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Cellular HTS Assays for Pharmacological Characterization of $Na_V1.7$ Modulators

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Abstract: Ion channels are challenging targets in the early phases of the drug discovery process, especially because of the lack of technologies available to screen large numbers of compounds in functionally relevant assays. The electrophysiological patch-clamp technique, which is the gold standard for studying ion channels, has low throughput and is not amenable to screening large numbers of compounds. However, for random high-throughput screening (HTS) of compounds against ion channel targets, a number of functional cellular assays have become available during the last few years. Here we use the sodium channel $Na_V1.7$ stably expressed in human embryonic kidney 293 cells and compare three HTS assays—a Li flux atomic absorption spectroscopy (AAS) assay, a fluorescent imaging plate reader (FLIPR™, Molecular Devices, Sunnyvale, CA) membrane potential assay, and a fluorescence resonance energy transfer (FRET)-based membrane potential assay—to an automated electrophysiological assay (the Ionworks™ HT [Molecular Devices] platform) and characterize 11 known Na_V inhibitors. Our results show that all three HTS assays are suitable for identification of $Na_V1.7$ inhibitors, but as an HTS assay the Li-AAS assay is more robust with higher Z' values than the FLIPR and FRET-based membrane potential assays. Furthermore, there was a better correlation between the Ionworks assay and the Li-AAS assay regarding the potency of the Na_V inhibitors investigated. This paper describes the first comparison between all the HTS assays available today to study voltage-gated Na_Vs, and the results suggest that the Li-AAS assay is more suited as a first HTS assay when starting an Na_V drug discovery campaign.

Introduction

VOLTAGE-GATED SODIUM CHANNELS (Na_Vs) play a critical role in the initiation and propagation of action potentials in excitable cells. The Na_Vs activate in response to membrane depolarization and are responsible for rapid influx of sodium ions during the rising phase of the action potential. Na_Vs are widely expressed in neuronal, neuroendocrine, skeletal muscle, and cardiac cells and are potential targets for a number of diseases. The role of the Na_V subtype $Na_V1.7$ (SCN9A) in pain has been demonstrated very convincingly in humans because of recent discoveries of loss-of-function mutations in the SCN9A gene in families with complete inability to sense pain[1] and gain-of-function mutations in the SCN9A gene leading to the painful inherited human neuropathy known as erythrothermalgia.[2] These findings in addition to animal knockout models have pointed to $Na_V1.7$ as a primary drug target, and a specific $Na_V1.7$ antagonist is expected to be a powerful analgesic.

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ABBREVIATIONS: AAS, atomic absorption spectrometry; EC_{50}, 50% effective concentration; FLIPR, fluorescent imaging plate reader; FRET, fluorescence resonance energy transfer; HEK, human embryonic kidney; IC_{50}, 50% inhibitory concentration; Na_V, sodium channel; SVqq, scorpion venom from *Leiurus quinquestriatus quinquestriatus*; TTX, tetrodotoxin; VSP, voltage-sensitive pair.

Until recently there have been very limited options in terms of HTS assays to identify Na_V inhibitors from large compound libraries, which is the starting point for a drug discovery program. Activation of Na_Vs leads to Na^+ influx across the cell membrane, resulting in concomitant transient changes in membrane potential. Both the actual ion flux and the change in membrane potential can be exploited for the development of functional assays for Na_V screening. The gold standard for studying the function of ion channels is traditional electrophysiological patch-clamp measurements, but this technique has limited utility in early phases of the drug discovery process due to a very low throughput. With the revolutionary developments in parallel-automated patch clamp and the introduction of the Ionworks™ HT (Molecular Devices, Sunnyvale, CA) instrument in 2003,[3,4] the opportunity of screening hundreds of compounds obtaining real electrophysiological data is now possible. The Ionworks HT instrument is an integrated platform consisting of computer-controlled fluid handling, recording electronics, and processing tools capable of voltage-clamp whole-cell recordings from up to 384 individual cells per experiment. To establish a recording, the system uses a planar, multiwell substrate (a PatchPlate™). The system effectively positions cells into a hole separating two fluid compartments in each well of the substrate. Voltage control and current recordings from the cell membrane are made subsequent to gaining access to the cell interior by applying a permeabilizing agent to the intracellular side. Based on the multiwell design of the PatchPlate, voltage-clamp recordings of up to 384 individual cells can be made in less than 20 min and are comparable to measurements made using traditional electrophysiology techniques.

Recent advances in atomic absorption spectroscopy (AAS) technology allowing detection of small volumes of biological samples and the development of fluorescent dyes sensitive to changes in membrane potential[5,6] have made it possible to develop these assays for higher-throughput screening of ion channel targets.

Traditionally ion flux assays have utilized radioisotopes such as ^{86}Rb or [^{14}C]guandinium for potassium and Na_V channels, respectively. AAS now allows for the use of nonradioactive rubidium (Rb) or lithium (Li) as surrogate ions in these assays. The use of conventional AAS to detect Rb in samples from Rb efflux assays has been described for a medium-throughput screening of compounds acting on potassium channels.[7,8] Similarly, Li can be used as a surrogate ion for Na to measure activity of Na_Vs, because Li has been shown to have permeability for Na_Vs similar to that of sodium.[9]

Fluorescent dyes sensitive to changes in membrane potential have been known for several years, but they have been improved significantly during the last few years, and with the fluorescent plate readers with integrated liquid handling like the fluorescence imaging plate reader FLIPR™ from Molecular Devices and the FDSS6000™ from Hamamatsu Photonics K.K. (Hamamatsu City, Japan), changes in membrane potential can be measured in HTS format. The FLIPR system uses a 488 nm argon laser for excitation of the fluorescent dye that exhibits an increase in the emission intensity at 550 nm when the membrane depolarizes. The Hamamatsu instrument measures a ratio of emission signals that increases when fluorescence resonance energy transfer (FRET) between a pair of dyes (one FRET donor and one FRET acceptor) is displayed in response to membrane depolarization.

In order to select the best HTS assay to discover $Na_V1.7$ inhibitors we developed three HTS assays: an Li-AAS influx assay, a FLIPR membrane potential assay, and a FRET membrane potential assay. For comparison of pharmacology we investigated 11 nonselective Na_V inhibitors in these three assays and compared them to the IonWorks HT patch-clamp assay. In addition, we screened a smaller ion channel-focused compound library (13,000 compounds) in the Li-AAS assay and the FLIPR membrane potential assay to assess the feasibility of using these assays for HTS.

Materials and Methods

Materials

A human embryonic kidney (HEK) cell line stably expressing $Na_V1.7$ was purchased from Cytomyx (Cambridge, UK). The voltage-sensitive dye pair (VSP) was purchased from Invitrogen Life Technologies (Paisley, UK), and blue membrane potential dye was purchased from Molecular Devices. Veratridine and scorpion venom from *Leiurus quinquestriatus quinquestriatus* (SVqq) along with Na_V blockers were purchased from Sigma-Aldrich (St. Louis, MO). Li calibration solution and wash buffer for AAS were purchased from Aurora Biomed Inc. (Vancouver, BC, Canada).

IonWorks HT electrophysiological assay

HEK-$Na_V1.7$ cells were grown to semiconfluence (maximum 75%) in T-75 Costar flasks (Corning, Corning, NY) at 37°C in a humidified environment (5% CO_2) in Dulbecco's Modified Eagle's Medium/F12 medium without L-glutamine, 10% fetal bovine serum, 1% L-glutamine, G418 sulfate, and 1% minimum essential medium nonessential amino acids. Just prior to use, the cells were washed with prewarmed (37°C) Dulbecco's phosphate-buffered saline without $CaCl_2$ and $MgCl_2$ (Invitrogen). After aspiration of this solution the flask was incubated at 37°C in an incubator with 2 ml of trypsin for a period of 3 min. Cells were then detached from the bottom of the flask by gentle tapping, 10 ml of Dulbecco's phosphate-buffered saline (Invitrogen) contain-

ing $CaCl_2$ (0.9 m*M*), $MgCl_2$ (0.5 m*M*), D-glucose (1 g/L), and L-pyruvate (36 mg/L) was then added to the flask, and the cell suspension was transferred to a 15-ml centrifuge tube prior to centrifugation (50 *g* for 1 min). The resulting supernatant was discarded, and the cell pellet was gently resuspended in 3 ml of Dulbecco's phosphate-buffered saline. A 0.3-ml aliquot of cell suspension was removed, and the number of viable cells (based on trypan blue exclusion) was determined in an automated cell counter (Cedex HiRes; Innovatis AG, Bielefeld, Germany) so that the cell resuspension volume could be adjusted with Dulbecco's phosphate-buffered saline to give the desired final cell concentration of ~500,000 cells/ml.

The principles and operation of the IonWorks HT instrument have been described by Schroeder *et al*.[3] and Kiss *et al*.[4] Test compounds (at threefold their final test concentration) were prepared in a 96-well plate (U-bottom, Greiner Bio-One, Kremsmuenster, Austria) with one compound per column to enable 12 eight-point concentration–response curves to be obtained from each experimental run. Cells were sealed in "internal" solution that had the following composition: 100 m*M* potassium gluconate, 40 m*M* KCl, 3.2 m*M* $MgCl_2$, 3 m*M* EGTA, and 5 m*M* HEPES (all from Sigma-Aldrich) adjusted to pH 7.25 using 10 *M* KOH. After sealing, the "internal" solution was changed to "access" solution that contained the internal solution plus 100 μg/ml amphotericin B (Sigma-Aldrich). After 9 min was allowed for patch perforation, the pre-compound response was recorded, followed by addition of test compounds. Compounds were incubated for 3.5 min before the post-compound response was recorded. The pre- and post-compound responses were evoked by a voltage train as follows: after a 10-s period holding at –90 mV, eight pulses, each consisting of a 50 ms step to –20 mV followed by a 320 ms step back to –90 mV, were applied at 3Hz. The current signal was sampled at 10 kHz. Currents were leak-subtracted based on the estimate of current evoked during the –10 mV step at the start of the voltage pulse protocol. Pre- and post-scan $Na_V1.7$ current amplitudes were measured automatically from the leak-subtracted traces by the IonWorks HT software through averaging a 10 ms current during the initial holding period at –90 mV (baseline current) and subtracting this from the peak of the current response for each of the eight voltage steps. Data shown are calculated only from pulse 8. Filters were set to a pre-scan seal resistance of $>$60 MΩ, pre-scan $Na_V1.7$ current amplitude of $>$150 pA, and post-scan seal resistance of $>$60 MΩ. Cells that did not meet these criteria were discarded from the measurements. Dividing the post-scan current amplitude by the respective pre-scan current amplitude for each well assessed the degree of inhibition of the $Na_V1.7$ current. In this way, noncumulative concentration–response curves were obtained based on recordings from one to four individual cells.

Li-AAS assay

HEK-$Na_V1.7$ cells were cultured and plated in growth medium containing Dulbecco's Modified Eagle's Medium/F12 medium without L-glutamine, 10% fetal bovine serum, 1% L-glutamine, G418 sulfate, and 1% minimum essential medium nonessential amino acids. Cells were plated (10,000 per well in 70 μl) in collagen-coated 384-well plates 3 days prior to the assay. First, cells were washed with sodium-free buffer (137 m*M* choline chloride, 5.4 m*M* KCl, 0.81 m*M* $MgSO_4$, 0.95 m*M* $CaCl_2$, 5.5 m*M* glucose, and 25 m*M* HEPES, pH 7.4) and incubated in this buffer for 15 min. To determine the effect of Na_V blockers the cells were pretreated (5–10 min) with compounds in LiCl-free buffer. Compounds were dissolved directly into buffer or diluted from 5$\times$ dimethyl sulfoxide (DMSO) stock. Cells were then incubated with LiCl buffer alone (45 m*M* choline chloride, 90 m*M* LiCl, 5.4 m*M* KCl, 0.81 m*M* $MgSO_4$, 0.95 m*M* $CaCl_2$, 5.5 m*M* glucose, and 25 m*M* HEPES, pH 7.4) or LiCl buffer with veratridine (25 μM) and scorpion venom (1.0 μg/ml) for 90 min. Cells were washed with LiCl-free buffer at the end of the incubation to remove any extracellular LiCl. Cells were then lysed by addition of 0.8% Triton X-100. Li concentration (in ppm) was determined by reading the samples in an ICR12000 atomic absorption spectrometer (Aurora Biomed Inc.).

For Li detection, the ICR12000 atomic absorption spectrometer detection parameters include lithium lamps with 2 mA current, 670.8 nm wavelength, 0.2 nm slit width, and 300–400 voltage range across the 12 photomultiplier tubes. Sample analysis solution was Aurora Biomed's proprietary solution. Integration settings were as follows: 2.5 s delay read time, 5 s integration time. Samples were injected in 75 μl of sample volume and 200 μl of wash solution. The atomic absorption spectrometer was calibrated each day. A standard curve of Li generated using 0.05, 0.25, 0.5, 1, and 1.5 ppm of Li in 17 ml of second wash buffer/83 ml of 0.8% Triton X-100 was used to calculate the ppm of Li in each well. Data were extracted by the atomic absorption spectrometer program as mg/ml of Li in samples in a 384-well plate format.

FLIPR membrane potential assay

HEK-$Na_V1.7$ cells were grown and plated in black poly-D-lysine-coated 384-well plates at 10,000 cells per well in 70 μl 3 days prior to testing. Cells were washed with Hanks' Balanced Salt Solution buffer (Invitrogen) with 1 m*M* $MgCl_2$ and 1 m*M* $CaCl_2$, leaving 20 μl of buffer per well after the last wash. Blue membrane potential dye (20 μl) was added, and the cells were incubated with dye for 45 min at 37°C. After incubation the dye-loaded cell plate was placed in the FLIPR workstation, and the experiment was started with imaging the whole plate every 2 s (excitation at 488 nm and emission

at 550 nm); after 10 s veratridine (25 μM) and scorpion venom (1 μg/ml) were added to the cells. The response elicited by veratridine and scorpion venom was taken as the maximal response. To determine the effect of Na_V blockers, cells were pretreated with compounds for 5–10 min before the plate was transferred to the FLIPR.

FRET membrane potential assay

HEK-Na_V1.7 cells were grown and plated in black poly-D-lysine-coated 384-well plates as described above. Cells were washed with assay buffer (Hanks-HEPES). The FRET donor, a coumarin-phospholipid (CC2-DMPE) dye (final concentration 2.5 μM), was added, and cells were incubated at room temperature for 30 min. Cells were then washed with assay buffer. The FRET acceptor, a hydrophobic oxonol [$DiSBaC_2(3)$] dye (20 μM), was added to cells and incubated for 20 min at 37°C. Na_V blockers were added to the cells 10 min prior to placing the cell plate in the FDSS6000. The cells were excited by light at 387 nm, and the ratio of the fluorescence intensity signals emitted at 460 and 580 nm was measured every 1.8 s. After baseline imaging was obtained, veratridine (25 μM) and scorpion venom (1 μg/ml) were added to measure a maximal depolarizing response.

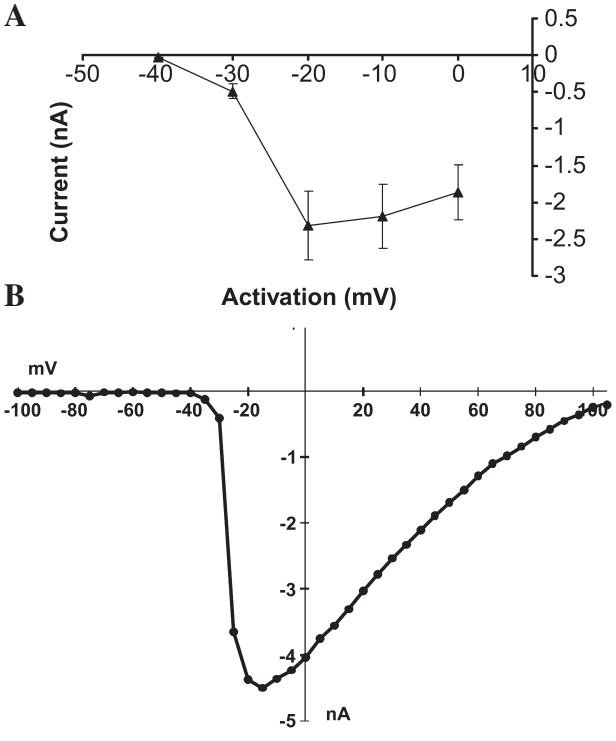

FIG. 1. Current–voltage curve for the HEK-Na_V1.7 cell line in (**A**) Ionworks HT and (**B**) conventional patch clamp. (A) Using the Ionworks HT, the holding potential was −90 mV. Data are mean ± SEM for 363 cells. (B) Using conventional patch clamp, the holding potential was –100 mV stepping 5 mV to +105 mV.

In resting cells the VSP FRET pair, the voltage-sensitive acceptor [$DiSBaC_2(3)$] and outer membrane-bound donor (CC2-DMPE), both bind to the outer surface of the cell membrane. When excited at 387 nm, the emission signal of the donor dye at 460 nm is relatively low, whereas the emission signal of the acceptor dye at 580 nm is relatively high because of efficient FRET. Upon depolarization of the cells the donor dye remains on the outer surface, but the mobile acceptor dye rapidly translocates to the inner surface of the cell membrane, resulting in diminished FRET and an increase in the 460/580 nm emission ratio.

Data analysis

The pIC_{50} (50% inhibitory concentration) values were determined from eight-point concentration–response curves fitted to the sigmoid modified Hill equation: $y =$ Bottom + (Top − Bottom)/(1 + 10^ [($LogEC_{50} - x$) × Hill slope]), where x is the logarithm of the concentration, y is the response, and EC_{50} is the 50% effective concentration. All curve fittings were performed using Graph Pad (San Diego, CA) Prism or custom software developed in-house at AstraZeneca (Wilmington, DE). Z' values were calculated according to the equation described by Zhang *et al.*[10]

Results

IonWorks HT electrophysiological assay

We first established the electrophysiological assay since this was the reference for comparing the pharmacology of the Na_V inhibitors investigated in this study. A major difference between conventional patch clamping and the Ionworks HT system is the cell handling. For successful recordings in the Ionworks HT, cells need to be detached from the bottom of the growth flask and suspended in solution for addition to the PatchPlate. This is a critical step when running this assay because the sealing of cells in the PatchPlate is sensitive to the handling of the cells. Initially we tried several procedures and optimized the handling according to seal rate (number of cells with a resistance >60 MΩ and average seal resistance for the whole plate) and number of cells where full recordings were obtained (sealing, pre-compound, and post-compound response), which was determined as the success rate for the experiment. Detaching the cells with trypsin prior to the experiment worked well, but the time was critical where trypsin incubations >5 min decreased the success rate from ~70% to <50% (data not shown). Furthermore, resuspending the cells in buffer also had to be done with care since this factor also had a major effect on the success rate. The initial voltage step protocols used for optimizing the cell handling procedures

TABLE 1. COMPARISON OF pIC_{50} OF KNOWN NA_V BLOCKERS IN THE MEMBRANE POTENTIAL ASSAY (FLIPR MEMBRANE POTENTIAL DYE [MPD] IN THE FLIPR AND FRET DYE PAIR IN THE HAMAMATSU ASSAY), LI-AAS INFLUX ASSAY, AND IONWORKS HT

	pIC_{50}			
	25 μM veratridine + 1 μg/ml SVqq			
	FLIPR (MPD blue dye)	*FRET (VSP dye pair)*	*AAS (Li influx)*	*IonWorks HT (current)*
Lidocaine	3.03	3.50	3.89	4.00
Bupivicaine	4.38	5.40	4.77	4.40
Quinidine	4.20	4.29	4.52	4.50
Flecainide	—	4.37	4.40	4.90
TTX	6.58	6.21	7.40	8.00
Flunarizine	5.58	5.35	5.59	6.70
Mexiletine	4.97	4.57	4.29	4.50
Phenytoin	5.03	4.81	3.19	4.50
Propafefenone	6.21	5.70	6.13	5.30
Tetracaine	6.00	5.76	5.26	6.55
CO-102862	5.70	5.06	4.21	>4.0

Compounds were tested in triplicate on two or three different days.

were current–voltage curves running from −90 mV and stepping to −50 to +20 at 10 mV intervals in order to determine the maximum response. As shown in Fig. 1A the maximal response was obtained at −20 mV, which is in agreement with the activation observed using conventional whole-cell patch clamp (Fig. 1B).

For screening, we designed the protocol as described in Materials and Methods, with multiple depolarization steps enabling the possibility of identifying use-dependent $Na_V1.7$ inhibitors. With the use-dependent inhibitor tetracaine we obtained almost 1 log unit shift in the pIC_{50} value when measuring the inhibition on pulse 1 (pIC_{50} ~5.8; data not shown) versus pulse 8 (pIC_{50} ~6.4; Table 1), *i.e.*, the compound is more potent when the channel is activated multiple times.

The current traces obtained with the Ionworks HT instrument are very similar to classical patch clamp, and the $Na_V1.7$ currents are characterized by fast inactivation as shown in Fig. 2B. The response is completely inactivated within 3–4 ms when holding the cells at –90 mV and stepping to –20 mV for 50 ms. Furthermore, when applying the first round of depolarization step (pre-compound) followed by 3 min of buffer addition the post-compound recordings were stable, with no decrease in current, while addition of 100 n*M* tetrodotoxin (TTX) gave almost full inhibition (Fig. 2A and B). To establish this screening protocol we tested three different sodium channel inhibitors (TTX, tetracaine, and lidocaine), representing different chemical classes with a broad range of potencies. All three types of antagonists showed full inhibition of the $Na_V1.7$ response, with IC_{50} values in agreement with conventional patch-clamp results (Fig. 2C and Table 1). The results shown in Fig. 2C also demonstrate that running the assay in screening format with one concentration of compound being applied to four wells in the PatchPlate resulted in a minimum of one successful cell recording for each concentration in the curve, but in most cases more than two cells produced successful recordings. With these results we screened 11 known nonselective sodium channel inhibitors in the Ionworks HT assay to establish the pharmacology of most common classes of Na_V inhibitors (Table 1). Again these pIC_{50} values were comparable to conventional patch clamp and, most importantly, demonstrated that all types of Na_V inhibitors tested could be detected with this assay.

Once we established the assay we used it to confirm the activity of hits obtained in the Li-AAS and membrane potential assays described below. After running 100 plates (~5 weeks of screening) we determined the average seal rate (in MΩ), and the success rate of cells was recorded (Fig. 3A). As shown the average sealing rate can vary substantially (150–350 MΩ), but the overall success rate is relatively stable around 70%, *i.e.*, 5 weeks of screening resulted in ~27,000 single-cell recordings. In addition, we wanted to determine the stability of the response in the HEK-$Na_V1.7$ cell line (cells were split ~15 times), and as shown in Fig. 3B the cell line showed a stable response around 2 nA in average response during 5 weeks of recordings.

Li-AAS assay

The nonradioactive Li influx assay is a direct measure of ion flux through Na_Vs. The permeability of Li ions through Na_Vs is almost the same as sodium ions,[7] which allows Li to be used as a surrogate ion for Na in the ion flux assay. The signal in this assay is robust because the

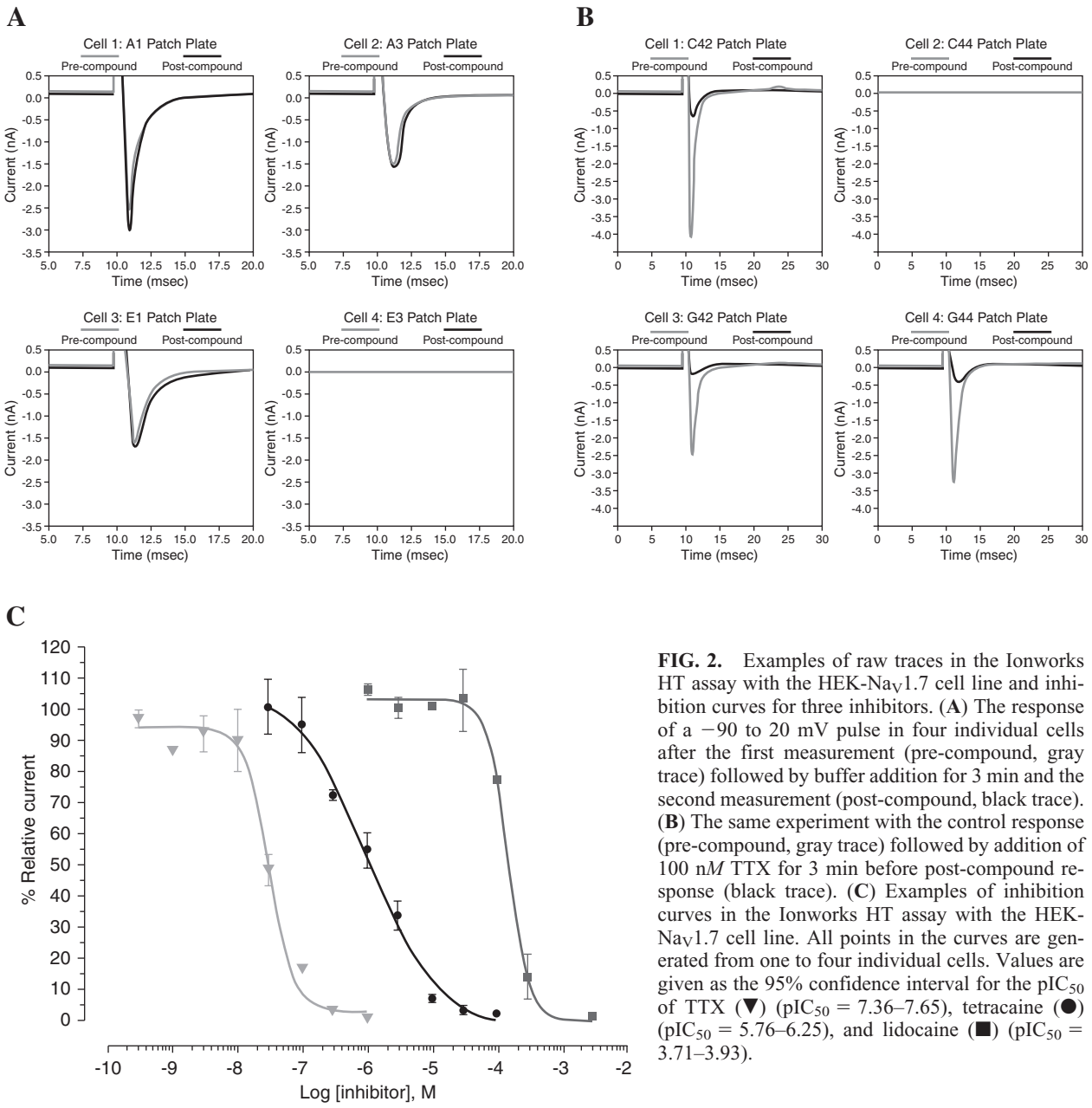

FIG. 2. Examples of raw traces in the Ionworks HT assay with the HEK-$Na_V1.7$ cell line and inhibition curves for three inhibitors. (**A**) The response of a −90 to 20 mV pulse in four individual cells after the first measurement (pre-compound, gray trace) followed by buffer addition for 3 min and the second measurement (post-compound, black trace). (**B**) The same experiment with the control response (pre-compound, gray trace) followed by addition of 100 n*M* TTX for 3 min before post-compound response (black trace). (**C**) Examples of inhibition curves in the Ionworks HT assay with the HEK-$Na_V1.7$ cell line. All points in the curves are generated from one to four individual cells. Values are given as the 95% confidence interval for the pIC_{50} of TTX (▼) (pIC_{50} = 7.36–7.65), tetracaine (●) (pIC_{50} = 5.76–6.25), and lidocaine (■) (pIC_{50} = 3.71–3.93).

absence of Li in biological fluids results in low background signal. Because of the inherent rapid inactivation of $Na_V1.7$ channels, veratridine and SVqq were used for Na_V activation. Veratridine slows the inactivation of Na_Vs, and SVqq prolongs channel opening. To identify $Na_V1.7$ channel blockers the HEK-$Na_V1.7$ cells are pretreated with test compounds prior to addition of veratridine and SVqq. Optimal cell density was found to be 10,000 HEK-$Na_V1.7$ cells suspended in 70 μl of culture medium. Plates were covered and placed in a Cytomat™ (Beckman Coulter, Fullerton, CA) incubator for 3 days prior to testing. The optimal concentration of Li was determined by incubating HEK-$Na_V1.7$ cells for different time periods with varying concentrations of LiCl in both the absence (basal influx) and presence of veratridine. The maximal net Li influx was observed upon incubation of HEK-$Na_V1.7$ cells with 80–100 m*M* LiCl for 90 min at 37°C (Fig. 4A). The EC_{50} of veratridine was between 21 and 25 μM when tested at various concentrations of LiCl (Fig. 4B).

When only veratridine was used to activate the channel, the increase in signal over background was small (twofold), and the absolute signal was within the lower limits of Li detection in AAS. To determine if a larger, more robust signal could be obtained, the effect of veratridine in the presence of various concentrations of SVqq was tested. Fig-

ure 4C shows near-maximal Li influx attained with 1 μg/ml SVqq in the presence of 25 μM veratridine (0.63 $\pm$ 0.037 ppm), which was reproducible and higher compared to 25 μM veratridine alone (0.169 $\pm$ 0.038 ppm).

Since the test compounds were dissolved in DMSO, the effect of DMSO concentration was also tested. Up to 1.3% of DMSO was well tolerated with no effect on Li influx (data not shown). However, 0.2% DMSO was used in the assay.

In order to determine if this assay is pharmacologically relevant, the pIC_{50} values for 11 known, nonselective sodium channel blockers were determined (Table 1), and inhibition curves of TTX, tetracaine, and lidocaine are shown in Fig. 5A. All the inhibitors gave full concentration–response curves, with TTX being most potent followed by tetracaine and lidocaine, in agreement with the results from the IonWorks HT assay.

FLIPR membrane potential assay

The increase in Na influx associated with activation of Na_Vs causes a concomitant transient change in membrane potential, which can be measured using fluorescent dyes sensitive to changes in membrane potential. The feasibility of a membrane potential assay to identify $Na_V1.7$ channel blockers was explored using Molecular Devices' blue membrane potential-sensitive dye detected with a FLIPR.

Optimal cell density was found to be 10,000 HEK-$Na_V1.7$ cells per well, plated 3 days prior to the experiment (data not shown). Activation of $Na_V1.7$ channels with 25 μM veratridine and 1 μg/ml SVqq resulted in a three- to fourfold increase in fluorescent signal using Molecular Devices' blue membrane potential dye, although the response showed relatively high variability (Fig. 6), with Z' between 0.15 and 0.45. To compare the pharmacology, the pIC_{50} values of the same sodium channel blockers tested in the Li-AAS flux assay were determined using this assay (Table 1). As demonstrated with the TTX, tetracaine, and lidocaine curve fits (Fig. 5B), the compounds showed concentration-dependent inhibition with the order of potency being TTX > tetracaine > lidocaine in agreement with the IonWorks HT and the Li-AAS assay. As shown in Fig. 5B, the SD for the data points ($n = 6$) is relatively large such that for a low potency inhibitor like lidodaine a full inhibition curve could not be obtained.

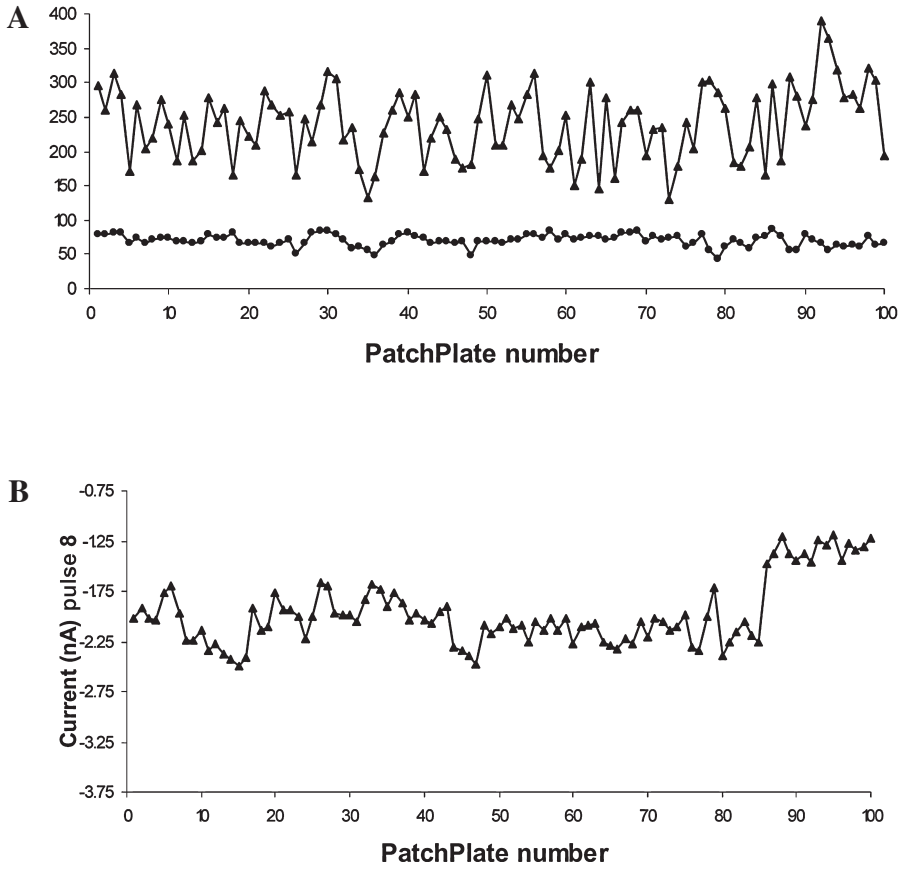

FIG. 3. The performance of the Ionworks HT assay with the HEK-$Na_V1.7$ cell line. (**A**) Success rate (●) and average seal (▲) for the assay, with an average seal of 240 MΩ and average success rate 70%. (**B**) Average current over time is 1.97 nA. There were 26,880 recordings from 100 plates over a 5-week period.

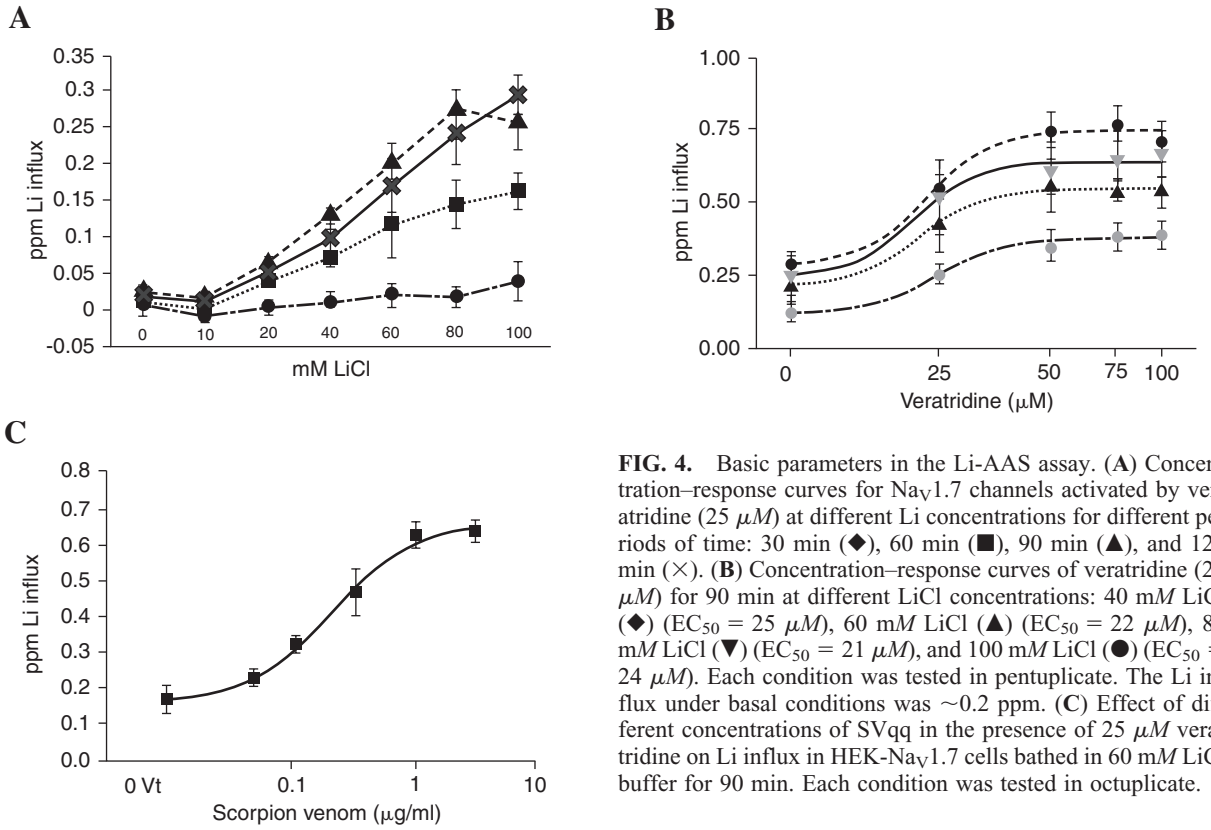

FIG. 4. Basic parameters in the Li-AAS assay. (**A**) Concentration–response curves for $Na_V1.7$ channels activated by veratridine (25 μM) at different Li concentrations for different periods of time: 30 min (◆), 60 min (■), 90 min (▲), and 120 min (×). (**B**) Concentration–response curves of veratridine (25 μM) for 90 min at different LiCl concentrations: 40 mM LiCl (◆) (EC_{50} = 25 μM), 60 mM LiCl (▲) (EC_{50} = 22 μM), 80 mM LiCl (▼) (EC_{50} = 21 μM), and 100 mM LiCl (●) (EC_{50} = 24 μM). Each condition was tested in pentuplicate. The Li influx under basal conditions was ~0.2 ppm. (**C**) Effect of different concentrations of SVqq in the presence of 25 μM veratridine on Li influx in HEK-$Na_V1.7$ cells bathed in 60 mM LiCl buffer for 90 min. Each condition was tested in octuplicate.

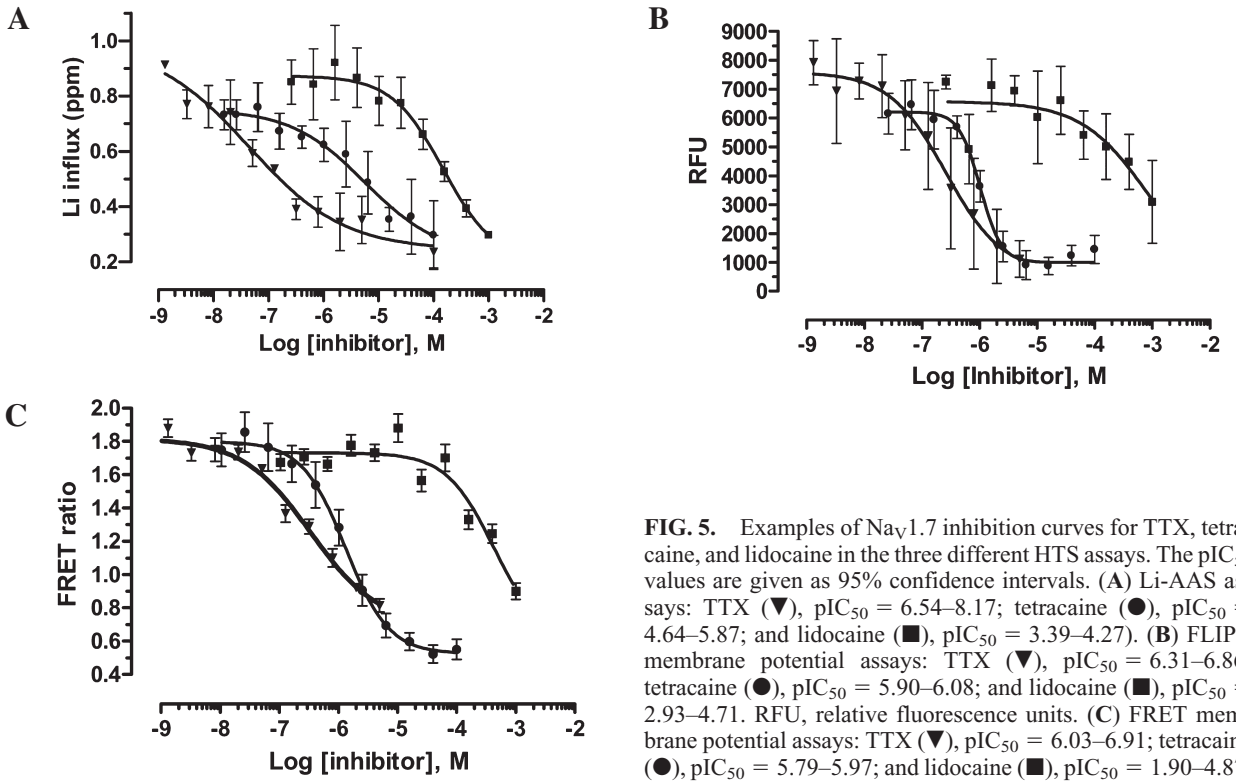

FIG. 5. Examples of $Na_V1.7$ inhibition curves for TTX, tetracaine, and lidocaine in the three different HTS assays. The pIC_{50} values are given as 95% confidence intervals. (**A**) Li-AAS assays: TTX (▼), pIC_{50} = 6.54–8.17; tetracaine (●), pIC_{50} = 4.64–5.87; and lidocaine (■), pIC_{50} = 3.39–4.27). (**B**) FLIPR membrane potential assays: TTX (▼), pIC_{50} = 6.31–6.86; tetracaine (●), pIC_{50} = 5.90–6.08; and lidocaine (■), pIC_{50} = 2.93–4.71. RFU, relative fluorescence units. (**C**) FRET membrane potential assays: TTX (▼), pIC_{50} = 6.03–6.91; tetracaine (●), pIC_{50} = 5.79–5.97; and lidocaine (■), pIC_{50} = 1.90–4.87.

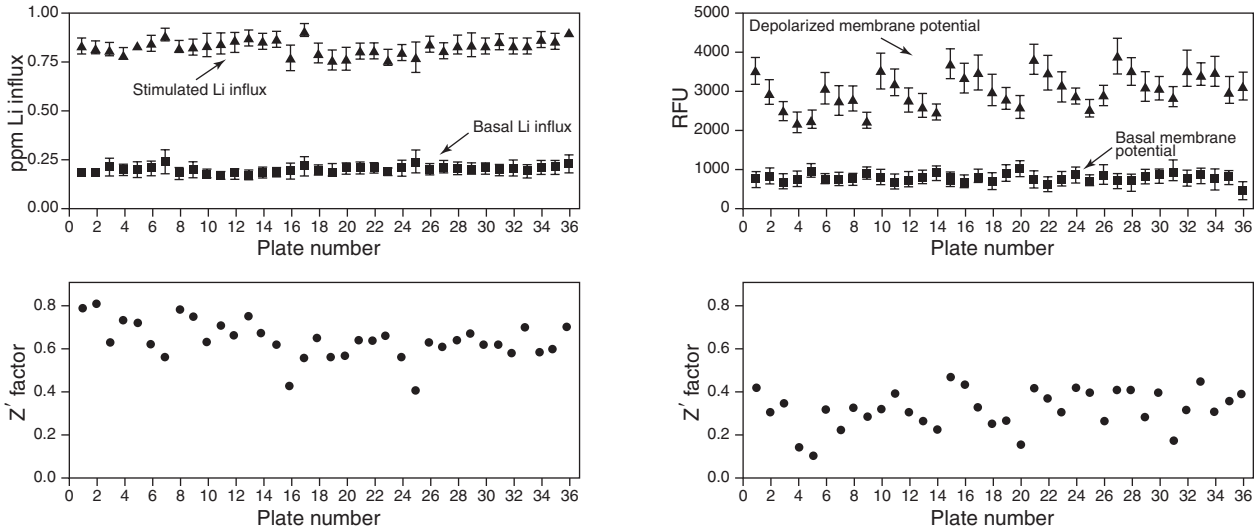

FIG. 6. Assay performance in screening mode (36 plates) of (**left panels**) the Li-AAS assay and (**right panels**) the FLIPR membrane potential assay. (**Top panels**) Basal Li influx or membrane potential as well as veratridine (25 μM) and SVqq (1 μg/ml)-evoked Li influx or depolarization. (**Bottom panels**) Z' factor for each plate tested in the respective screen as well as average Z factor: (left) average Z' factor = 0.62; (right) average Z' factor = 0.32. RFU, relative fluorescence units.

Screening an ion channel-focused compound library

In order to determine the feasibility of using the membrane potential or Li-AAS assays in HTS, 13,000 compounds (selected from the AstraZeneca collection using computational chemistry and pharmacophore modeling of various ion channel classes) were screened for inhibition of $Na_V1.7$ at a concentration of 10 μM in both assays.

The increase in signal upon treatment of HEK-$Na_V1.7$ cells with 25 μM veratridine and scorpion venom (1 μg/ml) was similar (fourfold) in both assays. However, the inter- and intraplate variabilities in the membrane potential assay signal were much higher, leading to an average Z' factor of 0.33 (Fig. 6). In the membrane potential assay the compounds were tested in sets of five plates at a time over which a decline in signal from plates 1 to 5 was observed, possibly due to the temperature sensitivity of the dye. In the Li-AAS assay the signal in all plates was robust and reproducible with low variability resulting in an average Z' factor of 0.62 (Fig. 6). Because the membrane potential assay is an indirect measure of $Na_V1.7$ channel activity and therefore prone to detection of false-positives, the hit rate (potential $Na_V1.7$ channel blockers) in this assay was higher compared to the more

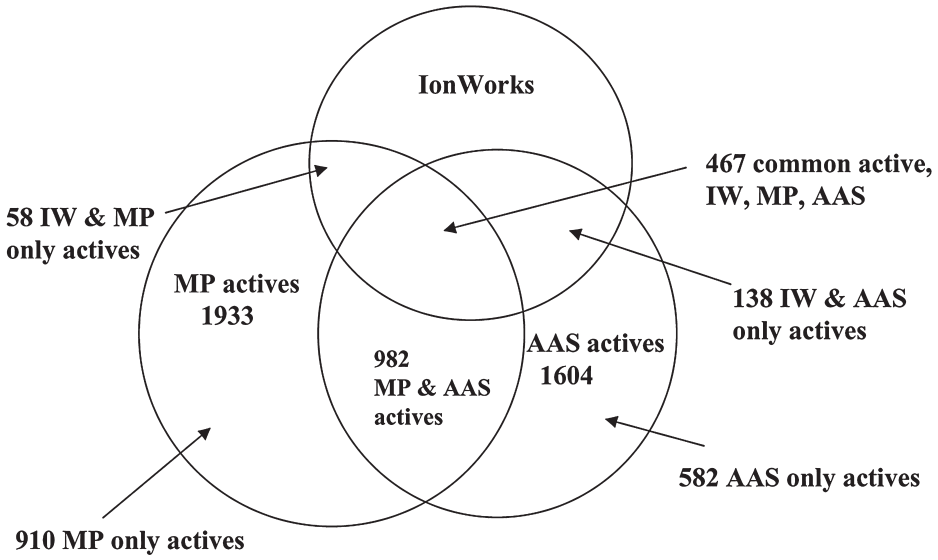

FIG. 7. Overlap of $Na_V1.7$ channel blockers in IonWorks HT (IW), Li-AAS (AAS), and membrane potential (MP) assays.

direct measure of channel activity monitoring Li ion flux. The overall hit rate in the Li-AAS assay was ~13%, while the FLIPR membrane potential assay gave a hit rate of ~23%.

Compounds were tested in triplicate in the membrane potential and Li-AAS assays with 40% inhibition used as a cutoff value. Out of the 982 compounds found active in both assays, 467 (48%) compounds were found active in the secondary IonWorks HT assay as well (Fig. 7). Additionally, 910 compounds were active only in the membrane potential assay, but only 58 of these compounds were confirmed active in the IonWorks HT assay. The majority of these 58 compounds showed borderline but subthreshold activity in the Li influx assay. Out of 582 compounds found active only in the Li-AAS assay, 138 of these were confirmed active in the IonWorks HT assay. Thus, the number of false-negatives in the Li-AAS assay (58) was less than half the number of false-negatives in the FLIPR membrane potential assay (138).

FRET membrane potential assay

Since the high variability and low Z' value of the FLIPR membrane potential assay made it unsuitable for HTS, an alternate FRET-based methodology using the VSP dye pair and Hamamatsu's FDSS6000 plate reader was investigated. As with the other two assays, 10,000 HEK-$Na_V1.7$ cells plated 3 days prior to experiment were found to be the optimal cell density. The ratio of the donor emission to acceptor emission increased (~10-fold) as the $Na_V1.7$ channels were activated with veratridine and SVqq. The variability in the signal was much lower compared to that seen with FLIPR membrane potential dye (Fig. 8). The IC_{50} values of 11 known Na_V blockers generated in this assay are reported in Table 1 and shown in Fig. 5C with TTX, tetracaine, and lidocaine. As demonstrated, the compounds show concentration-dependent inhibition with the order of potency being the same as the other assays. Although the SD for the data points (n = 6) was very tight, the low potency inhibitor lidocaine did not give a full inhibition curve.

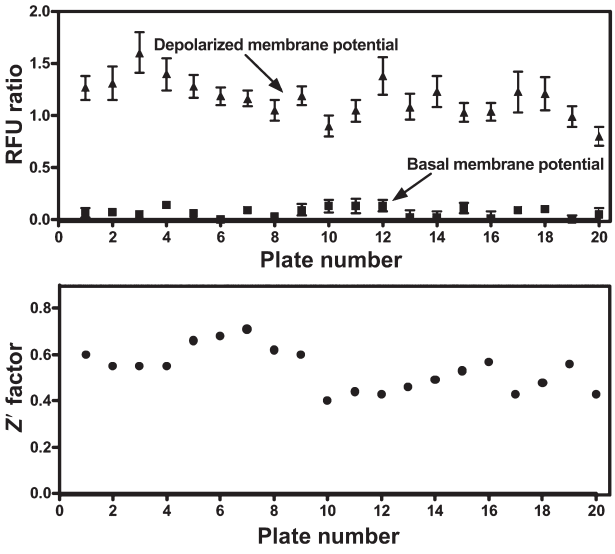

FIG. 8. Assay performance of 20 plates in the membrane potential assay using the VSP dye pair in the Hamamatsu FDSS6000. (**Top panel**) Basal membrane potential and veratridine (25 μM) and SVqq (1 μg/ml)-evoked depolarization. RFU, relative fluorescence units. (**Bottom panel**) Z' factor for each plate and average Z factor: average Z' factor = 0.54.

To determine the inter- and intraplate variability and reproducibility, 20 plates with cells were treated with veratridine (25 μM) and SVqq (1 μg/ml). The performance of the assay was measured by calculating the Z' factor for each plate tested, and, as shown in Fig. 8, the average Z' was 0.54 compared to 0.32 for the FLIPR assay.

Discussion

Voltage-gated ion channels can exist in multiple conformational states, including resting (closed but able to open), open, and inactivated (closed and not able to open). Channels exist mainly in the resting state in hyperpolarized membranes but predominantly in the inactivated state in depolarized membranes. Channel activation occurs when the conformation transitions from the resting to the open state as a result of membrane potential changes. Therefore, in order for an assay to detect channel activity, the resting membrane potential of the cell must be sufficiently hyperpolarized to maintain channels in the resting state prior to activation by membrane depolarization. This can easily be accomplished in voltage-clamp electrophysiological assays where membrane potential can be precisely controlled. However, in the Li influx and membrane potential assays the resting membrane potential of the cell cannot be controlled. Also, HEK cells have less hyperpolarized resting membrane potentials than excitable cells, and, as a consequence, a smaller population of channels will exist in the resting state. Therefore in the absence of voltage control it is necessary to enhance the measured response to Na_V activation by prolonging the channel open state with SVqq[11] while simultaneously blocking channel inactivation using veratridine.[12,13] Using these agents to activate Na_Vs in a screening protocol carries the risk that they may obscure or interfere with the pharmacological action of inhibitors and may not be appropriate for determining structure–activity relationship of inhibitors. However, since the majority of the compounds found active in these biochemical assays were subsequently confirmed active by electrophysiology, the technique is useful as an enabling method for detecting activity in screening.

The correlation between IC_{50} values of known Na_V blockers in the Li influx and IonWorks HT electrophysiology assays was good (r^2 = 0.8762 excluding

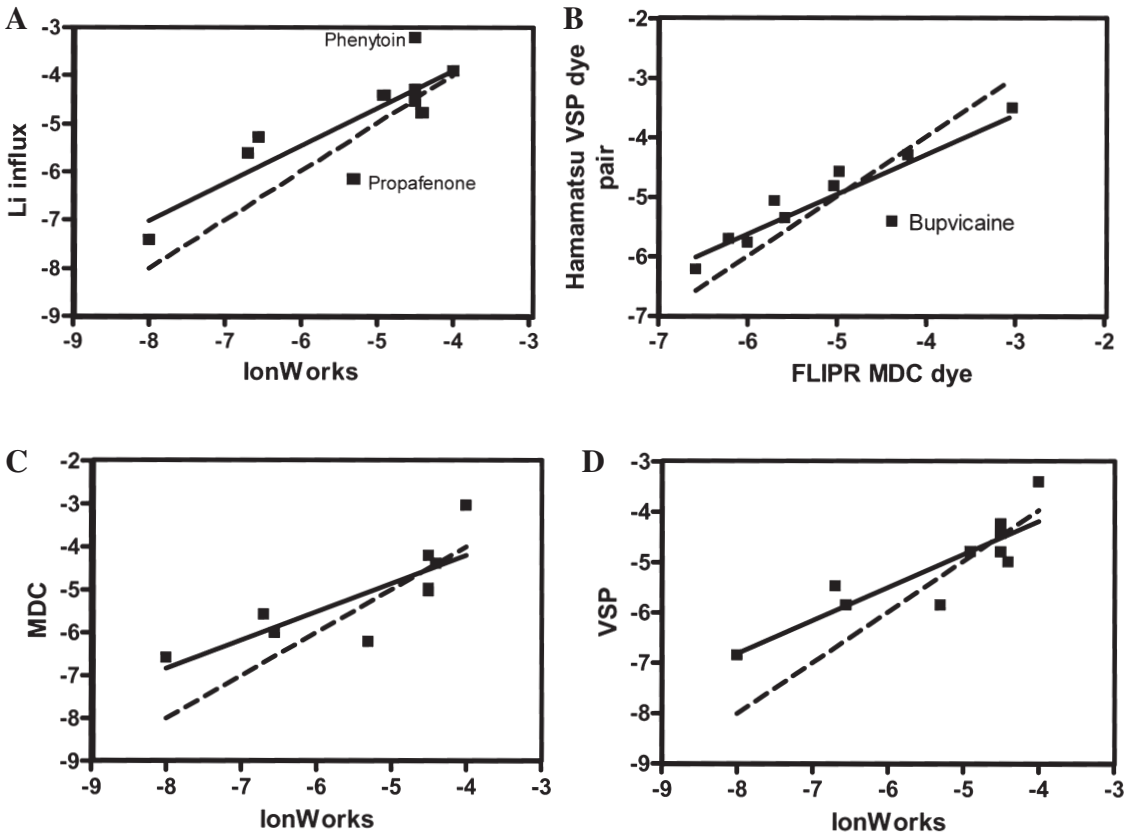

FIG. 9. Correlation of potency of known Na_V blockers in the different assays. (**A**) The Li influx assay and the IonWorks HT assay, $r^2 = 0.8762$, not including phenytoin and propafenone. (**B**) The membrane potential assay using blue membrane potential dye from Molecular Devices Corp. (MDC) in the FLIPR and the VSP dye pair in the FSDD6000, $r^2 = 0.8037$. (**C**) The IonWorks HT and membrane potential assay using blue membrane potential dye (MDC) in the FLIPR, $r^2 = 0.6535$. (**D**) The IonWorks HT and the membrane potential assay using the VSP dye pair in the FDSS6000, $r^2 = 0.7695$. The solid line is the line of correlation, and the dotted line is the line of correspondence.

phenytoin and propafenone; Fig. 9A). Propafenone, with a lower IC_{50} in Li influx assay (0.83 log unit), has been reported to block potassium channels ($K_V1.5$ and K_{ATP} channels) in addition to Na_Vs.[14,15] Phenytoin, tetracaine, and flunarizine were found to be more potent in the IonWorks HT assay than in the Li influx assay (>1 log unit). All three compounds have been reported to block Na_Vs as well as calcium channels.[16–19] Activity at these other channels as well as different mechanism of action at $Na_V1.7$ channels may be the reason for the differences obtained in the pIC_{50} values for these compounds in the two assays. In addition in the IonWorks HT assay we used multiple pulses and could identify use-dependent blockers being more potent inhibitors at pulse 8 compared to pulse 1. In the Li-AAS assay it is not possible to activate the channel multiple times, and this could be a reason why the potency of use-dependent blockers like tetracaine (~1 log unit more potent at pulse 8 in the IonWorks HT assay) is underestimated in the Li-AAS assay. However, none of the tested reference Na_V inhibitors was ineffective in the Li-AAS assay, demonstrating that although use dependence cannot be determined with this assay, it is still capable of identifying active inhibitors.

Furthermore, the different time resolutions of the assays are probably giving rise to different potencies of the inhibitors. The IonWorks HT assay was performed with millisecond resolution, whereas the membrane potential assays have a resolution of seconds. Both assays were performed with compound incubations of 1–2 min. The Li-AAS assay is an end point measurement done after 90 min of channel activation in the presence of inhibitor. The role of these time aspects is difficult to predict since they will depend on the binding properties of the inhibitors, but it is something to consider when comparing such diverse assays as described here.

Using the IonWorks HT assay to confirm compounds active in the Li-AAS and membrane potential assay, we found that the Li-AAS assay identified more confirmed active compounds (605 out of 663) compared to the FLIPR assay (525 out of 663). Since Li flux is a more direct measure of channel activation, this signal is less amplified than that measuring the change in membrane potential and hence may be more sensitive to detect in-

hibition of channel function. Although there were false-positives and -negatives in both assays, the number of each in the Li-AAS assay was lower. The higher false-positive rate in the membrane potential assay is likely due to the inherent susceptibility of the assay to detect compounds that alter membrane potential via mechanisms other than Na_V blockade.

The use of the ratiometric FRET dye pair provides a more robust membrane potential assay. Although the signal window is relatively small, the ratio of emission at 460 and 540 nm resulted in a normalized and more reproducible signal, giving a Z' factor of ≥ 0.5. While it does not overcome the inherent problem of a high false-positive rate in a membrane potential assay, the robustness and throughput of this assay provide another option for HTS of ion channel targets. The rank order of potency for known Na_V blockers in the two different assay systems measuring changes in membrane potential (Fig. 9B) was the same except for bupivacaine (1 log unit difference in IC_{50}). The correlation ($r^2 = 0.8037$) between the pIC_{50} values was good. However, the assay performance of the membrane potential assay using the FRET dye pair was more robust ($Z' = 0.54$) with much lower inter- and intraplate variability compared to that using the FLIPR membrane potential dye ($Z' = 0.32$). When correlating the pIC_{50} values obtained in these two membrane potential assays with the IonWorks HT assay (Fig. 9C and D) it is also noticeable that the FRET assay was performing better, with a correlation factor of 0.77, compared to 0.65 for the FLIPR assay. Furthermore, the IC_{50} curves produced with the FRET membrane potential assay are very tight compared to the FLIPR assay, suggesting that this assay could be useful as a indirect but robust secondary HTS assay with significantly higher throughput than the IonWorks HT assay.

Conclusions

This study shows that the Li influx assay with detection by AAS is a robust and reliable assay for HTS for Na_V targets. Li influx is a direct measure of ion channel activity as it measures the flow of Li through Na_Vs. It provides an alternative to low-throughput electrophysiology assays in the early phase of the drug discovery process to screen large number of compounds in order to identify "hits." The assay is suitable for pharmacological characterization of $Na_V1.7$ inhibitors, as confirmed in a patch-clamp electrophysiology assay. In addition, the Li-AAS assay showed lower false-positive and false-negative hit rates compared to the FLIPR membrane potential assay, thus making this our preferred HTS assay to identify Na_V inhibitors.

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