Validation of an Atomic Absorption Rubidium Ion Efflux Assay for KCNQ/M-Channels Using the Ion Channel Reader 8000

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Abstract: M-channels (M-current), encoded by KCNQ2/3 K^+ channel genes, have emerged as novel drug targets for a number of neurological disorders. The lack of direct high throughput assays combined with the low throughput of conventional electrophysiology (EP) has impeded rapid screening and evaluation of K^+-channel modulators. Development of a sensitive and efficient assay for the direct measurement of M-current activity is critical for identifying novel M-channel modulators and subsequent investigation of their therapeutic potential. Using a stable CHO cell line expressing rat KCNQ2/3 K^+ channels confirmed by EP, we have developed and validated a nonradioactive rubidium (Rb^+) efflux assay in a 96-well plate format. The Rb^+ efflux assay directly measures the activity of functional channels by atomic absorption spectroscopy using the automated Ion Channel Reader (ICR) 8000. The stimulated Rb^+ efflux from KCNQ2/3-expressing cells was blocked by the channel blockers XE991 and linopirdine with IC_{50} values of 0.15 μM and 1.3 μM, respectively. Twelve compounds identified as KCNQ2/3 openers were further assessed in this assay, and their EC_{50} values were compared with those obtained with EP. A higher positive correlation coefficient between these two assays ($r = 0.60$) was observed than that between FlexStation membrane potential and EP assays ($r = 0.23$). To simplify the assay and increase the throughput, we demonstrate that EC_{50} values obtained by measuring Rb^+ levels in the supernatant are as robust and consistent as those obtained from the ratio of Rb^+ in supernatant/lysate. By measuring the supernatant only, the throughput of ICR8000 in an eight-point titration is estimated to be 40 compounds per day, which is suitable for a secondary confirmation assay.

Introduction

KCNQ2 AND KCNQ3 K^+ channel subunits coassemble as M-channels in neurons to produce native M-current.[1] The M-current is a low-threshold, noninactivating voltage-gated K^+ current that exerts an inhibitory control over neuronal excitability by repolarizing the membrane back toward the K^+ equilibrium potential during action potentials. This is a common mechanism by which neuronal activity is controlled. Mutations of either *KCNQ2* or *KCNQ3* genes cause neonatal epilepsy, or deletion of one in mice enhances sensitivity to epileptogenic agents, indicating that disruption of the M-current leads to

disordered neuronal excitability.[2–5] Due to their importance in regulating neuronal excitability, KCNQ2/3 K^+ channels have emerged as novel drug targets, and modulators of KCNQ2/3 K^+ channels have been under clinical investigations for a number of neurological disorders, such as epilepsy and pain.[6–10] Selective M-current blockers linopirdine and XE991 have been shown to promote acetylcholine release and improve learning abilities in animal models of cognition.[11–13] The M-current activator retigabine has been shown to be effective in preventing seizures presumably by opening KCNQ channels.[6,14,15]

Development of a sensitive and efficient assay for the direct measurement of M-current activity is a critical step

ABBREVIATIONS: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle medium; EP, electrophysiology; ICR, Ion Channel Reader; Rb^+, rubidium; TEA, tetraethylammonium chloride.

for identifying novel KCNQ/M-channel modulators and subsequent investigation of their therapeutic potential. The lack of direct, high throughput assays and the low throughput of conventional electrophysiology (EP) have impeded a rapid screening and evaluation of K^+-channel modulators. FLIPR and FlexStation assays, with voltage-sensitive fluorescent dyes to detect membrane potential changes, are indirect measures of channel function and subject to interference by autofluorescent compounds, which results in high false-positive rates.[16] Therefore, it is necessary to seek an alternative assay that offers advantages of higher throughput, more direct measurement, and convenience with low cost.

The recent development and application of nonradioactive rubidium (Rb^+) efflux assay using atomic absorption spectrometry have demonstrated that the assay represents a safe and convenient screening tool with a capacity of medium to high throughput.[17,18] The atomic absorption Rb^+ efflux assay also provides an attractive alternative to replace the radioactive format of ^{86}Rb and scintillation counting.[16–18] Here, we describe the validation of an atomic absorption of Rb^+ efflux assay that can be utilized to screen KCNQ/M-channel modulators. Using the Rb^+ efflux assay, we have characterized compounds from an in-house chemical library that increase Rb^+ efflux through KCNQ2/3 channels. EC_{50} values obtained by Rb^+ efflux were compared with those obtained by the gold standard patch-clamp EP. A positive correlation coefficient was achieved ($r = 0.60$) between the two assays, whereas no correlation for the same set of compounds was observed between FlexStation membrane potential and EP assays ($r = 0.23$).

Materials and Methods

Materials

All chemical reagents, including KCNQ-channel blockers linopirdine and its analogue XE991, were purchased from Sigma (St. Louis, MO) unless otherwise stated. Poly-D-lysine-coated 96-well microplates (catalogue no. 356461) purchased from Becton–Dickinson (Franklin Lakes, NJ) were used in the Rb^+ efflux assay. Dulbecco's modified Eagle medium (DMEM) for cell culture and all antibiotics were purchased from Invitrogen (Carlsbad, CA).

Stable expression of KCNQ2 and KCNQ3 channels in Chinese hamster ovary (CHO) cells

Both cDNAs of KCNQ2 (accession no. AF087453) and KCNQ3 (accession no. AF091247) were amplified from a rat brain cDNA library by polymerase chain reactions.

Open reading frames of rKCNQ2 and rKCNQ3 cDNAs were subcloned in frame into mammalian expression vectors of pcDNA3.1/Neo (+) or Hygro (−) vectors (Clontech) for selections. All sequences were confirmed by an in-house automated sequencer (ABI3700, Applied Biosystems). For stable expression of rKCNQ2/3 channels, CHOK1 cells were transfected with both rKCNQ2 and rKCNQ3 constructs and selected based on antibiotic selection markers. Cells were grown in suspension or attached on 96-well plates at 37°C and 5% CO_2. The culture medium consisted of high-glucose DMEM with a supplement of 10% fetal bovine serum (Sigma).

Whole-cell patch-clamp recordings

Stable CHO cell lines that express both rKCNQ2/3 channels were functionally confirmed by electrophysiological recordings. The whole-cell configuration of the patch-clamp technique was used to record K^+ currents from the cells at room temperature using an EPC-9 amplifier (HEKA Instrutech Corp.) with the acquisition program and analysis program Pulse-PulseFit from HEKA (Lambrecht, Germany). Electrodes were fabricated using a P-87 puller (Sutter Instrument). Electrodes had a resistance of 1–5 MΩ when filled with recording solution that consisted of the following (in mM/L): 120 potassium aspartate, 20 KCl, 10 EGTA, 5 HEPES (pH 7.2 with KOH), 5 Mg-ATP, 5 phosphocreatine-Na, 1 $MgCl_2$. The standard bath recording solution consisted of the following (in mM/L): 140 NaCl, 5.4 KCl, 5 HEPES (pH 7.2 with NaOH), 15 dextrose, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 0.4 KH_2PO_4, 0.3 Na_2HPO_4. Series resistance compensation was used for current over 1 nA, and currents were filtered at 3 kHz. Outward KCNQ2/3 currents were recorded from cells held at −80 mV by depolarizing voltage steps for 1 s. The amplitude of current sensitive to either openers (WAY compounds) or blockers (XE991 or linopirdine) was measured at the end of pulses, and EC_{50} or IC_{50} values of compounds were determined. Untransfected blank CHO cells did not show any detectable endogenous M-type currents in the recording conditions.

Atomic absorption Rb^+ efflux assay

Stable CHO cells expressing rKCNQ2/3 channels were grown in DMEM supplemented with 10% fetal calf serum at 37°C with 5% CO_2. Cells were plated at a density of 20,000 cells/well in poly-D-lysine-coated 96-well microplates and incubated overnight at 37°C with 5% CO_2. For Rb^+ loading, cell culture medium was discarded by gently inverting the plate, and then cells were loaded by application of 200 μl of Rb^+ loading buffer per well and incubated for 3 h at 37°C, 5% CO_2. The

Rb⁺ loading buffer contained: 5.4 mM RbCl, 5 mM glucose, 25 mM HEPES, 150 mM NaCl, 1 mM MgCl$_2$, 0.8 mM NaH$_2$PO$_4$, 2 mM CaCl$_2$ (pH adjusted to 7.4 with NaOH). For measurement of Rb⁺ efflux block, blockers were added to the loading buffer in the final 30 min. Following the 3-h incubation, the Rb⁺ loading buffer was removed and cells were washed gently three times with wash buffer before drug/compound application. Wash buffer contained: 5.4 mM KCl, 25 mM HEPES, 150 mM NaCl, 1 mM MgCl$_2$, 0.8 mM NaH$_2$PO$_4$, 2 mM CaCl$_2$ (pH adjusted to 7.4 with NaOH). In experiments screening for KCNQ openers, the depolarization buffer (20 mM K⁺) contained 20 mM KCl, 25 mM HEPES, 130 mM NaCl, 1 mM MgCl$_2$, 0.8 mM NaH$_2$PO$_4$, 2 mM CaCl$_2$ (pH adjusted to 7.4 with NaOH). In experiments screening for KCNQ blockers, the depolarization buffer (50 mM K⁺) differs from opener depolarization buffer with KCl increased to 50 mM and NaCl decreased to 100 mM.

For Rb⁺ efflux measurements, supernatant (200 μl from each well) was collected and transferred to a new 96-well plate after 10 min of compound incubation. Cells were then lysed by adding 200 μl of lysis buffer (Aurora Biomed, Inc., Vancouver, BC, Canada) to each well, and incubated at 37°C for 20 min for complete cell lysis before the measurement. The concentration of Rb⁺ in cell supernatants (Rb⁺ supern) and cell lysates (Rb⁺ lysate) was determined using an automated Ion Channel Reader (ICR) 8000 flame atomic absorption spectrometer (Aurora Biomed). One hundred cell supernatant or lysate samples were processed automatically from 96-well plates and injected into an air-acetylene flame followed by 150 μl of Rb⁺ sample analysis buffer (Aurora Biomed). The amount of Rb⁺ in the sample was measured by absorption at 780 nm using a hollow cathode lamp as a light source and a PMT detector. A calibration curve covering the range of 0–5 mg/L (or ppm) Rb⁺ in sample analysis buffer was generated with each set of two 96-well plates. Reading of a whole 96-well plate takes ~30 min.

FlexStation assay

The FlexStation (Molecular Devices Corp., Sunnyvale, CA) with excitation wavelength set at 535 nm, detection wavelength at 565 nm, and a cutoff at 550 nm was used to measure voltage changes in KCNQ2/3-expressing CHO cells in 96-well plates. The assay was performed according to the procedure by the manufacturer using the membrane potential assay kit (Molecular Devices Corp., catalogue no. R8034). In brief, the cells were plated at a density of 20,000 cells/well into poly-D-lysine-coated 96-well plates (COSTAR 3603) and incubated for 24 h prior to the assay in a 37°C/5% CO$_2$

incubator. On the day of the assay, one vial of dye was dissolved in low K⁺ assay buffer that gives a better ratio of signal to noise [0.5 mM KCl, 160 mM NaCl, 10 mM glucose, 1 mM MgCl$_2$, 2 mM CaCl$_2$, and 10 mM HEPES (pH to 7.4 with NaOH)] and the overnight culture medium was removed from wells by aspiration before 200 μl of membrane potential dye was added to each well. The plates were left at room temperature for 45 min to 2 h before reading on the FlexStation. After a 20-sec baseline determination, the FlexStation added 50 μl of 5X test compound to each well containing 200 μl of dye solution, resulting in a final concentration of 1× test compound.

Statistical analysis

All experimental results are presented as means ± SEM. For analysis of Rb⁺ release, the Rb⁺ efflux (F) at any given time/concentration point was defined by

$$F = [\text{Rb}^+_{\text{supern}}/(\text{Rb}^+_{\text{supern}} + \text{Rb}^+_{\text{lysate}})] \times 100 \quad (1)$$

and the effect (E) of compound was defined by

$$E = [(F_{\text{cpd}} - F_{\text{basal}})/(F_{\text{d}} - F_{\text{basal}})] \times 100 \quad (2)$$

where F_{cpd} is the efflux in the presence of compound in depolarization buffer, F_{basal} is the efflux in basal buffer, and F_{d} is the efflux in depolarization buffer.

For simplified analysis of compound effect on Rb⁺ efflux in supernatant only (F_{supern}), the Rb⁺ efflux was defined by

$$F_{supern} = (\text{Rb}^+_{\text{supern/cpd}}/\text{Rb}^+_{\text{supern/d}}) \times 100 \quad (3)$$

where the Rb⁺$_{\text{supern/cpd}}$ is the supernatant efflux only in the presence of compound and Rb⁺$_{\text{supern/d}}$ is the supernatant efflux in depolarization buffer only in the absence of compound.

For evaluation of the assay quality, the assay performance was assessed by calculating the Z' factor based on the equations as published by Zhang et al.[19]:

$$Z' \text{ factor} = 1 - 3 \times (\text{SD}_2 + \text{SD}_1)/|\text{M}_2 - \text{M}_1| \quad (4)$$

where M_2 and M_1 represent the means of positive control (in the presence of compound) and negative control (in the absence of compound), respectively, and SD_2 and SD_1 represent the standard deviations of the positive and negative controls.

All dose-response curves for compound EC$_{50}$ values obtained by either Rb⁺ efflux assay or EP were fitted by logistic equation using OriginPro 7.0 program (Microcal Software, Inc.).

Results and Discussion

Functional expression of KCNQ2/3 channels in CHO cells

To be certain that the stable CHO cell line used in the Rb$^+$ efflux assay expressed heteromeric KCNQ2/3 K$^+$ channels, we started by electrophysiological confirmation using whole-cell configuration of the patch-clamp technique. It has been shown that coexpression of KCNQ2 and KCNQ3 increased the current amplitude of KCNQ2 ∼10 times.[1] In the CHO cells stably expressing KCNQ2 and KCNQ3, depolarizing voltage steps elicited typical outward whole-cell currents of KCNQ/M-channels that displayed slow activation and no inactivation during a second pulse. The current amplitude at 20 mV is ∼4.0 nA, an eight-fold increase compared with the current amplitude (∼0.5 nA) of KCNQ2 alone (Fig. 1). It has also been reported that coexpression of KCNQ2 and KCNQ3 channels alters the channel pharmacology, compared with the KCNQ2 channel alone with respect to sensitivity to the nonselective K$^+$-channel blocker tetraethylammonium chloride (TEA).[20,21] To confirm the pharmacology of heteromeric KCNQ2/3 channels expressed in the CHO cells, we applied TEA to the recording solution to block the channel. The lower panels of Fig. 1 show the different sensitivities of KCNQ2/3 current and KCNQ2 current to 1 mM TEA block. Heteromeric KCNQ2/3 channels are less sensitive to TEA block with an IC$_{50}$ of 3.8 ± 0.3 mM, com-

pared with an IC$_{50}$ of 0.13 ± 0.02 mM for KCNQ2 alone. Similar currents were not observed in untransfected CHO cells (data not shown). The results are consistent with the predominant expression of heteromeric KCNQ2/3 channels with properties similar to native KCNQ2/3 channels that are thought to underlie the M-current in neurons.[1,21]

Rb$^+$ efflux assay

We started by optimizing the assay conditions. To determine an optimal concentration of extracellular KCl that could best enhance Rb$^+$ efflux from KCNQ2/3 CHO cells seeded at 20,000 cells/well, we used a known opener WAY-1 compound as a tool in this optimization. The WAY-1 compound previously identified from electrophysiological studies is known to activate KCNQ2/3 channels in CHO cells with an EC$_{50}$ of 0.71 μM. KCNQ2/3 CHO cells were loaded with 5.4 mM RbCl for 3 h, and then challenged with different concentrations of extracellular KCl ranging from 10 to 90 mM in the presence of WAY-1 compound from 0.01 to 30 μM. As shown in Fig. 2A, the amount of Rb$^+$ released from the cells was increased with an increasing concentrations of the KCNQ2/3 channel opener WAY-1 compound. Maximal Rb$^+$ efflux was induced by extracellular 20 mM KCl in the presence of WAY-1 compound with an assay window of 200%, compared with that of 175% for 10 mM KCl or 150% or below for 30–90 mM KCl un-
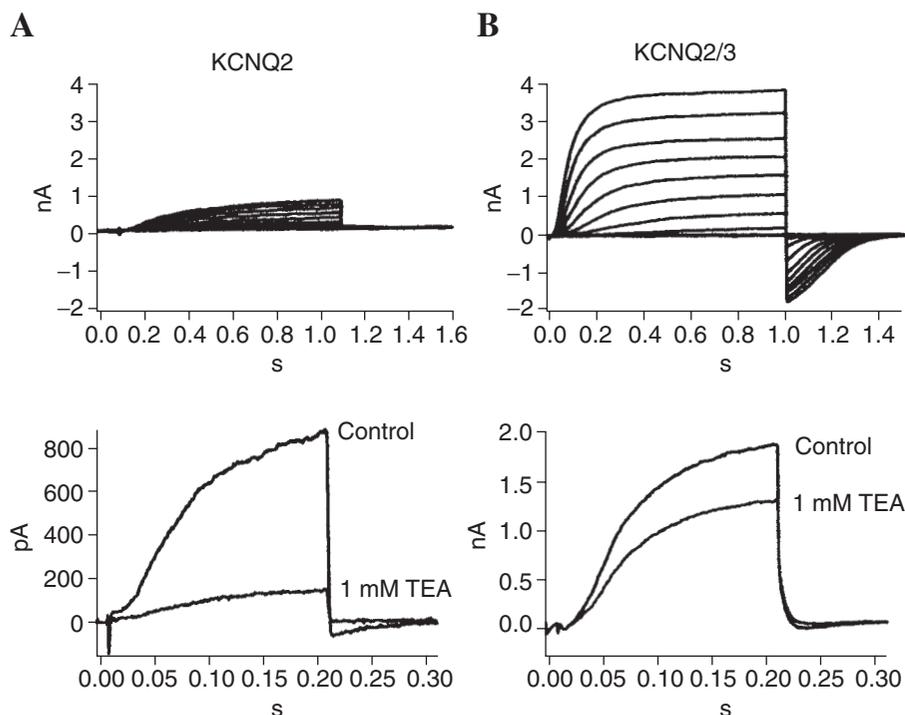
FIG. 1. Functional and pharmacological confirmation of stable KCNQ2/3 CHO cells. (**A**) KCNQ2 current traces in a cell held at −80 mV (top panel) were elicited with 1.0-s depolarizing pulses in 10-mV increments from −80 to +20 mV. The bottom panel shows the block of KCNQ2 current by TEA (1.0 mM). (**B**) KCNQ2/3 current traces were elicited with the same protocol used in A. The bottom panel displays the current block by TEA (1.0 mM).

A

B
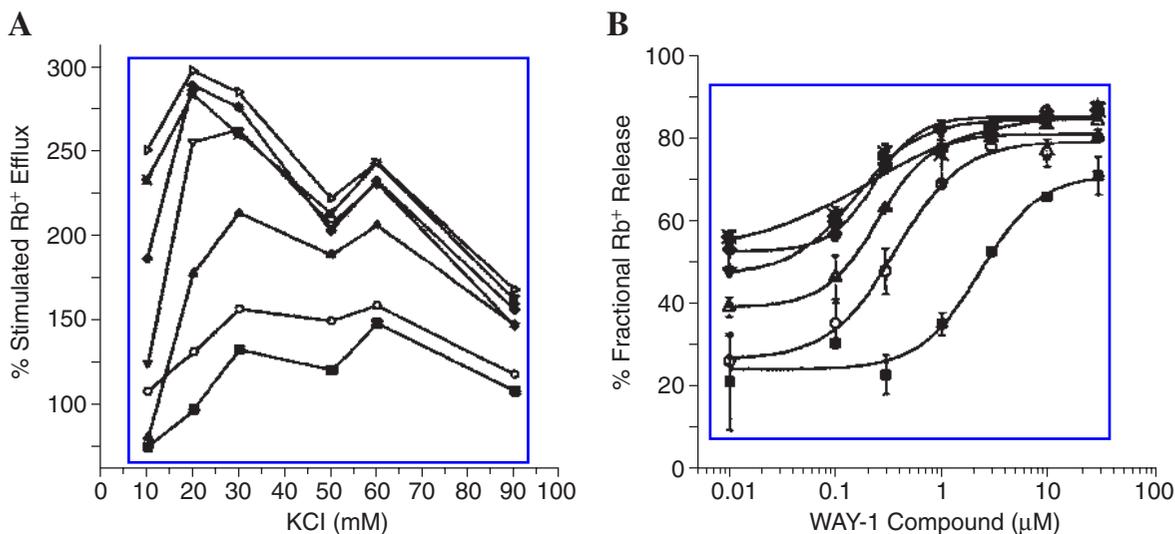
FIG. 2. Effect of extracellular K$^+$ concentration on Rb$^+$ release in the presence of a KCNQ channel opener (WAY-1 compound). (**A**) Rb$^+$ efflux stimulated by extracellular K$^+$ from 10 to 90 mM in the presence of WAY-1 compound from 0.01 to 30 μM (■, 0.01 μM; ○, 0.1 μM; △, 0.3 μM; ▽, 1.0 μM; ⊕, 3.0 μM; ⦻, 10 μM; △, 30 μM). Depolarization with 20 mM KCl produces the maximal Rb$^+$ efflux in the presence of the WAY-1 with the biggest assay window (200%), compared with 175% for 10 mM or lower for 30–90 mM KCl under isosmotic conditions. (**B**) EC$_{50}$ values (μM) of WAY-1 compound determined from A and plotted for different concentrations of KCl are 2.29, 0.37, 0.25, 0.16, 0.24, and 0.22 for 10 mM (■), 20 mM (○), 30 mM (△), 50 mM (▽), 60 mM (⊕), and 90 mM (⦻), KCl, respectively.

der isosmotic conditions (Fig. 2A). In Fig. 2B, the stimulated fractional release of Rb$^+$ was plotted against concentrations of the WAY-1 compound, and its EC$_{50}$ values were determined for different concentrations of extracellular KCl. The 20 mM KCl depolarization so-

lution achieved the best concentration-response relationship for the WAY-1 compound with an EC$_{50}$ of $\sim$0.37 μM, similar to the value obtained by patch-clamp experiments. The result suggested that depolarizing KCNQ2/3 CHO cells with 20 mM KCl resulted in the

A

B
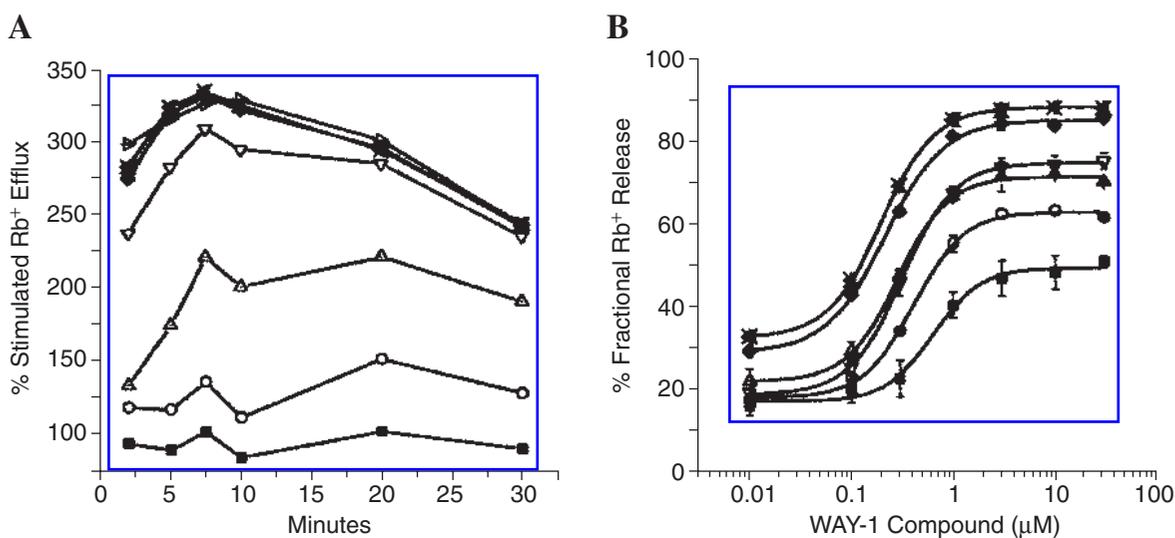
FIG. 3. Time course of Rb$^+$ release following depolarization (by 20 mM KCl) in the presence of WAY-1 compound. (**A**) Time course of Rb$^+$ efflux at different concentrations of WAY-1 compound from 0.01 to 30 μM (■, 0.01 μM; ○, 0.1 μM; △, 0.3 μM; ▽, 1.0 μM; ⊕, 3.0 μM; ⦻, 10 μM and ▷, 30 μM). (**B**) Concentration-response curves for WAY-1 compound at different time points from 2 to 30 min (■, 2 min; ○, 5 min; △, 7.5 min; ▽, 10 min; ⊕, 20 min, and ⦻, 30 min) after KCl depolarization. EC$_{50}$ values for 7.5 and 10 min were 284 nM and 315 nM, respectively, and are consistent with that obtained by EP.
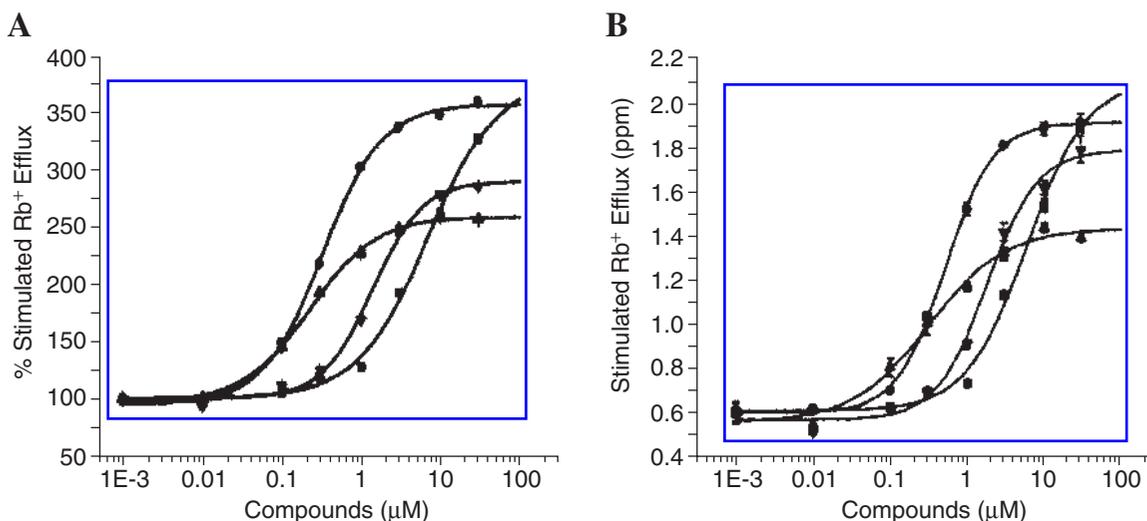
FIG. 4. Dose-dependent effects of KCNQ-channel openers on stimulated Rb^+ efflux. Compounds were measured in quadruplicates. **(A)** EC_{50} values (μM) obtained from measurements of supernatant/lysate were 6.9 for WAY-2 (■), 0.33 for WAY-3 (○), 0.22 for WAY-4 (▲), and 1.4 for WAY-5 (▽). **(B)** Data from A were plotted for supernatant measurement only, and absolute release of Rb^+ was detected in the range of 0.6–2.0 ppm in response to different concentrations of the same set of compounds. EC_{50} values (μM) of WAY-2 (■), WAY-3 (○), WAY-4 (▲), and WAY-5 (▽) were 6.0, 0.52, 0.32, and 1.82, respectively.

best ratio of signal to noise. Using the depolarization solution of 20 mM KCl, we further determined the time course of Rb^+ efflux in response to different concentrations of the KCNQ/M-channel opener WAY-1 compound, and EC_{50} values were determined. Figure 3A shows the time course of Rb^+ efflux from 2 to 30 min in the presence of different concentrations of WAY-1 compound. Depolarization with 20 mM KCl for 7.5 or 10 min produced the largest increase in Rb^+ release in response to the WAY-1 compound from 0.01 to 30 μM. The plot of EC_{50} values further showed that the time points of both 7.5 and 10 min displayed optimal dose-dependent responses with minimal background noise at 0.01 μM WAY-1, and maximal fractional release of Rb^+ at 30 μM WAY-1 compound (Fig. 3B). These results suggested that depolarization with 20 mM KCl for 10 min allowed for the maximal release in the presence of KCNQ2/3 openers combined with the lowest background of K^+-stimulated Rb^+ release, the optimized conditions of which were used for the remainder of the study.

Evaluation of KCNQ/M-channel openers

Development of a sensitive and efficient assay for the direct measurement of M-current activity is a primary goal of this assay validation. The anticonvulsant retigabine, a KCNQ-channel opener, has been reported to activate KCNQ2/3 or KCNQ2 channels by shifting the membrane potential to the more hyperpolarized direction

and increasing the open channel probability.[14,15] It also has been shown that retigabine induces a concentration-dependent increase of Rb^+ efflux through KCNQ2 channels.[18] Using the optimized assay conditions, we investigated the dose-dependent relationship of Rb^+ efflux by determining EC_{50} values for a number of WAY compounds that are known to activate KCNQ2/3 channels from our electrophysiological recordings. Figure 4A
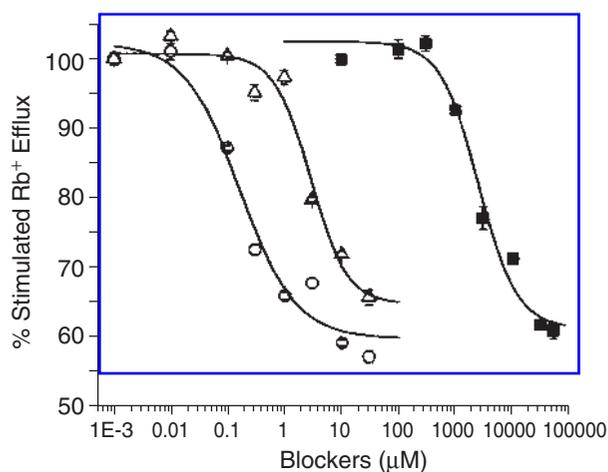
FIG. 5. Rb^+ efflux through KCNQ2/3 channels is blocked by K^+-channel blockers. TEA (■), linopirdine (△), and XE991 (○) were added to KCNQ2/3 CHO cells during the last 30 min of Rb^+ loading (3 h). IC_{50} values for TEA, linopirdine, and XE991 were 2.52 mM, 2.85 μM, and 0.16 μM, respectively.
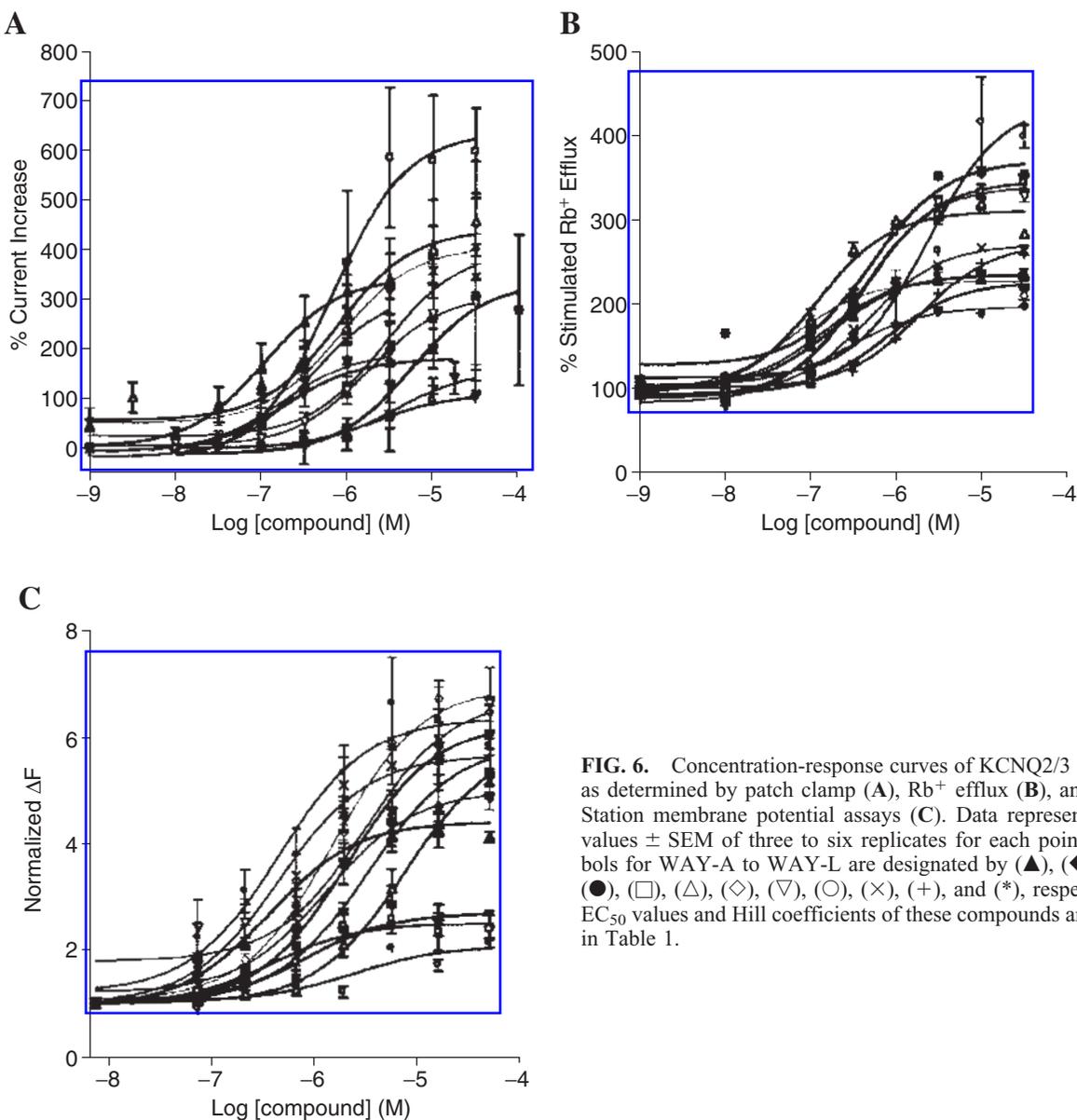
FIG. 6. Concentration-response curves of KCNQ2/3 openers as determined by patch clamp (**A**), Rb$^+$ efflux (**B**), and Flex-Station membrane potential assays (**C**). Data represent mean values $\pm$ SEM of three to six replicates for each point. Symbols for WAY-A to WAY-L are designated by ($\blacktriangle$), ($\blacklozenge$), ($\blacktriangledown$), ($\bullet$), ($\square$), ($\triangle$), ($\diamondsuit$), ($\triangledown$), ($\bigcirc$), ($\times$), ($+$), and ($*$), respectively. EC$_{50}$ values and Hill coefficients of these compounds are listed in Table 1.

shows a typical Rb$^+$ efflux profile of four KCNQ channel openers (WAY compounds) with EC$_{50}$ values ranging from 0.3 to 7 μM. WAY-3 and WAY-4 compounds showed the same potency with similar EC$_{50}$ values of 0.3 μM, but different maximal release of Rb$^+$ (250% versus 350%). WAY-2 compound showed a low potency with an EC$_{50}$ of 6.9 μM and no saturating effect at 30 μM. These results indicate that the assay is capable of identifying M-channel openers with various potencies. In Fig. 4B, data from Fig. 4A were plotted for the supernatant measurement only, and absolute release of Rb$^+$ was detected in the range of 0.6–2.0 ppm in response to different concentrations of the compounds. EC$_{50}$ values from supernatant measurements were found to be identical

to those obtained by measurement of both supernatant and lysate, indicating that the simplified measurement of supernatant only is sufficient to obtain consistent EC$_{50}$ values.

Evaluation of the channel blockers

To characterize further the Rb$^+$ efflux through KCNQ2/3 channels expressed in stable CHO cells, we evaluated the channel pharmacology with three well-known blockers and examined their effects on the M-channel activity. Both XE991 and linopirdine are structurally related analogues that have been shown to potently block native M-current and heteromeric

TABLE 1.   COMPARISON OF EC_{50} VALUES AND HILL COEFFICIENTS OF KCNQ2/3 OPENERS
OBTAINED IN EP, RB^+, EFFLUX, AND FLEXSTATION MEMBRANE POTENTIAL ASSAYS

	EP		Rb^+ efflux		FlexStation	
Compounds	EC_{50}	*HC*	EC_{50}	*HC*	EC_{50}	*HC*
WAY-A	0.12	0.94	0.2	1.1	1.1	1.4
WAY-B	0.2	1.7	0.27	1.5	1.48	1.1
WAY-C	0.3	1.0	0.14	0.94	0.52	0.9
WAY-D	0.46	1.0	0.65	0.88	2.06	0.88
WAY-E	0.61	1.5	0.14	1.5	7.4	1.3
WAY-F	0.66	1.0	0.48	1.6	0.38	0.92
WAY-G	0.68	0.6	0.062	1.1	1.5	1.1
WAY-H	1.98	0.97	0.35	1.6	4.5	1.0
WAY-I	2.1	0.88	2.1	0.74	2.3	0.51
WAY-J	2.4	1.3	0.42	1.2	0.47	1.1
WAY-K	2.9	1.2	1.6	1.2	3.3	1.3
WAY-L	4.7	1.0	0.66	1.3	1.5	1.0

Note: Four inactive compounds determined by EP were tested in Rb^+ efflux and FlexStation and
found to have no effect in both assays. HC, Hill coefficient.

KCNQ2/3 channels in electrophysiological record-ings.[1,21] Figure 5 shows that XE991 and linopirdine blocked the Rb^+ efflux through KCNQ2/3 channels in a dose-dependent manner with IC_{50} values of 0.16 and 2.85 μM, respectively, which are also in agreement with published results obtained in electrophysiological recordings. In contrast, TEA exhibited weak potency against KCNQ2/3 channels with an IC_{50} value of 2.52 mM. These results are also in agreement with published results obtained from recordings indicating that the known pharmacological profile of the M-channel defined by electrophysiological studies is faithfully repro-duced in the Rb^+ efflux assay. The result also indicates that the assay can be used for screening of channel blockers. It is noticeable that the stimulated Rb^+ efflux can only be blocked ~40% with presumably saturating concentrations of blockers. The reason for the incomplete block is not quite known, and is probably due to a non-specific release of Rb^+ through activation of other endogenous K^+ channels by the 50 mM KCl depolarization that causes a higher basal release as shown in Fig. 2B. We also tried to use lower concentrations of K^+, and we were unable to improve the dose-dependent inhibition of Rb^+ efflux by those blockers.
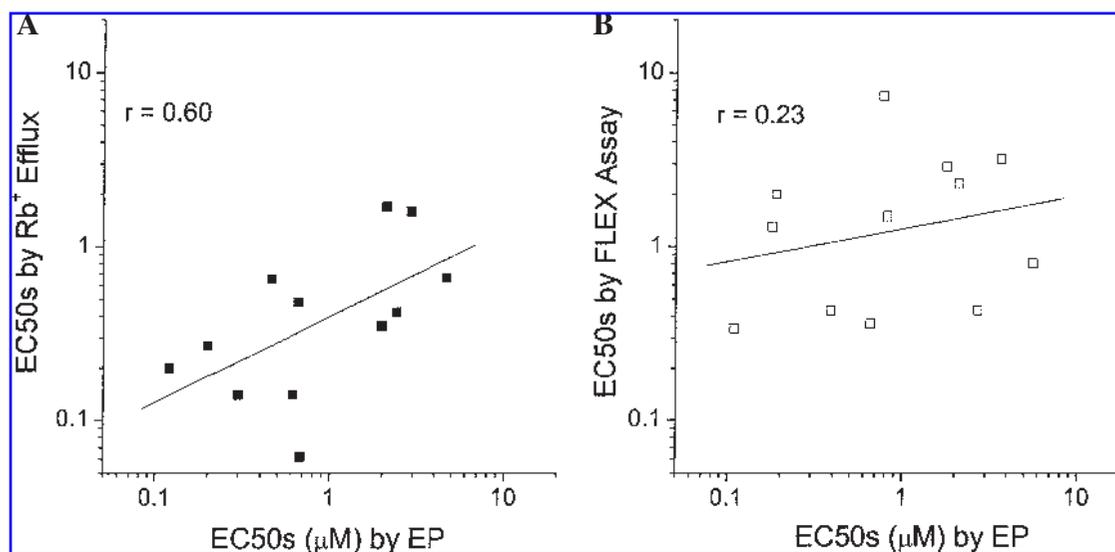
FIG. 7. EC_{50} correlation between Rb^+ efflux assay and EP. (**A**) Correlation of EC_{50} values of WAY compounds determined by whole-cell recordings and Rb^+ efflux assay. The EC_{50} values from both assays agreed each other with a correlation coefficient of 0.6. (**B**) The same set of compounds was also evaluated using membrane potential dye-based FlexStation assay. The comparison of values from EP and FlexStation assays showed no correlation ($r = 0.23$).
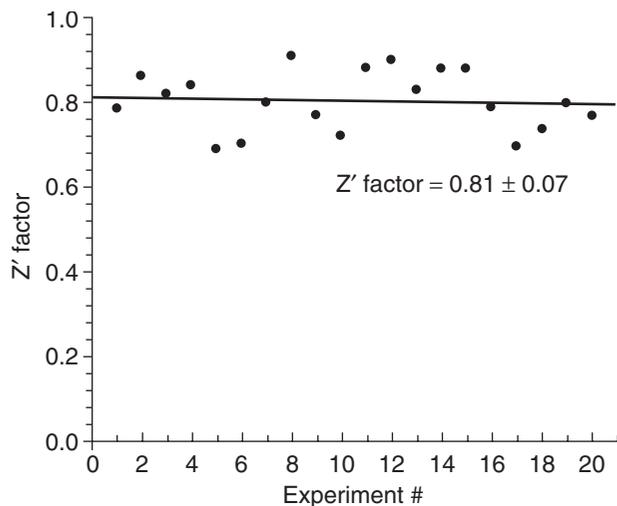
FIG. 8. Performance of atomic absorption Rb$^+$ efflux assay. Z' factor for each experiment was determined from positive (in the presence of 1 μM KCNQ-channel opener compound) and negative (in the absence of compound) controls. The values of Z' ranged from 0.7 to 0.91 with an average of 0.81 $\pm$ 0.07.

Correlation between EP and Rb$^+$ efflux assays

The Rb$^+$ efflux assay is a functional assay that specifically detects K$^+$ ion flowing through K$^+$ channels. Patch-clamp recordings as a gold standard reveal a rich content of information about the activity of channel function. To validate further the Rb$^+$ efflux assay, we compared the effects of 16 compounds on Rb$^+$ efflux with electrophysiological recordings of KCNQ2/3 current. Four compounds that are inactive in EP experiments have no effect on Rb$^+$ release using the Rb$^+$ efflux assay. EC$_{50}$ values for the remaining 12 compounds determined by electrophysiological recordings were then compared with those obtained from the Rb$^+$ efflux assay and FlexStation (Fig. 6 and Table 1). Figure 7A plots the EC$_{50}$ values determined by both EP and Rb$^+$ efflux assay and shows a positive correlation coefficient ($r = 0.60$) between the two assays. By contrast, there is no correlation between EP and the FlexStation assay ($r = 0.23$) (Fig. 7B). As discussed earlier, the Flexstation assay using a voltage-sensitive dye represents an indirect index of KCNQ2/3-channel activity, whereas the Rb$^+$ efflux assay provides a direct measurement of K$^+$ flow through KCNQ2/3 channels. In addition, these openers may have activity at other ion channels detected in the membrane potential dye FlexStation assay, but not the Rb$^+$ assay. Our results indicate that, in the evaluation of KCNQ2/3 openers, it is necessary to use an assay that provides a more direct measurement of channel activity to best correlate with definitive data obtained from patch-clamp experiments.

Assay performance

To evaluate the robustness and reproducibility of the atomic absorption Rb$^+$ efflux assay, we assessed the assay performance by calculating the Z' factor for 20 separate experiments. The Z' factor, as a screening window coefficient and tool to evaluate assay quality, compares the assay dynamic range to data variation.[19] An average Z' value of 0.81 was obtained from a total of 20 experiments, consisting of four data points of each experiment for positive control (1.0 μM WAY compounds) and an equal number of data points for negative control (no compound). A Z' factor equal to 1.0 is an ideal and perfect assay. In general, Z' factors greater than or equal to 0.5 indicate a robust assay that is suitable for HTS and automation. Z' values ranged from 0.70 to 0.91 in 20 experiments conducted on different days (Fig. 8). These results show that the atomic absorption Rb$^+$ efflux assay is adequately optimized with high quality and suitable for screening of KCNQ2/3 modulators.

Conclusions

In this study, we developed and validated a nonradioactive Rb$^+$ efflux assay to measure directly the activity of functional channels by atomic absorption using a stable CHO cell line expressing KCNQ2/3 channels confirmed by EP. The Rb$^+$ efflux assay performed in a 96-well plate format using the automated ICR8000 was used to screen compounds that modulate the M-channel activity, with average Z' factor of 0.81. EC$_{50}$ values from in-house compounds characterized as KCNQ openers by a Rb$^+$ efflux assay were compared with those obtained with EP. A positive correlation coefficient was achieved ($r = 0.60$) between the two assays. To simplify the assay and increase the throughput, we also compared EC$_{50}$ values obtained from measurements of only supernatant versus supernatant/cell lysate. The results show that measurement of only supernatant is sufficient to obtain consistent EC$_{50}$ values compared with that of supernatant/lysate. The throughput of the ICR8000 by supernatant measurement only in an eight–point titration is estimated to be 40 compounds per day, which is suitable for a secondary confirmation assay.

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