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A medium-throughput functional assay of KCNQ2 potassium channels using rubidium efflux and atomic absorption spectrometry

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Abstract

Heterologous expression of KCNQ2 (Kv7.2) results in the formation of a slowly activating, noninactivating, voltage-gated potassium channel. Using a cell line that stably expresses KCNQ2, we developed a rubidium flux assay to measure the functional activity and pharmacological modulation of this ion channel. Rubidium flux was performed in a 96-well microtiter plate format; rubidium was quantified using an automated atomic absorption spectrometer to enable screening of 1000 data points/day. Cells accumulated rubidium at 37 °C in a monoexponential manner with $t_{1/2} = 40$ min. Treating cells with elevated extracellular potassium caused membrane depolarization and stimulation of rubidium efflux through KCNQ2. The rate of rubidium efflux increased with increasing extracellular potassium: the $t_{1/2}$ at 50 mM potassium was 5.1 min. Potassium-stimulated efflux was potentiated by the anticonvulsant drug retigabine ($EC_{50} = 0.5\,\mu M$). Both potassium-induced and retigabine-facilitated efflux were blocked by TEA (IC_{50}s = 0.4 and 0.3 mM, respectively) and the neurotransmitter release enhancers and putative cognition enhancers linopirdine (IC_{50}s = 2.3 and 7.1 μM, respectively) and XE991 (IC_{50}s = 0.3 and 0.9 μM, respectively). Screening a collection of ion channel modulators revealed additional inhibitors including clofilium (IC_{50} = 27 μM). These studies extend the pharmacological profile of KCNQ2 and demonstrate the feasibility of using this assay system to rapidly screen for compounds that modulate the function of KCNQ2.

Keywords: KCNQ2; Kv7.2; K^+ channel; Rubidium flux; Atomic absorption spectrometry

Potassium channels are structurally and functionally diverse, ubiquitously distributed among cell types, and intimately involved in the regulation of membrane potential. In addition, potassium channel defects give rise to several clinically relevant syndromes. These properties render potassium channels important potential therapeutic targets in numerous areas including cardiovascular, central nervous, endocrine, and immune systems [1–3].

Lack of fast, efficient, and sensitive screening methods is a major impediment to the development of novel potassium channel ligands and subsequent exploitation of their therapeutic potential. Electrophysiological techniques have been the methods of choice, but their throughput is still very limited. Voltage-sensitive fluorescent probes in conjunction with appropriate plate readers have relatively high throughput, have been shown to be effective in some systems but not others, carry a high cost, and are subject to interference by fluorescent compounds frequently encountered in chemical libraries [4–6].

In many biological processes the Rb^+ ion can substitute for K^+, and Rb^+ fluxes have proven to be reliable indicators of K^+ channel activity. The use of ^{86}Rb for this application is well established [7,8] and the technique has been updated to a relatively high-throughput format [9]. However, the safety and disposal issues surrounding the use of ^{86}Rb in intensive screening

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environments are unacceptable. Atomic absorption spectrometry has been shown to be sufficiently sensitive and reliable for monitoring Rb^+ fluxes from a number of cell types, but the throughput has been rather low [5,10]. The recent introduction of fully automated atomic absorption spectrometers that can be integrated into existing laboratory robotics systems promises to radically improve the throughput capability of this method.

Homomultimeric expression of KCNQ2 ($K_V7.2$) results in the formation of a slowly activating, noninactivating, TEA[1]-sensitive, voltage-gated potassium channel [11–15]. KCNQ2 can also form heteromeric channels with KCNQ3 [12,13,16]. Mutations in the genes encoding KCNQ2 and KCNQ3 cause a form of epilepsy in newborns termed benign familial neonatal convulsions [17–19], indicating an important role for these channel proteins in the control of neuronal firing properties. KCNQ2 is expressed in human hippocampus where, either as a homomeric channel or as possibly a heteromeric complex with KCNQ3 [20], it may be responsible for the M current, a noninactivating K^+ current that is inhibited by M_1 muscarinic acetylcholine receptor [21] and which plays a key role in the regulation of neuronal excitability [22]. To test the hypothesis that the new generation of atomic absorption spectrometers can play a useful role in K^+ channel drug discovery we have developed a medium-throughput assay to quantify KCNQ2 channel activity.

Materials and methods

Materials

RbCl, linopirdine, TEA, and fibronectin-coated microtiter plates were purchased from Sigma. Cell culture media was purchased from Mediatech, Inc. All other materials were of the highest quality commercially available.

Stable expression of KCNQ2 in HEK293 cells

HEK293 cells were transfected with KCNQ2L cDNA in pcDNA3 vector as previously described [15]. A stable clone (H17) was derived and maintained in culture media containing Dulbecco's modified Eagle's medium, 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 250 μg/ml G418.

Rubidium efflux assay

H17 cells were seeded in 96-well fibronectin-coated microtiter plates at a density of 20,000 cells/well and placed in an incubator at 37 °C with 5% CO_2. All incubation steps occurred under these conditions. After 48 h the cells were washed three times in wash buffer (25 mM Hepes, 150 mM NaCl, 1 mM $MgCl_2$, 0.8 mM NaH_2PO4, 2 mM $CaCl_2$, pH adjusted to 7.4 with NaOH) and then incubated for 2.5 h in 200 μl/well of Rb^+ loading buffer (wash buffer plus 5.4 mM $RbCl_2$, 5 mM glucose). Half of the loading buffer sample was then removed from each well and replaced with fresh loading buffer containing either drug or DMSO. After an additional 30-min incubation, the plates were washed three times in wash buffer to remove extracellular Rb^+. The wells then received compound or DMSO (0.5% final concentration) in depolarization buffer (25 mM Hepes, 100 mM NaCl, 50 mM KCl, 1 mM $MgCl_2$, 0.8 mM NaH_2PO_4, 2 mM $CaCl_2$, pH adjusted to 7.4 with NaOH) or basal buffer (wash buffer plus 5.4 mM KCl). The cells were incubated for 10 min at room temperature and then the supernatant was removed and transferred to another 96-well plate. The cells were lysed by the addition of 200 μl of 0.1% Triton X-100 in depolarization buffer.

The concentration of Rb^+ in the supernatants (Rb_{supern}) and cell lysates (Rb_{lysate}) was quantified using an ICR8000 flame atomic absorption spectrometer (Aurora Biomed Inc., Vancouver, B.C.) under conditions defined by the manufacturer. One hundred-microliter samples were processed automatically from microtiter plates by dilution with an equal volume of Rb sample analysis buffer (Aurora Biomed Inc.) and injection into an air–acetylene flame. The amount of Rb^+ in the sample was measured by absorption at 780 nm using a hollow cathode lamp as light source and a PMT detector. This automated system was capable of reading two 96-well plates in 90 min. A calibration curve covering the range 0–2 mg/L Rb^+ in sample analysis buffer was generated with each set of samples.

Data analysis

Nonlinear least squares regression analysis was used to fit the time course of Rb^+ uptake to one- and two-phase exponential association models according to Eqs. (1) and (2), respectively,

$$Y = Y_{max} * (1 - \exp(-k_{up} * t)), \quad (1)$$

$$Y = Y_{max}1 * (1 - \exp(-k_{up}1 * t)) + Y_{max}2 * (1 - \exp(-k_{up}2 * t)), \quad (2)$$

where Y represents the Rb^+ uptake at time t, Y_{max}, $Y_{max}1$, and $Y_{max}2$ represent the Rb^+ uptake by the respective components at equilibrium, and k_{up}, $k_{up}1$, and $k_{up}2$ are the corresponding rate constants for uptake.

Rb^+ efflux (F) at any given point was defined by

$$F = [Rb_{supern}/(Rb_{supern} + Rb_{lysate})] \times 100 \quad (3)$$

[1] *Abbreviations used:* TEA, tetraethylammonium chloride; DMSO, dimethyl sulfoxide.

and the effect (E) of compound was defined by

$$E = [(F_c - F_{basal})/(F_s - F_{basal})] \times 100, \quad (4)$$

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

Concentration–response curves were fitted to the equation

$$E = E_{min} + (E_{max} - E_{min}) /(1 + 10^{\wedge}[(-p\mathrm{EC}_{50} - \log C) * n]) \quad (5)$$

or, in terms of the EC_{50},

$$\mathrm{EC}_{50} = 10^{\wedge}((\log[((E_{min} + (E_{max} - E_{min}))/E) - 1]/n) + \log C),$$

where E_{max} and E_{min} are the maximum and minimal effects of the compound, respectively. $p\mathrm{EC}_{50}$ is the negative log of the molar concentration of compound that produces a half- maximal effect, $\log C$ is the log of the molar concentration of compound, and n is the Hill coefficient.

For kinetic analysis of efflux, Rb^+ release from the cells was expressed as the percentage remaining (R) using the following equation:

$$R = [\mathrm{Rb}_{lysate}/(\mathrm{Rb}_{supern} + \mathrm{Rb}_{lysate})] \times 100. \quad (6)$$

Depolarization and agonist-stimulated Rb^+ efflux (R_s) at each time point were determined according to

$$R_s = (1 - [(R_{tot} - R_{basal})/ - R_{basal}]) \times 100, \quad (7)$$

where R_{tot} is the percentage remaining in the presence of stimulus and R_{basal} is the percentage remaining in untreated cells. The data obtained were fitted to one- and two-phase exponential decay models according to Eqs. (4) and (5), respectively,

$$R_s = R_{max} * \exp(-k_e * t), \quad (8)$$

$$R_s = R_{max}1 * \exp(-k_e 1 * t) + R_{max}2 * \exp(-k_e 2 * t), \quad (9)$$

where R_{max}, $R_{max}1$, and $R_{max}2$ are the maximum stimulus-induced effluxes for the respective processes and k_e, $k_e 1$, and $k_e 2$ are the rate constants of efflux. The applicability of one- or two-phase models was assessed using the F test [23]. Half-lives were determined from the appropriate rate constants according to

$$t_{1/2} = 0.693/k. \quad (10)$$

The Z' factor, which measures the overall assay quality, was determined using the equation

$$Z' = 1 - (3\sigma_{c^+} + 3\sigma_{c^-})/(|\mu_{c^+} - \mu_{c^-}|), \quad (11)$$

where μ_{c^+} and μ_{c^-} represent the mean efflux values under stimulated and basal conditions, respectively, and σ_{c^+} and σ_{c^-} represent the SD of the stimulated and basal efflux values [24].

All nonlinear regression analyses were performed using GraphPad Prism (GraphPad Prism Software, San Diego, CA). Data expressed as mean ± SE represent results from at least three experiments, each performed with triplicate samples.

Results and discussion

An HEK293 clone stably expressing KCNQ2 (clone H17) was used in the Rb efflux studies. The biophysical characteristics of KCNQ2 expressed in this cell line are the same as those previously published using transiently transfected HEK293 cells [15]. KCNQ2 causes membrane repolarization by gating the efflux of K^+ ions. Thus, for Rb^+ flux studies, one measures the release of Rb^+ from cells preloaded with this surrogate ion and can express the amount released as a percentage of the total Rb^+ loaded in the cell sample. Experiments to determine whether any of the buffers or cell lysate contributed significantly to background absorbance or quenched the Rb^+ signal were performed. The absorbance readings from depolarization buffer, basal buffer, lysis buffer, and a cell lysate sample were not statistically different from that obtained with sample analysis buffer ($p \geqslant 0.19$). When spiked with 50 ng of Rb^+, each of these samples had absorbance values indistinguishable from the 0.5 mg/L standard ($p \geqslant 0.22$). None of the buffers or cell lysate caused significant quenching or background absorbance, therefore corrections to the absorbance readings of the spent media and cell lysates were not needed.

H17 cells were loaded with Rb^+ by incubating in a cell culture media in which KCl is replaced with 5.4 mM RbCl. As shown in Fig. 1, a 3-h incubation period is sufficient to reach maximal loading. Results from three experiments were each best fit by the single phase association equation (Eq. (1)), with a resulting mean

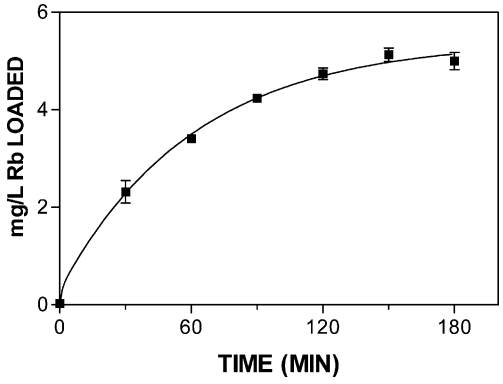

Fig. 1. Time course of Rb^+ loading in KCNQ2-expressing HEK293 cells. Clone H17 cells were incubated with Rb loading buffer for various times. The cells were then rapidly washed three times to remove extracellular Rb^+ and then lysed with 0.1% Triton X-100. The amount of Rb^+ in the cell samples was quantified by atomic absorption spectrometry as described under Materials and methods.

maximal loading of 4 ± 0.7 mg/L and mean $t_{1/2}$ of 40 ± 4 min.

KCNQ2, like other voltage-dependent K^+ channels, is activated in response to membrane depolarization. For Rb^+ efflux studies, KCNQ2 activation is achieved by treating cells with high extracellular potassium. As shown in Fig. 2A, H17 cells incubated under basal conditions (5 mM KCl) had minimal efflux of Rb over a 30-min time period. Increasing the extracellular KCl concentration to 20 mM resulted in a time-dependent increase in Rb^+ efflux. Higher concentrations of KCl further enhanced the rate of Rb^+ efflux. These data were best fit to the single exponential decay equation (Eq. (8)) with $t_{1/2}$ values of 36.2, 15.5, and 7.0 min for 20, 30, and 50 mM KCl treatments, respectively (Fig. 2B). Exposing untransfected HEK293 cells to 50 mM KCl resulted in no increase in Rb^+ efflux compared to basal conditions,

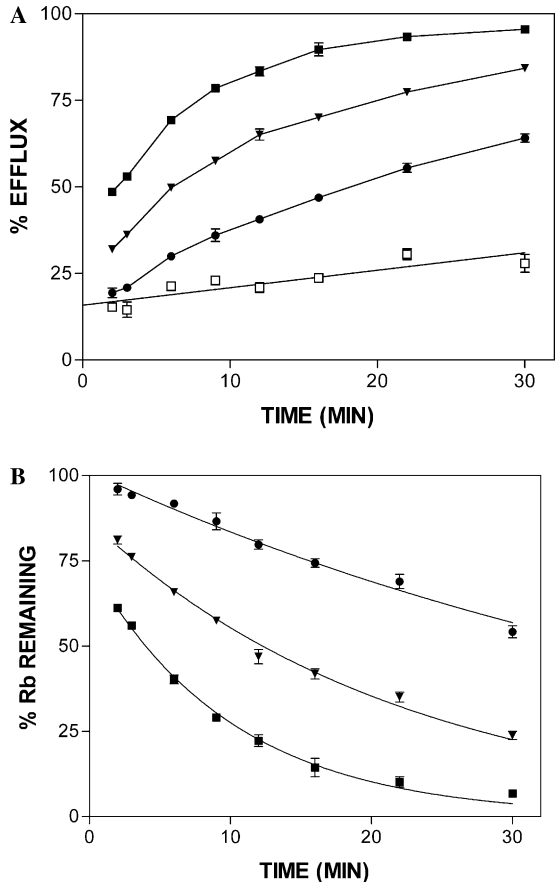

Fig. 2. Elevating extracellular KCl induces Rb^+ efflux from KCNQ2-expressing cells. H17 cells were loaded with Rb^+ for 3 h, rapidly washed three times to remove extracellular Rb^+, and then incubated with 5.4 mM (□), 20 mM (●), 30 mM (▼), or 50 mM (■) KCl. At various times the supernatant was removed from individual wells and the cell samples were lysed with Triton X-100. The supernatant and cell lysate samples were processed and efflux values were determined as described under Materials and methods. (A) Data are expressed as percentage of Rb^+ released from the cells at each time point. (B) Data represent the percentage of Rb^+ remaining in the cells and were calculated using Eq. (7). Data are fitted to a single phase exponential decay equation.

suggesting that the depolarization-induced Rb^+ efflux in H17 cells was due to the expression of KCNQ2. For subsequent experiments, standard assay conditions included exposing H17 cells to 50 mM KCl for 10 min.

The Z' factor has been widely adopted as a measure of assay performance and suitability for screening compounds [24]. A Z' factor between 0.5 and 1.0 indicates that the assay has large separation between the stimulated and the basal values, with small variation in these values. Using a 10-min exposure to 50 mM KCl as the stimulation condition, the Z' factor for this assay is 0.73, indicating that it is capable of providing robust, high-precision data suitable for SAR studies. A Z' factor of 0.60 was obtained when Rb^+ efflux was measured in a 384-well format, demonstrating good sensitivity and precision in this higher-density format that utilizes smaller sample volumes.

Treating H17 cells with 50 mM KCl for 10 min resulted in a 400% increase in Rb^+ efflux. This is significantly higher than the 30% increase in Rb^+ efflux reported using cells expressing Kv1.1 or Kv1.4 channels [10]. Key parameters influencing the magnitude of Rb^+ flux in response to depolarization are channel expression levels, unitary K^+ conductances (Kv1.1 = 10 pS, Kv1.4 = 5 pS, KCNQ2 = 5.8 pS [3]), degree of inactivation (Kv1.1 and Kv1.4 are inactivating channels, whereas KCNQ2 is not [3]), and relative permeability for Rb^+ (currently unknown).

The pharmacology of KCl-induced Rb^+ efflux was evaluated using TEA, linopirdine, and XE991, compounds previously shown by electrophysiological analyses to modulate KCNQ2 activity. TEA is a nonselective K^+ channel blocker with weak potency for KCNQ2. IC_{50} values ranging from 100 to 300 μM have been reported for KCNQ2 expressed in HEK293, CHO, and tsA-201 cells [14,15,21,25]. Linopirdine blocks the neuronal M current and heterologously expressed homomeric and heteromeric KCNQ channels which are believed to be the molecular determinant of this K^+ current. XE991 is a structurally related analog of linopirdine and a more potent inhibitor of KCNQ2 channels (0.71 versus 4.8 μM, respectively, against KCNQ2 expressed in *Xenopus* oocytes) [16]. As shown in Fig. 3, TEA, linopirdine, and XE991 each reduced Rb^+ efflux in a concentration-dependent fashion, with IC_{50} values of 400, 2.3, and 0.3 μM, respectively. In addition, K^+ channel blockers reported to be ineffective against KCNQ2 by electrophysiology including 4-aminopyridine, E-4031, MCD, DTXI, and charybdotoxin [11,13,14] were also inactive in the Rb^+ efflux assay. Thus, the known pharmacological profile of KCNQ2 defined by electrophysiological studies is faithfully reproduced in the Rb^+ efflux assay.

Retigabine is an anticonvulsant compound, capable of increasing potassium conductance in numerous cell types of neuronal origin [26,27]. It has been shown to

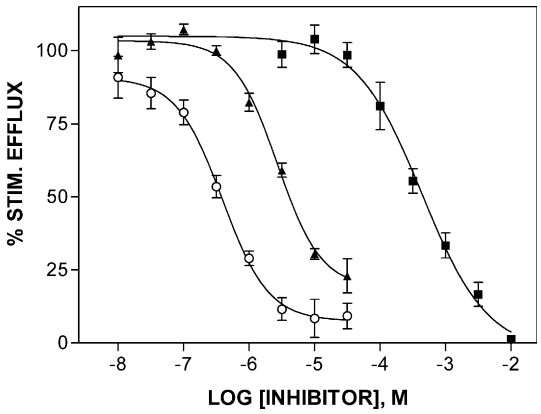

Fig. 3. K^+ channel blockers inhibit KCl-induced Rb^+ efflux. H17 cells were incubated with various concentrations of TEA (■), linopirdine (▲), or XE991 (○) for the last 30 min of the 3 h Rb^+ loading time. The cells were washed three times and then incubated for 10 min with the same concentrations of inhibitor prepared in depolarization buffer. The amount of Rb^+ released from the cells was quantified and expressed as a percentage of Rb^+ efflux observed in the absence of inhibitor. The pIC_{50} values for TEA, linopirdine, and XE991 were 3.42 ± 0.05, 5.5 ± 0.06, and 6.46 ± 0.06, respectively.

enhance currents through heterologously expressed monomeric and heteromeric channels of the KCNQ family including KCNQ2 and KCNQ2/3 [28–31]. Based on its reported mechanism of action, one would expect retigabine to increase Rb^+ efflux in H17 cells in response to membrane depolarization. As shown in Fig. 4, cells treated with 20 mM KCl plus retigabine showed a faster rate of Rb^+ efflux than that achieved with 20 mM KCl alone. Half-lives were 36.2, 16.7, 8.1, 6.6, and 6.8 min for control, 0.3, 1, 3, and 10 μM retigabine, respectively.

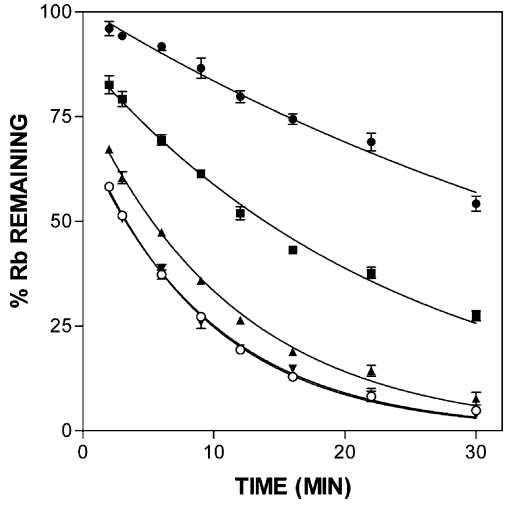

Fig. 4. Retigabine enhances Rb^+ efflux from KCNQ2-expressing cells. H17 cells were treated with depolarization buffer containing 20 mM KCl plus 0 (●), 0.3 μM (■), 1 μM (▲), 3 μM (○), or 10μM (▼) retigabine. Samples were processed, and data were obtained and fitted as described under Materials and methods. Data represent the percentage of Rb^+ remaining in the cells and are fitted to a single phase exponential decay equation.

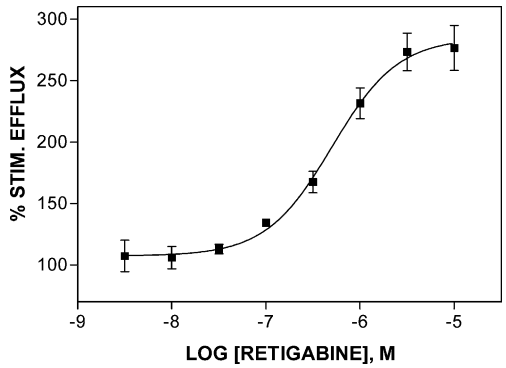

Fig. 5. Concentration-dependent induction of Rb^+ efflux by retigabine. H17 cells were treated for 10 min with 20 mM KCl plus concentrations of retigabine ranging from 3 nM to 10 μM. Data are expressed as the percentage of Rb^+ efflux seen with 20 mM KCl alone. The pEC_{50} value was 6.3 ± 0.05.

The concentration dependence of retigabine's effect was investigated further by monitoring the increase in Rb^+ efflux observed at 10 min (Fig. 5). Under these treatment conditions, the EC_{50} for retigabine was 0.5 μM.

Treating H17 cells with retigabine caused a leftward shift in the KCl activation curve (Fig. 6), i.e., retigabine increased the sensitivity of this channel to depolarization-induced activation. These results are consistent with voltage clamp studies that demonstrated a leftward shift in the voltage dependence for KCNQ2 activation induced by retigabine [28].

To verify the selectivity of retigabine's effect, we tested the ability of TEA, linopirdine, and XE991 to block Rb^+ efflux in cells treated with 1 μM retigabine plus 20 mM KCl. All three compounds completely blocked Rb efflux and did so with IC_{50} values of 300, 7.1, and 0.9 μM, respectively (data not shown). These values are the same rank order and of comparable potencies as those observed when blocking KCl-induced activation

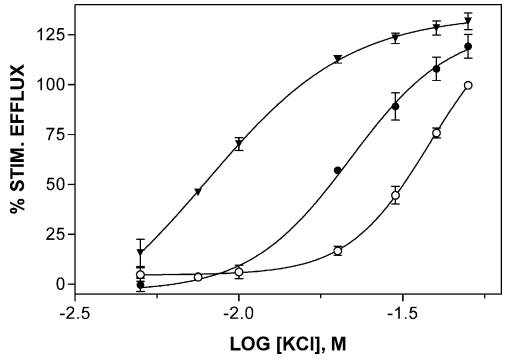

Fig. 6. Retigabine causes a leftward shift in the KCl concentration response curve. H17 cells were treated with concentrations of KCl ranging from 5.4 to 50 mM in the absence (○) or presence of 1 μM (●) or 10 μM (▼) retigabine. After 10 min the supernatants were isolated, cells were lysed with Triton X-100, and Rb^+ content was quantified as described under Materials and methods. The data are expressed as the percentage of Rb^+ efflux observed with 50 mM KCl.

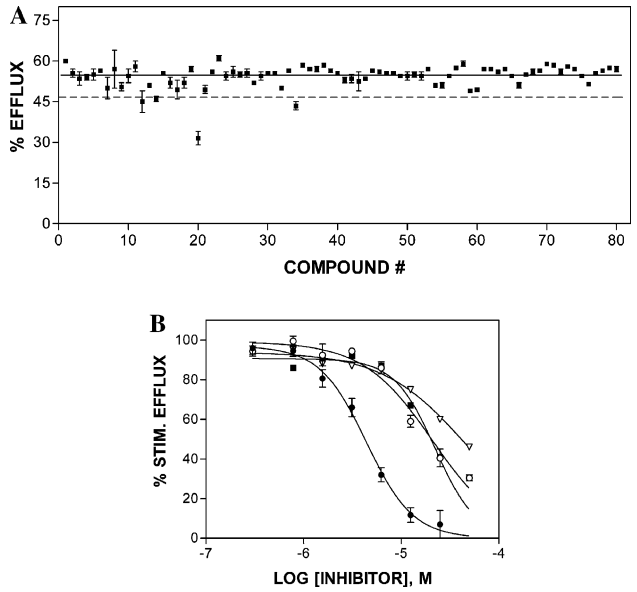

Fig. 7. Testing a collection of ion channel modulators for activity against KCNQ2. (A) Compounds were assayed in duplicate at 10 μM, and the results are expressed as mean ± range. The solid line represents the average percentage efflux of all samples and the dashed line represents 20% inhibition of stimulated efflux. The basal efflux in this experiment was 15%. (B) Concentration–response curves for compound A (●), compound B (■), compound C (▽), and clofilium (○), compounds that demonstrated >20% inhibition in (A). The pIC_{50} values for these compounds were 5.35 ± 0.05, 4.65 ± 0.12, 4.23 ± 0.05, and 4.59 ± 0.06, respectively.

of KCNQ2 (Fig. 3). Retigabine did not increase Rb^+ efflux in untransfected HEK293 cells, indicating that retigabine's effect was mediated via KCNQ2. Thus, the Rb^+ efflux assay is capable of quantifying the functional response of KCNQ2 to both inhibitors and openers and provides data that are consistent with the more time-intensive electrophysiological methods.

The H17 Rb^+ flux assay was used to test a collection of small-molecule compounds known to modulate the function of different ion channel families. The results from this limited screen provided additional insight as to the ability and reproducibility of detecting weak inhibitors of KCNQ2 in "screening mode." Eighty compounds were tested at 10 μM in duplicate (Fig. 7). The average efflux with this test set was 54 ± 4.2% (SD), which was not significantly different from control wells without compound. In general, the variation in duplicate data for each of the compounds was minimal. Seventy-five of the 80 compounds had a range of ⩽5 in their duplicate efflux values. This provides confidence that the assay can reliably detect the activity of inhibitors in screening mode. Four compounds inhibited Rb^+ efflux by 20% or more, and all four of these compounds were active upon retest, producing concentration-dependent inhibition profiles (Fig. 7b).

Of the four hits identified in the screening set, one compound—clofilium—had been previously reported to weakly inhibit KCNQ2 expressed in *Xenopus* oocytes [13]. In our Rb^+ efflux assay, clofilium had an $IC_{50} = 27\,\mu M$. Clofilium blocks a number of voltage-gated K^+ channels with IC_{50} values ranging from 840 nM for Kv1.5 [32] to 80 μM for HisK [33]. The results from this limited screen provide confidence that the Rb^+ efflux assay combined with atomic absorption spectrometry detection can be used to reliably test for small-molecule modulators of the KCNQ2 channel.

Conclusion

In 1999, Terstappen [10] demonstrated the feasibility of using atomic absorption spectrometry to measure the functional activity of some ion channel classes including voltage-gated K^+ channels. We utilized this detection system to develop a relatively simple experimental protocol capable of quantifying KCNQ2 activity. The applicability of this assay system to KCNQ2 was demonstrated by achieving the same rank order and potency of inhibition with three K^+ channel blockers previously validated in electrophysiological studies and by inducing Rb^+ efflux with retigabine, a known positive modulator of K^+ channels within the KCNQ family. Neither retigabine nor the K^+ channel blockers showed activity on nontransfected cells. These results indicate that one can reliably measure the functional activation and pharmacological modulation of KCNQ2 channels using Rb^+ efflux with atomic absorption spectrometry detection. This biological response was of suitable magnitude for highly precise measures in both 96- and 384-well microtiter plate formats. Using these methods we were able to identify four active compounds from a collection of small-molecule ion channel modulators. Combining a Rb^+ efflux assay with the new generation of atomic absorption instrumentation provides a much higher throughput opportunity for assessing compounds for their ability to modulate KCNQ2 function as compared to electrophysiological methods. Finally, this detection system provides an attractive alternative to ^{86}Rb and scintillation counting, with the safety and disposal concerns associated with the radioactive assay format.

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