

I. Abstract

Ion channels are emerging as important molecular targets for high throughput functional assays in drug discovery and safety pharmacology. Compounds that modulate ion channel function have therapeutic potential for a variety of cardiovascular and CNS disorders. However, the search for such modulators has been limited by the lack of available functional assays in a HTS format. Moreover, membrane excitability in cell-based assay systems is a dynamic phenomenon that requires precision and accuracy in measurement.

Ion channels like KCNQ4 and the tandem pore domain (2P) channel are also considered important drug targets for modulation. Development and validation of functional assays for the aforementioned targets is assessed in recombinant mammalian cell lines using Aurora Biomed's Ion Channel Reader (ICR) technology platform. Utilization of the assays and technology platform provide precise, accurate, and physiologically relevant data at HTS speeds.

II. Introduction

The pressure to develop HTS assays to meet the growing demands of the pharmaceutical industry is enormous. Aurora Biomed offers a unique technology that can be applied to both voltage-gated and ligand-gated ion channels. The Ion Channel Reader (ICR) series uses Flux Assays and Atomic Absorption Spectroscopy (AAS) to detect ion channel activity. The Flux Assays can be optimized to accommodate different channel kinetic properties. The technology was originally designed to address drug safety needs with the hERG voltage-gated potassium channel, but is now widely being used in drug discovery for other potassium channels and applications are underway for sodium, chloride and calcium channels.

The slowly activating and inactivating KCNQ potassium channel family (1-5) have been associated with several diseases such as epilepsy, QT syndrome and deafness¹. KCNQ4 has been shown to play a role in both vestibular and auditory systems and thus an important target for drug discovery. Using Aurora Biomed's ICR technology, the Rb⁺ Flux Assay was optimized in order to study these channels stably expressed in a HEK cell line. Compounds that induced both activation and inhibition were detectable with the ICR, indicating the flexibility of the methodology. Alternatively, the 2P channels (KCNK 1-8) do not exhibit voltage-sensitive activation and thus do not inactivate and function to set membrane potential near K⁺ equilibrium. The 2P channels are inward rectifying and can also be studied using Aurora Biomed's ICR technology by adjusting the Rb⁺ Flux Assay protocol to account for different channel properties. This channel has a larger open probability at positive potentials and with the reversal potential around 0 mV, outward current can occur at positive potentials.

Overall, Aurora Biomed has a unique approach to studying ion channels for direct functional interactions that exceeds the existing alternatives. The results generated can be used to acquire accurate drug rank orders in screening platforms to increase productivity and advance the drug discovery process.

III. Materials and Methods

KCNQ4

A stable HEK cell line expressing KCNQ4 was grown in DMEM (Gibco) supplemented with 10% FCS, 50 mg/L Pen/Strep (Sigma) at 37°C, in 5% CO₂. Cells were plated at a density of 25,000 cells/well in 96-well microplates and incubated at 37°C, 5% CO₂, attaining 90% confluency. Cells are Rb⁺ loaded by application of 180 ml Rb⁺ Load Buffer (Aurora Biomed) and incubated for 3 hours at 37°C, 5% CO₂. Compounds are added at 20 ml/well of a 10 X stock solution followed by incubation for 30 minutes at 37°C, 5% CO₂. The 10 X compound solution was diluted in Rb⁺ Load Buffer and contains 0.1% DMSO. Excess Rb⁺ and compound are removed by three successive washes with 200 ml of Rb⁺ Wash Buffer (Aurora Biomed). For blockers, the Rb⁺ Wash Buffer was replaced with 180 ml of Channel Open Buffer (Aurora Biomed) containing 40 mM KCl and 20 ml of 10 X compound (diluted in Channel Open Buffer). For activators, the Rb⁺ Wash Buffer was replaced with 180 ml of Rb⁺ Wash Buffer containing 20 ml of 10 X compound. The activation effect of the compound may be studied by using 10 mM or 20 mM or 40 mM KCl Channel Open Buffer in place of Rb⁺ Wash Buffer if the activator is weak. Compound is diluted in the buffer used. Channel activation is recommended for 4 minutes for detection of both blockers and activators. A 200 ml extracellular sample was collected from the supernatant and stored in 96-well microplates. Intracellular samples were obtained by whole cell lysis with the application of 200 ml Lysis Buffer (Aurora Biomed). The level of Rb⁺ in both the intracellular and the extracellular samples (100 ml samples) were measured by Aurora Biomed's ICR series.

2P

A stable mammalian fibroblast cell line expressing a 2P 4TMD channel was grown in DMEM (Sigma): Nutrient Mixture F-12 at 1:1, supplemented with 10% FCS, 50 mg/L Pen/Strep (Sigma) at 37°C, in 5% CO₂. Cells were plated at a density of 50,000 cells/well in 96-well microplates and incubated overnight at 37°C, 5% CO₂. Cells were Rb⁺ loaded by application of 180 ml Rb⁺ Load Buffer (Aurora Biomed) and incubated for 3 hours at 37°C, 5% CO₂. Compounds were added at 20 ml/well of a 10 X stock solution followed by incubation for 30 minutes at 37°C, 5% CO₂. The 10 X compound solution was diluted in Rb⁺ Load Buffer and contains less than 0.1% DMSO. Excess Rb⁺ and compound were removed by three successive washes with 200 ml of Rb⁺ Wash Buffer (Aurora Biomed).

ICR8000	ICR12000
Medium Throughput Up to 5000 wells/day	High Throughput >12-fold Increase Throughput
Single Channel 1 Sample at a Time	Multi Channel 12 Samples at a Time
50 µl Sample Volume	10-20 µl Sample Volume
Accommodates 96/384-well Plates	Accommodates 96/384/1536-well Plates
Foot print: 65 cm X 55 cm X 37 cm	Foot print: 120 cm X 95 cm X 135 cm
Acetylene Gas - Compressed Air	Natural Gas or Acetylene Gas - Compressed Air
Manual with Optional Plate Stacker/Bar Code Reader	Plate Stacker/ Bar Code Reader
Sensitivity: 0.05 ng/L Detection Limit Precision: <3% CV	

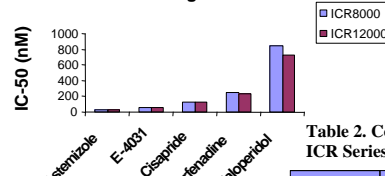
Table 1. Comparison of ICR 8000 and ICR 12000

Aurora Biomed's ICR series is a powerful analytical tool for HTS of ion channels. The ICR 12000 has a 12-fold capacity to that of the ICR 8000, yet maintains the accuracy with a 0.05 mg/L detection limit for Rb⁺. A number of compounds were tested for IC₅₀ values with the HEK/hERG cell line and was shown to be reproducible with both instruments.

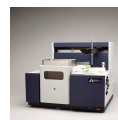
Figure 2. Comparison of ICR 8000 and ICR 12000 Performance

Using a HEK/hERG cell line and the Rb⁺ flux assay, several known inhibitors were tested to detect ICR IC₅₀ values. As shown here, both instruments have generated similar IC₅₀ values. These findings confirm that both instruments are highly sensitive and are performing optimally. This is reiterated in Table 2.

IC-50 & Drug Rank Order



ICR 8000



ICR 12000

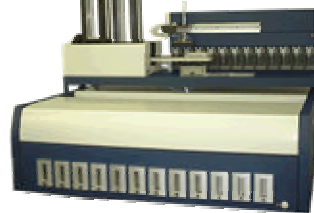
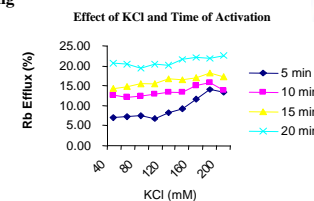
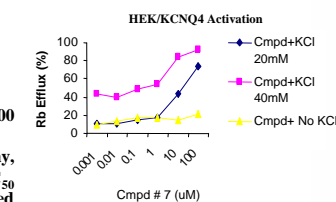
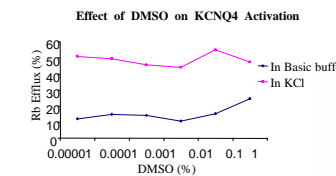
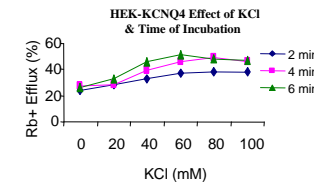


Table 2. Comparison of the ICR Series Performance

DRUG	IC ₅₀ (nM)		Electro physiology
	ICR 8000	ICR 12000	
Astemizole	39 27 26 42*	34 31 24 52*	1.6
Cisapride	130 207 249*	123 306 263*	4.9
E-4031	56	60	10
Terfenadine	243 217 253 220	233 274 307 307	79
Haloperidol	855	728	-



KCNQ4

Figure 3. Effect of KCl Concentration and Time

KCNQ4 ion channels stably expressed in HEK cells are activated chemically by changes in membrane potential. Here, after cells are loaded with Rb⁺, we demonstrate a concentration range of external KCl from 40 mM to 100 mM, resulting in Rb⁺ efflux ($[Rb^+]_{out}/[Rb^+]_{total}$) which was detected with Aurora Biomed's Ion Channel Reader (ICR) 8000. Cells were incubated at different time lengths. Six minute incubation in 50 mM KCl appears to give the highest level of Rb⁺ Efflux. The maximum efflux level was approximately 55 - 60%. The basal efflux level (ion flow without addition of KCl) was approximately 20%.

Figure 4. Effect of DMSO on Channel Activation

HEK/KCNQ4 cell line was analysed using Aurora Biomed's ICR 8000 with the Rb⁺ Flux Assay to determine the effects of DMSO. Here, channels are activated with 60 mM KCl for four minutes. No significant change in basal efflux or maximum efflux was observed with up to 1% DMSO.

Figure 5. Detection of Channel Activation

The Rb⁺ Flux Assay was carried out with HEK/KCNQ4 using an agonist at six concentrations to generate a dose response curve. The maximum Rb⁺ Efflux ($[Rb^+]_{in}/[Rb^+]_{total} \times 100$) was 80% and the basal Rb⁺ Efflux was 40% when channel activation was induced by application of 40 mM KCl for 4 minutes. The calculated ICR EC₅₀ value was not determined as more experiments need to be carried out.

2P

Figure 6. Effect of KCl Concentration and Time

The Rb⁺ Flux Assay was carried out with the Fibroblast/2P cell line with varying concentrations of KCl for different incubation times to determine optimal conditions for channel activation.

V. Conclusion

Both activators and blockers of HEK/KCNQ4 were easily identified using Aurora Biomed's ICR 8000 and Flux Assays. As described in the literature, flux assays can be optimized to identify channel activity for both ligand-gated and voltage-gated ion channels². Here, the Rb⁺ Flux Assay has been shown to accurately identify compounds that change channel ion flow in both delayed and inward rectifying potassium channels. Although more experiments are required here, it is clear to see that Aurora Biomed technology is advanced and adaptable to many ion channel HTS needs.

References

- Robbins, J. 2001. KCNQ potassium channels: physiology, pathophysiology and pharmacology. *Pharmacology & Therapeutics* 90:1-19.
- Terstappen, G. 1999. Functional analysis of native and recombinant ion channels using a high-capacity non-radioactive rubidium efflux assay. *Analytical Biochemistry*, 272:149-155.

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