder characterized by difficulties with communication, learning, and motor coordination. Of relevance to the present discussion, the *MECP2*-null mouse exhibits increased expression of Kir4.1 in the locus coeruleus, a brainstem region involved in regulation of respiratory activity by carbon dioxide (CO_2).¹⁸ Zhang *et al.* (2011) proposed that an increase in CO_2 /pH-insensitive Kir4.1 homotetrameric channels and loss of CO_2 /pH-sensitive Kir4.1/5.1 heteromeric channels lead to a blunted respiratory response to CO_2 and dysregulation of respiratory rhythmogenesis in Rett syndrome patients. If this is correct, and barring untoward general effects on neurotransmission, then small-molecule antagonists of homotetrameric Kir4.1 channels may help correct breathing abnormalities in Rett syndrome patients.^{19,20}

Evaluating the therapeutic potential of Kir4.1 awaits the development of potent, specific, and bioavailable small-molecule modulators, as the molecular pharmacology of Kir4.1 is limited to a small number of neurological drugs (*e.g.*, selective serotonin re-

uptake inhibitors and tricyclic antidepressants) exhibiting weak off-target activity toward the channel. Here we report the development and validation of a fluorescence-based thallium (Tl^+) flux assay to enable high-throughput library screening and an automated patch clamp electrophysiology assay that utilize an IonFlux HT workstation to facilitate hit validation and characterization.

MATERIALS AND METHODS

Molecular Biology and Stable Cell Line Generation

The human Kir4.1 channel open-reading frame was purchased from OriGene Technologies (NM_002241.4; Origene) and subcloned into pcDNA5/TO (Life Technologies) using standard molecular biology techniques. Polyclonal T-REx-HEK293 cell lines stably transfected with pcDNA5/TO-Kir4.1 were established essentially as described previously.^{21,22} Monoclonal cell lines were isolated through limiting dilution in 384-well plates and tested for tetracycline inducible Tl^+ flux, as outlined below (see *Tl^+ Flux Assay Development and Library Screening*). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μ g/mL streptomycin, 5 μ g/mL blasticidin S, and 250 μ g/mL hygromycin in a 5% CO_2 incubator at 37°C.

Manual Patch Clamp Electrophysiology

T-REx-HEK293-Kir4.1 cells were used for patch clamp analysis following overnight induction with tetracycline (1 μ g/mL). The standard pipette solution contained (in mM) 135 KCl, 2 $MgCl_2$, 1 EGTA, 10 HEPES-free acid, and 2 Na_2ATP (Roche), pH 7.2, 275 mOsmol/kg. The standard bath solution contained (in mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 10 HEPES-free acid, pH 7.4, 290 mOsmol/kg. Electrodes pulled from capillary glass had resistances of 3.0–3.5 M Ω when filled with pipette solution. Voltage and current signals were generated and acquired using an Axopatch 200B amplifier and Digidata 1322A analog-to-digital converter (Molecular Devices). Data acquisition and analysis were performed using pClamp 9.2 software (Molecular Devices). All recordings were made at room temperature (20°C–23°C).

Western Blot Analysis

Monoclonal T-REx-HEK293-Kir4.1 cells were plated at a density of 1×10^5 cells/cm² and cultured overnight in the absence or presence of tetracycline (0.01–1.0 μ g/mL), washed three times with ice-cold phosphate-buffered saline (PBS), and solubilized in ice-cold RIPA buffer containing (in mM) 150 NaCl, 100 NaF, 50 Tris-HCl (pH 8.0), 35 sodium deoxycholate, 5 EDTA, 1% Triton X-100, 0.1% SDS, and 1 $\times$ protease inhibitor cocktail. Cell lysates were scraped into Eppendorf tubes, sonicated, rotated end-over-end for 30 min at 4°C, and then centrifuged for 10 min to pellet insoluble material. The protein concentration of cleared lysates was quantitated using BCA reagent (Thermo Scientific). Cell lysates were either used immediately or frozen as single-use aliquots at –80°C for subsequent immunoblot analysis.

Proteins were separated electrophoretically using 10% Bis-Tris NuPAGE gels (Life Technologies) and transferred to 0.45- μ m-pore nitrocellulose membranes (BioRad) for immunoblot analysis. Membranes were blocked overnight at 4°C in Tris-buffered saline (25 mM

Tris, 1.3 mM KCl, and 137 mM NaCl) containing 0.1% TWEEN-20 (TBST) and 5% nonfat milk. Membranes were incubated for 1 h at room temperature in polyclonal Kir4.1 antiserum (Alomone Labs) diluted 1:500 in milk-TBST and subsequently for 1 h at room temperature in milk-TBST containing HRP-conjugated secondary antiserum diluted 1:40,000. Protein bands were visualized on film using enhanced chemiluminescence detection methods (Thermo Scientific). Membranes were stripped in buffer containing 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.7), and 2% SDS (50°C for 30 min) and reprobed with β -actin antiserum.

TI⁺ Flux Assay Development and Library Screening

A TI⁺ flux assay of Kir4.1 function was developed essentially as described previously (Table 1).²³ Briefly, T-REx-HEK293-hKir4.1 cells (20,000 cells/20 μ L per well) were plated in clear-bottomed, amine-coated, and black-walled 384-well plates (BD Biosciences) and cultured overnight in DMEM containing 10% dialyzed FBS, 50 U/mL penicillin, 50 μ g/mL streptomycin, 5 μ g/mL blasticidin S, and 250 μ g/mL hygromycin, and 1 μ g/mL tetracycline (assay media) at 37°C in the presence of 5% CO₂. The following day, a dye stock was prepared fresh by dissolving 50 μ g of FluoZin-2 (Life Technologies) in 100 μ L of

anhydrous dimethyl sulfoxide (DMSO). To prepare the dye-loading solution, 50 μ L of 20% pluronic acid is added to the earlier dye stock and the dye stock was diluted to 2 μ M with assay buffer (AB; Hank's balanced salt solution + 20 mM HEPES, pH 7.3). Twenty microliters was added to each well that contains cells. The cells were incubated for 60 min at room temperature (RT), followed by automated washing using the ELx405 plate washer (BioTek) primed with AB. A final aspiration step resulted in 20 μ L residual volume. The plate was loaded into the Functional Drug Screening System 6000 (FDSS6000; Hamamatsu) to obtain an initial baseline image (F_0)—10 images at 1 Hz (excitation, 470 $\pm$ 20 nm; emission, 540 $\pm$ 30 nm). The FDSS-integrated 384-channel pipettor simultaneously added 20 μ L of DMSO vehicle control, fluoxetine, or test compounds to each plate for final concentrations of 0.1%, 100, and 10 μ M, respectively. Test compounds in 40 μ L of AB were prepared by transferring 80 nL into a 384-well polypropylene plate using the ECHO 550 acoustic formatter (Labcyte) from a 10 mM stock (BioFocus DPI). Four columns in each plate were filled with DMSO vehicle control or fluoxetine-containing buffer for determination of Z' (e.g., Fig. 3B), while the remaining 20 columns were used for compound screening. The compounds were allowed to incubate on the cells for 20 min at RT; then, the plate was

reloaded into the FDSS and a second baseline was acquired. The FDSS-integrated pipettor added 10 μ L of thallium stimulus (125 mM sodium gluconate, 12 mM thallium sulfate, 1 mM magnesium sulfate, 1.8 mM calcium gluconate, 5 mM glucose, and 10 mM HEPES, pH 7.3) while imaging of the flux continued at 1 Hz for 4 min total sampling time.

Concentration–Response Curves

Compound master plates were created by serially diluting compounds 1:3 from 10 mM stock in 100% DMSO using the BRAVO liquid handler (Agilent Technologies). Assay daughter plates were created using the ECHO 555 liquid handler (Labcyte), transferring 240 nL from the master plate to the daughter plate for each well followed by addition of 40 μ L of AB resulting in antagonist compound concentration–response curves (CRCs) starting at 60 μ M (2 $\times$ final concentration). Concentration–response series were done in triplicate in the daughter plate. Cell plates and daughter compound plates were loaded onto an FDSS 6000. Baseline readings were taken (10 images at 1 Hz; excitation, 470 $\pm$ 20 nm; emission, 540 $\pm$ 30 nm) and 20 μ L of test compounds was added followed by 50 images at 1 Hz additional baseline. Following a 20-min incubation period, baseline readings were taken for 30 s followed by addition of TI⁺ stimulus buffer, followed by a 4-min read.

Data were analyzed using Excel (Microsoft Corp.). Raw data were opened in Excel and each data point in a given trace was divided by the first data point from that trace (static ratio). For experiments in which antagonists were added, data were again normalized by dividing each point by the fluorescence value immediately before the thallium addition to correct for any subtle differences in the baseline traces after the compound incubation period. The slope of the

Table 1. Thallium Flux Assay Protocol

Step	Parameter	Value	Description
1	Plate cells	20 μ L	20,000 T-REx-HEK293 cells
2	Reporter reagent	20 μ L	Fluozin-2 reporter dye
3	Incubation	1 h	Ambient temperature
4	Reporter removal	20 μ L	Remove dye and replace with assay buffer
5	Controls	20 μ L	Fluoxetine (100 μ M) or DMSO (0.1%) final assay concentration
6	Library compounds	20 μ L	10 μ M final assay concentration
7	Incubation	20 min	Ambient temperature
8	Stimulus reagent	10 μ L	Add thallium stimulus buffer
9	Assay readout	470 and 540 nm	CCD imager, fluorescent mode

Step Notes

1. Plate induced/uninduced cells in 384-well assay plates with multichannel pipettor (Biohit).
2. Assay buffer with 0.5 μ g/mL stock concentration of Fluozin-2 and 20% weight per volume Pluronic F-127 added with multidrop combi reagent dispenser (Thermo Scientific).
3. Plates lidged and maintained at room temperature.
4. Excess dye was removed with ELx405 microplate washer (BioTek) and replaced with assay buffer.
5. Columns 1–2 and 23–24, assay buffer $\pm$ fluoxetine or DMSO added with multichannel pipettor in checkerboard pattern.
6. FDSS-integrated 384-channel pipettor (Hamamatsu) dispenses compounds in all wells in columns 3–23.
7. Plates maintained at room temperature.
8. FDSS-integrated 384-channel pipettor dispenses thallium stimulus in all wells.
9. Reading at 1 Hz exposure, baseline read for 10 s, 4-min total sampling time.

fluorescence increase that begins 5 s after TI^+ addition and ends 15 s after TI^+ addition was calculated. The data were then plotted in Origin software (OriginLab Software, Inc.) to generate CRCs after correcting for the slope values determined for baseline waveforms generated in the presence of vehicle controls. Potencies were calculated from fits using a four-parameter logistic equation.

Automated Patch Clamp Electrophysiology

T-REx-HEK293-Kir4.1 cultured cells were induced overnight with 0.1 $\mu\text{g}/\text{mL}$ tetracycline prior to IonFlux automated electrophysiology. For cell isolation, cultured cells were washed with Ca- and Mg-free HBSS buffer, followed by Detachin™ solution (Genlantis) to detach them. The cells were resuspended in extracellular solution at 3×10^6 cells/mL. The extracellular solution contained 135 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 5 mM glucose, and 10 mM HEPES-free acid, pH 7.4, 290 mOsmol/kg. The intracellular solution for the whole-cell voltage clamp contained the following (mM): 5.4 CaCl_2 , 1.8 MgCl_2 , 120 KCl, 4 Na_2ATP , 10 HEPES, and 10 EGTA, pH 7.2 with KOH. The cell suspension in extracellular solution was dispensed into an IonFlux plate for recording. The IonFlux plate has been described previously.^{24,25} In brief, the IonFlux HT plate consists of 32 unique experimental patterns with each experimental pattern being comprised of 12 wells in the form of an 8-well plate with a unique microfluidic bottom. Within a 12-well experimental pattern, two wells that are referred to as “traps” are filled with intracellular solution, eight wells are designated for compounds that can be applied at any time during the experiment, one well is filled with cell suspension in extracellular solution, and the remaining “waste” well contains the solution outflow during the experimental run. For each experimental pattern, there are two recordings. Each recording is from an ensemble of 20 cells patched in parallel on a single “trap,” such that the current measured is the sum of all 20 cells of the ensemble. Given the variability in the quality of the seal for each given cell, the ensemble recording ends up being similar to “loose seal” recording conditions.

For recording Kir4.1 current, cells were voltage clamped at -80 mV (which is near E_K for the solution pair used), stepped to -120 mV and then ramped at 0.5 mV/s to $+120$ mV, and finally stepped back to -80 mV. Given that the cells are being held near E_K , the current flow at the holding potential is primarily nonspecific leak current, due in large part to the “loose seal” recording conditions. Kir4.1-specific current was estimated using the following formula:

$$I_{\text{Kir4.1}} = I_{-120 \text{ mV}} - (I_{-80 \text{ mV}} \times 1.5)$$

This equation is based on the assumption that the current measured at -80 mV is exclusively nonspecific leak. Data analysis and graphical presentation were performed using a combination of IonFlux, Excel, and Origin software.

Animals

Experiments were performed on Sprague–Dawley rats and were approved by the University of Alabama Institutional Animal Care and Use Committee.

Astrocyte Cultures

Spinal cord astrocytes were cultured in phenol red-free medium as described previously.⁵ Briefly, postnatal day 0 (P_0) pups were decapitated and spinal cords were dissected into ice-cold serum-free Eagle's minimum essential medium (EMEM) (Gibco) containing 20 mM glucose. Once the meninges were stripped, the cords were minced and placed into an O_2 -saturated papain solution (Worthington) for 20 min. The tissue was washed twice with spinal cord astrocyte media (EMEM supplemented with 10% fetal calf serum, 20 mM glucose, and penicillin/streptomycin) and then triturated. Cells were plated at a density of 1.0×10^6 cells/mL on polyornithine- and laminin-coated coverslips. The media were changed every day for the first 3 days and then every fourth day thereafter. Electrophysiology was performed on astrocytes that had been in culture for 7–10 days.

Astrocyte Electrophysiology

Whole-cell voltage-clamp recordings were made as described previously.⁵ Coverslips were transferred to a Zeiss Observer D1 microscope (Zeiss) equipped with DIC optics. Signals were acquired using an Axopatch 200B amplifier, controlled by Clampex 10.2 software via a Digidata 1320 interface (Axon Instruments). Signals were filtered at 2 kHz and digitized at 5 kHz. Data acquisition and storage were conducted with the use of pClamp 10.2 (Axon Instruments). Whole-cell capacitance and series resistances were measured directly from the amplifier, with the upper limit for series resistance being $12 \text{ M}\Omega$ and series resistance compensation adjusted to 80% to reduce voltage errors. Coverslips were continuously perfused with artificial cerebral spinal fluid (ACSF) containing (in mM) 120 NaCl, 3.0 KCl, 1.0 MgCl_2 , 2.0 CaCl_2 , 26.2 NaHCO_3 , 11.0 glucose, and 5.0 HEPES, bubbled with 95% O_2 and 5% CO_2 . The pipette solution contained (in mM) 125 K-gluconate, 10 KCl, 10 HEPES, 10 creatine phosphate, 2 MgATP , 0.2 NaGTP , and 0.5 EGTA. Drugs were diluted in DMSO to a stock concentration and added directly to the bathing solution at a concentration of $1 \mu\text{L}$ per milliliter (1:1,000).

RESULTS

Cell Line Characterization

A monoclonal T-REx-HEK293 cell line that expresses Kir4.1 from a tetracycline-inducible promoter was developed and characterized. Tetracycline-inducible cell lines are useful because (1) they can limit potential cell line degeneration and loss of assay performance due to K^+ channel overexpression, and (2) they provide a convenient internal control for distinguishing effects of pharmacologically active compounds on endogenous TI^+ flux pathways versus the overexpressed Kir channel of interest. Manual whole-cell patch clamp electrophysiology was used to characterize the functional and pharmacological properties of Kir4.1 channels expressed in T-REx-HEK293 cells. Under current clamp conditions, the resting membrane potential of cells cultured overnight in the absence or presence of tetracycline ($1 \mu\text{g}/\text{mL}$) was -23.8 ± 3.9 mV ($n=8$) and -72.5 ± 0.6 mV ($n=5$), respectively. The cells were voltage clamped at -75 mV, and then stepped between -120 mV and $+120$ mV in 10-mV increments for 250 ms to evoke whole-cell currents. Uninduced

cells exhibited relatively small-amplitude outward currents at positive test potentials (Fig. 1A) carried by voltage-gated K^+ and Cl^- channels endogenously expressed in HEK293 cells.^{26,27} In sharp contrast, tetracycline induced the expression of large-amplitude inward and outward currents, exhibiting weak rectification at positive test potentials (Fig. 1B). The current was inhibited by barium (not shown) as well as the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Fig. 1C), as reported by Kurachi and colleagues.^{28,29} The mean $\pm$ SEM current-voltage (I-V) relationships recorded from uninduced cells (open circles; $n=5$) or induced cells in the absence (closed circles; $n=8$) or presence (open triangles; $n=4$) of 100 μ M fluoxetine are shown in Figure 1D. Fluoxetine also inhibited the endogenous outward current in HEK cells (Fig. 1C, D), consistent with reports that the drug inhibits other ion channels.^{30–32} Thus,

overnight induction with tetracycline gives rise to robust, fluoxetine-inhibitable Kir4.1 channel activity in this stable cell line. As shown in Figure 1E, Western blot analysis revealed that overnight incubation with tetracycline led to the dose-dependent induction of Kir4.1 channel expression that reached a maximum level with 0.1 μ g/mL tetracycline. No Kir4.1-immunoreactive bands were observed in lysates prepared from nontransfected “parental” T-Rex cells, indicating that the antiserum is specific for Kir4.1.

Tl⁺ Flux Assay

A quantitative Tl⁺ flux assay was developed to enable high-throughput screening (HTS) for small-molecule Kir4.1 modulators. The assay employs the fluorescent dye FluoZin-2 to report the inwardly directed movement of the K^+ congener Tl⁺ through the channel pore, as reported by us for other Kir channels previously.^{22,23,33,34} Monoclonal T-Rex-HEK293-hKir4.1 cells were plated in clear-bottomed 384-well plates, cultured overnight in the absence or presence of tetracycline, and used in Tl⁺-flux assays the following day (see **Materials and Methods** for details). Figure 2A and B shows FluoZin-2 fluorescence emission traces recorded from wells that contain uninduced or induced cells, respectively, before and after addition of various Tl⁺ concentrations (arrow; see below). The Tl⁺-induced fluorescence increase in uninduced cells (Fig. 2A) was relatively small, reflecting the movement of the cation through endogenous pathways. However, induction of Kir4.1 expression led to a dramatic increase in the rate and extent of Tl⁺ flux (Fig. 2B). Indeed, we were concerned that Tl⁺ flux was so high that the indicator dye may become saturated and limit the assay's ability to detect weak inhibitors or agonists, and therefore we established a Tl⁺ CRC to identify a submaximal

Fig. 1. Tetracycline-inducible Kir4.1 expression in stably transfected T-Rex-HEK293 cells. (A–C) Representative whole-cell currents recorded from monoclonal T-Rex-HEK293-Kir4.1 cells cultured without tetracycline (A), or with tetracycline before (B) and after (C) bath addition of the Kir4.1 channel blocker fluoxetine. Cells were voltage clamped between -120 mV and $+120$ mV for 500 ms in 10 mV increments from a holding potential of -75 mV. (D) Mean $\pm$ SEM current-voltage (I-V) relationships from uninduced (U) or induced cells bathed in control buffer (I) or buffer containing 100 μ M fluoxetine (I+F). Current amplitude was normalized to cell capacitance and is expressed as pA/pF ($n=5-6$). (E) Western blot analysis of Kir4.1 expression in lysates prepared from parental T-Rex or T-Rex-Kir4.1 cells cultured overnight with the indicated concentrations of tetracycline. Tet (0.1 and 1 μ g/mL) induced in T-Rex-Kir4.1 cells, but not in the parental cells (T-Rex), the expression of a prominent ~ 42 kDa band and higher-molecular-weight bands corresponding to differentially glycosylated forms of the channel. The membrane was stripped and reprobed for β -actin to ensure equal protein loading.

Fig. 2. Thallium flux reporter assay of Kir4.1 channel function. **(A, B)** Representative Ti^+ -induced changes in FluoZin-2 fluorescence recorded from T-REx-HEK293-Kir4.1 cells cultured overnight without **(A)** or with **(B)** tetracycline. Baseline fluorescence was recorded for 4 min before Ti^+ addition (arrow). Mean $\pm$ SEM ($n=3$) concentration–response curve (CRC) and 4-parameter logistic fit for Ti^+ flux recorded from tetracycline-induced cells is shown in the inset of panel **(B)**. **(C)** Dimethyl sulfoxide (DMSO) tolerance of Kir4.1-mediated Ti^+ flux. Data are means $\pm$ SEM Ti^+ flux normalized to that recorded in the presence of DMSO ($n=9$). **(D)** Representative checkerboard assay using DMSO (vehicle) or 100 μM fluoxetine. The mean peak fluorescence amplitude of each sample population is indicated with a solid line. Three standard deviations from the mean are shown with a dashed line. The mean $\pm$ SD Z' calculated over nine plates on 3 separate days was 0.75 ± 0.06 .

concentration that still provided a robust signal window. The inset of Figure 2B shows that mean $\pm$ SEM change in FluoZin-2 fluorescence exhibited a sigmoidal, dose-dependent relationship that saturated around 2 mM Ti^+ . The concentration required to evoke $\sim 80\%$ of the maximal response was determined from the fit of a four-parameter logistic function to be 0.84 mM. This concentration was used for assay development and the pilot screen to provide “headroom” for identifying small-molecule activators of Kir4.1.

The sensitivity of the assay to the small-molecule solvent DMSO was determined by assessing Ti^+ flux across a twofold dilution series of DMSO ranging from 0.1% to 5% v/v. As shown in Figure 2C, Ti^+ flux was unaffected at concentrations up to 0.3% v/v, but it dropped off dose dependently at higher concentrations. DMSO had no effect on Kir4.1-mediated Ti^+ flux at the screening concentrations of 0.1% and 0.3% v/v.

“Checkerboard” assays were performed on 3 separate days to determine the well-to-well uniformity within a plate and day-to-day reproducibility. Every other well of a 384-well plate that contains tetracycline-induced T-REx-HEK-293-Kir4.1 cells was treated for 20 min with DMSO (vehicle) or 100 μM fluoxetine (nonspecific

blocker) before initiating Ti^+ flux. In Figure 2D, Ti^+ -induced FluoZin-2 fluorescence from each well of one plate is plotted, with the mean fluorescence and three standard deviations from the mean indicated with a μ and 3σ , respectively. The assay conditions afforded clear separation of the two cell populations, yielding a mean $\pm$ SEM Z' value for nine plates on 3 separate days of 0.75 ± 0.06 . Thus, the assay is robust, reproducible, and suitable for HTS.

Discovery of a Novel Kir4.1 Inhibitor

The assay was validated with a screen of 3,655 small molecules and bioactive lipids at the Vanderbilt HTS center. Small molecules and lipids were tested at a nominal concentration of 10 μM (0.1% DMSO) and 3 μM (0.3% DMSO), respectively. The mean Z' for the entire 19-plate run, calculated from the peak fluorescence amplitude in vehicle or fluoxetine-treated wells, was 0.82 ± 0.04 . The hit rate was 0.4% (16/3,655 compounds). All of the hits were inhibitors; none of which were lipids. The most potent inhibitor identified was 3,3-diphenyl-*N*-(1-phenylethyl) propan-1-amine, which we termed VU717. The chemical

structure is shown in Figure 3A. In Ti^+ flux assays, VU717 dose-dependently inhibited Kir4.1 activity with an IC_{50} of 6.3 μM and Hill coefficient of 4.5 (Fig. 3B, C).

Development of an Automated Patch Clamp Assay for Kir4.1 Using the IonFlux HT

Patch clamp electrophysiology is the gold standard technique for interrogating the pharmacology of ion channels. However, manual patch clamping is low-throughput and thus it imposes a considerable bottleneck. We therefore set out to develop an automated patch clamp assay to minimize this bottleneck and facilitate the development of Kir4.1 channel modulators.

We first established the time required to induce Kir4.1 channel expression and function in T-REx-HEK293-Kir4.1 cells following induction with tetracycline. Figure 4A shows a representative Western blot of Kir4.1 (top panel) or actin (bottom panel, loading control) expression in lysates prepared from cells cultured with tetracycline (1 $\mu\text{g}/\text{mL}$) for 0, 2, 4, 6, or 18 h. Kir4.1 expression was time dependent and reached a maximum following 18 h of induction.

Fig. 3. Discovery of a novel Kir4.1 channel inhibitor in a Ti^+ flux-based pilot screen. **(A)** Chemical structure of 3,3-diphenyl-N-(1-phenylethyl)propan-1-amine (VU717). **(B)** Representative FluoZin-2 fluorescence traces recorded from induced T-REX-HEK293-Kir4.1 cells treated with the indicated doses of VU717. **(C)** Mean $\pm$ SEM CRC for VU717-dependent inhibition of Kir4.1-mediated Ti^+ flux. Data are from three independent experiments performed in triplicate.

We then assessed Kir4.1 channel activity in cells induced for the same time period using an IonFlux HT automated patch clamp workstation. The IonFlux HT utilizes a network of microfluidic channels that enables fully automated cell introduction, trapping, sealing, whole-cell formation, and voltage clamp using 64 indepen-

dent voltage clamp amplifiers. Recordings are made in the “loose patch” configuration from an ensemble of 20 cells per channel. This approach has the advantage of increasing the robustness and throughput of recordings, but may suffer from significant “leak” current introduced by sub-Giga-Ohm-resistance seals. We therefore employed Ba^{2+} to discriminate

Fig. 4. Automated patch clamp electrophysiology assay for Kir4.1. **(A)** Time-course of Kir4.1 protein expression in lysates prepared from cells cultured for the indicated times with tetracycline ($0.1 \mu\text{g/mL}$). β -Actin was used as a loading control. **(B)** Corresponding time-course analysis of Ba^{2+} -inhibitable Kir4.1 current amplitude at -120 mV recorded using an IonFlux HT automated patch clamp workstation. Following overnight induction with tetracycline, the cells were voltage clamped at a holding potential of -80 mV and then ramped between -120 mV and $+120$ mV in the absence (control) and presence of $300 \mu\text{M}$ barium (Ba^{2+}). Data are mean $\pm$ SEM current amplitude at -120 mV. **(C)** Representative leak-subtracted (see the “Materials and Methods” section), whole-cell currents recorded in the absence or presence of Ba^{2+} .

Kir4.1-mediated current from leak current. Cells were voltage clamped at a holding potential of -80 mV, which is near the calculated Nernst equilibrium potential for K^+ , and then the voltage was stepped to a test potential of -120 mV. Currents were leak subtracted as described in **Materials and Methods**. The mean $\pm$ SEM of Ba^{2+} -sensitive current at -120 mV is shown in Figure 4B. In contrast to the Western blot results that show abundant Kir4.1 protein expression after only 6 h of tetracycline induction, no Ba^{2+} -sensitive currents were detectable at this time point. Following 18 h of induction, however, robust Ba^{2+} -inhibitable currents were clearly observed. Cells were therefore induced overnight with tetracycline in all subsequent experiments.

Figure 4C shows representative, leak-subtracted current traces recorded in the absence or presence of $300 \mu\text{M}$ Ba^{2+} from cells subjected to a voltage ramp between -120 mV and $+120$ mV. In control solution, the current exhibited a steep voltage-

Fig. 5. The IonFlux HT assay is reproducible. **(A)** Leak-subtracted whole-cell current amplitude recorded at -120 mV from individual microfluidic patterns in the absence or presence of $300 \mu\text{M}$ Ba^{2+} . Data recorded on two separate days are shown. **(B)** Mean $\pm$ SEM Ba^{2+} CRC recorded on two separate days ($n=10$ – 30).

dependent relationship between -120 mV and $+50$ mV, a plateau around $+60$ mV, and rectification at potentials more positive than $+70$ mV. This current waveform is similar to that recorded from T-REx-HEK293-Kir4.1 cells using the manual patch clamp technique (Fig. 1D). The inward current was abolished by $300 \mu\text{M}$ Ba^{2+} .

Figure 5A summarizes the current amplitudes recorded in the absence or presence of Ba^{2+} from an entire 384-well format plate on two separate days. Each plate consists of 32 “experimental patterns,” each containing 2 cell “traps.” Each data point represents the average current amplitude from two daughter traps. The mean $\pm$ SD current amplitudes in control and Ba^{2+} -treated cells were, respectively, -5857 ± 1438 and 886 ± 478 pA. Out of 64 patterns tested, 59 of these led to successful cell trapping, whole-cell formation, and recordings of Ba^{2+} -inhibitable Kir4.1 channel activity (92% success rate).

The mean $\pm$ SEM Ba^{2+} CRC recorded on two separate days is shown in Figure 5B. The IC_{50} values derived from 4-parameter logistic functions for days 1 and 2 were 3.59 ± 0.12 and $2.37 \pm 0.12 \mu\text{M}$, respectively. Taken together, these data indicate that robust Kir4.1-dependent channel activity can be readily measured using the IonFlux HT workstation (Table 2).

Characterization of Small-Molecule Kir4.1 Antagonists with the IonFlux HT

VU717 and fluoxetine share some chemical features that likely contribute to their activity toward Kir4.1, including two hydrophobic benzene rings and an ionizable secondary amine group (Fig. 6A). In an effort to identify other inhibitors of Kir4.1, a search of the National Center for Biotechnology Information PubChem database for structurally related substances revealed that the calcium channel blocker prenylamine (3,3-diphenyl-N-(1-phenylpropan-2-yl)propan-1-amine) shares these chemical features (Fig. 6A). Prenylamine was formerly used clinically as a vasodilator in the treatment of angina pectoris, but it

was discontinued because of its propensity to induce *torsades de pointes* by blocking hERG channels.³⁵ The potencies of VU717, prenylamine, and fluoxetine were compared across a threefold dilution series ranging from 300 nM to $100 \mu\text{M}$. The mean $\pm$ SEM CRCs for the three compounds are shown in Figure 6B. The IC_{50} values for VU717 ($n=6$ – 43), prenylamine ($n=4$ – 20), and fluoxetine ($n=2$ – 17) were 6.0 ± 1.3 , 6.2 ± 1.0 , and $10.4 \pm 3.2 \mu\text{M}$, respectively, and the Hill coefficients were 1.9, 2.0, and 2.3, respectively. For comparison, CRCs were established in Kir4.1 Ti^+ flux assays for VU717, prenylamine, and fluoxetine, which yielded IC_{50} values

of 9.72 ± 2.0 , 14.8 ± 1.9 , and $22.8 \pm 0.9 \mu\text{M}$, respectively. Thus, although the potencies derived from electrophysiology and Ti^+ flux assays differ by ~ 2.5 -fold, the Ti^+ flux assay assigned the correct rank-order potency across this series.

VU717 Inhibits Native Kir4.1 Channels in Astrocytes

Given that VU717 was discovered in a screen against a heterologously expressed channel, we assessed the effects of the compound on astrocyte currents to determine whether VU717 is active against native Kir4.1 channels. Astrocytes that exhibit significant arborization were selected under DIC microscopy for voltage clamp recording (Fig. 7A, inset). In response to a linear voltage ramp (-160 mV to $+160$ mV over 300 ms) these cells responded with a large amplitude current that reversed at -80 mV (Fig. 7B). This current was nearly, completely inhibited in the presence of $250 \mu\text{M}$ Ba^{2+} containing ACSF, identifying this current as Kir4.1-mediated current as previously described.^{5,36} Barium was then washed off and the currents quickly returned to baseline. ACSF that contains $10 \mu\text{M}$ or $30 \mu\text{M}$ VU717 was then applied to the voltage clamped cell until

Table 2. IonFlux-HT Assay Protocol Table

Step	Parameter	Value	Description
1	Test compounds	$70 \mu\text{L}$	Add to designated compound wells of microfluidic plate
2	Plate priming		Use IonFlux microfluidic plate priming protocol
3	Transfer cells	90 – $100 \mu\text{L}$	2 – 5 million cells/mL, cell suspension in microfluidic inlet well
4	Assay readout		Run IonFlux experimental protocol

Step Notes

1. DMSO stock compounds prepared in external buffer to appropriate final concentrations.
2. Microfluidic plate (Fluxion) transferred to IonFlux-HT (Fluxion) station for priming.
3. Cell suspension in serum-free medium added to designated microfluidic inlet well for readout.
4. Assay readout initiated as soon as cells are transferred to microfluidic plate.

Fig. 6. Characterization of small-molecule Kir4.1 inhibitors using the IonFlux HT. **(A)** Chemical structures of VU717, prenylamine, and fluoxetine. **(B)** Mean $\pm$ SEM CRCs for VU717 (Δ , $n=6-43$), prenylamine ($\circ$, $n=4-20$), and fluoxetine ($\blacksquare$, $n=2-17$). A fit of the CRC with a single-site four-parameter logistic function yielded IC_{50} values of 6.0 ± 1.3 , 6.2 ± 1.0 , and 10.4 ± 3.2 μ M, respectively, for VU717, prenylamine, and fluoxetine.

maximum inhibition was achieved. Figure 7B shows that 10–30 μ M VU717 inhibited $\sim 90\%$ of the whole-cell current at -120 mV, which is nearly identical to that blocked by 250 μ M barium. This is comparable to that observed in conventional patch clamp recordings from Kir4.1-expressing HEK293 cells in which VU717 inhibited $94\% \pm 1.8\%$ ($n=5$) of current at -120 mV. This data clearly indicates

Like most of the TI^+ flux assays developed by our group,^{22,23,34,35} the Kir4.1 assay employs a tetracycline-inducible HEK293 cell expression system. This offers a number of experimental advantages over constitutive expression systems for assay development and HTS. For example, the ability to quantitate “background” TI^+ flux through endogenous pathways and a K^+ channel target of interest is useful

that the TI^+ flux assay is capable of identifying inhibitors of native Kir4.1 channels.

DISCUSSION

The assays developed herein should support a robust small-molecule probe discovery effort for Kir4.1. The TI^+ flux assay developed originally by Weaver *et al.* and later commercialized by Invitrogen and TEFlabs under the FluxOR[®] and Thallos labels, respectively, has now been used to establish fluorescence-based functional assays for a variety of potassium channels, including Kir1.1, Kir2.1, Kir2.3, Kir3.1/3.2, Kir7.1, hERG, KCNQ4,^{22,33,34,37-43} as well as the potassium chloride cotransporter KCC2.⁴⁴ Most of the Kir channels are well suited for the TI^+ flux assay, because they typically exhibit a large open probability near the resting membrane potential of the cell and therefore do not require activation by depolarization or an agonist. This is not true of G protein-coupled Kir3 channels, which require activation with a Gi/o-coupled receptor before TI^+ addition.^{34,38}

Fig. 7. VU717 inhibition of native Kir4.1 activity channels in astrocytes. **(A, B)** Representative I-V relationships recorded by voltage ramping a cell from -160 mV to $+160$ mV in control artificial cerebral spinal fluid (ACSF) or ACSF containing 30 μ M VU717 or 250 μ M Ba^{2+} . A DIC image of a cultured astrocyte is shown in the inset. **(B)** Mean $\pm$ SEM % inhibition of astrocyte whole-cell current at -120 mV by VU717 (10 and 30 μ M; $n=5$, $n=6$, respectively) or Ba^{2+} (250 μ M; $n=16$).

for selecting stably transfected clones affording the most robust signal window for screening. Dozens of clones cultured in the absence or presence of tetracycline can be assayed simultaneously, and those exhibiting the strongest tetracycline-inducible signal can be selected for assay development. In the absence of suitable reference compounds (*e.g.*, fluoxetine used in this study), uninduced cells could be included in assay plates as a “blocked” control for calculating Z' scores. Finally, false-positive hits that act on endogenous TI^+ flux pathways, as opposed

to the K⁺ channel target, can be rapidly identified and triaged in screens against uninduced cells. As shown in *Figure 2A and B*, Kir4.1-mediated Tl⁺ flux dominates the signal window, which increases the ability of the assay to identify specific Kir4.1 modulators.

In pilot experiments, we noted that the fluorescence emission of FluoZin-2 approached its asymptotic value within ~60 s of adding Tl⁺ to the cell plate. Such robust Tl⁺ flux was not surprising given that (1) the cell line was selected for high Kir4.1 expression and function, and (2) Kir4.1 is a “weak rectifier” that exhibits a high open-state probability across a broad range of membrane potentials, unlike some voltage-gated K⁺ channels that require depolarization for channel opening and Tl⁺ flux.³⁷ However, the robustness of Tl⁺ flux raised concerns that the dye would become saturated and fail to report subtle changes in Kir4.1 activity caused by pharmacologically weak modulators in a screen. We reasoned that using a lower Tl⁺ concentration would prevent saturation, increase the sensitivity of the assay, and provide “headroom” for identifying activators. From dose–response experiments, an EC₈₀ concentration of 0.84 mM was determined, which represents only 15% of that used previously by our group.^{22,23,34,35} Despite the use of lower Tl⁺ concentrations, which tended to slightly increase the well-to-well variability in fluorescence and reduce the mean Z' score (data not shown), the assay generated robust screening statistics, indicating that it is suitable for HTS of chemical libraries. Our studies of VU717 show that the assay is clearly capable of identifying a ~6 μM IC₅₀ inhibitor of Kir4.1 in a primary screen, which is a reasonable starting point to developing a submicromolar inhibitor suitable for *in vitro* and ideally *in vivo* pharmacology.

With an anticipated hit rate of 0.4%, it would take several weeks to characterize a hit collection from a large compound screen using “gold standard” manual patch clamp electrophysiology techniques. We therefore evaluated the IonFlux HT as an automated patch clamp workstation for characterizing Kir4.1 modulators in a shorter time frame while maintaining fidelity. The IonFlux platform technology is based on the Fluxion's proprietary microfluidic system, which enables ensemble cell recording in a continuous manner. While already validated for the study of ligand-gated, voltage-gated, sodium, and hERG channel pharmacology,^{24,25} this is the first study to show that the IonFlux platform can be adapted for the study of Kir channel pharmacology. Kir4.1 is considered a “weak rectifier” since a decrease in channel conductance (*i.e.*, rectification) is only observed at relatively large positive potentials (*e.g.*, *Fig. 4A*). This presented a challenge for discriminating Kir4.1-derived current in “loose patch” recordings contaminated by “leak” current, because both channel-mediated and leak currents exhibit Ohmic (linear) current–voltage relationships across most of the voltage range tested. To circumvent this problem, we reasoned that all of the Kir4.1-mediated current would reverse directions at the Nernst equilibrium potential for potassium (E_K), which was approximately –80 mV under the recording conditions used as opposed to 0 mV for leak-mediated currents. Leak current, defined as the offset current at –80 mV multiplied by 1.5 (*i.e.*, –80 mV × 1.5 = –120 mV), was subtracted from the whole-cell current amplitude at –120 mV to yield values for leak-subtracted

Kir4.1 current amplitude. The results suggest that this platform is sufficiently robust (>90% success rate) to provide an initial electrophysiological characterization of small-molecule inhibitors of Kir4.1. With a runtime of ~30 min per plate and the ability to characterize up to 30 compounds along with controls on a given plate, the system will be capable of producing CRCs for hundreds of compounds per day.

In conclusion, we have developed and validated a robust Tl⁺ flux assay to support a primary screen for small-molecule modulators of Kir4.1. A pilot screen of 3,655 small molecules and lipids and a subsequent structural homology search led to the discovery of the novel Kir4.1 antagonists VU717 and prenylamine. In addition, we demonstrated for the first time that Kir4.1 channel pharmacology can be characterized in automated loose patch clamp electrophysiology assays using the IonFlux HT workstation. The development of small-molecule Kir4.1 modulators using these assay platforms will provide critically needed tools for investigating the integrative physiology and therapeutic potential of Kir4.1 in temporal lobe epilepsy and hypertension.

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No competing financial interests exist.

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Abbreviations Used

AB = assay buffer
ACSF = artificial cerebral spinal fluid
CRC = concentration-response curve
DCT = distal convoluted tubule
DMEM = Dulbecco's modified Eagle's medium
DMSO = dimethyl sulfoxide
EMEM = Eagle's minimum essential medium
FBS = fetal bovine serum
FDSS = functional drug screening system

HBSS = Hank's balanced salt solution
HTS = high-throughput screening
NCC = sodium-chloride co-transporter
NCI = National Cancer Institute
PBS = phosphate-buffered saline
pHi = intracellular pH
Tl⁺ = thallium
T-Rex-HEK293 = tetracycline inducible human embryonic kidney
VU717 = 3,3-diphenyl-*N*-(1-phenylethyl)propan-1-amine