State-Dependent Compound Inhibition of $Na_{1.2}$ Sodium Channels Using the FLIPR $V_m$ Dye: On-Target and Off-Target Effects of Diverse Pharmacological Agents

ELFRIDA R. BENJAMIN, FARHANA PRUTHI, SHAKIRA OLANREWAJU, VICTOR I. ILYIN, GREGG CRUMLEY, ELENA KUTLINA, KENNETH J. VALENZANO, and RICHARD M. WOODWARD

Voltage-gated sodium channels (NaChs) are relevant targets for pain, epilepsy, and a variety of neurological and cardiac disorders. Traditionally, it has been difficult to develop structure-activity relationships for NaCh inhibitors due to rapid channel kinetics and state-dependent compound interactions. Membrane potential ($V_m$) dyes in conjunction with a high-throughput fluorescence imaging plate reader (FLIPR) offer a satisfactory 1st-tier solution. Thus, the authors have developed a FLIPR $V_m$ assay of rat $Na_{1.2}$ NaCh. Channels were opened by addition of veratridine, and $V_m$ dye responses were measured. The IC$_{50}$ values from various structural classes of compounds were compared to the resting state binding constant ($K_r$) and inactivated state binding constant ($K_i$) obtained using patch-clamp electrophysiology (EP). The FLIPR values correlated with $K_i$ but not $K_r$. FLIPR IC$_{50}$ values fell within 0.1- to 1.5-fold of EP $K_i$ values, indicating that the assay generally reports use-dependent inhibition rather than resting state block. The Library of Pharmacologically Active Compounds (LOPAC, Sigma) was screened. Confirmed hits arose from diverse classes such as dopamine receptor antagonists, serotonin transport inhibitors, and kinase inhibitors. These data suggest that NaCh inhibition is inherent in a diverse set of biologically active molecules and may warrant counterscreening NaChs to avoid unwanted secondary pharmacology. (Journal of Biomolecular Screening 2006: 29-39)

Key words: sodium, channel, membrane potential, inactivation, state dependent, fluorescent imaging plate reader

INTRODUCTION

Voltage-gated sodium channels (NaChs) play key roles in determining neuronal excitability. These channels are critically involved in the generation of the neuronal action potential, as they mediate the initial inward current during depolarization. Similarly, they are responsible for this same process in cardiac tissue and other excitable cells. They represent the molecular site of action of various neurotoxins, local anesthetics, anticonvulsants, and antiarrhythmics.

NaChs are composed of an $\alpha$ pore-forming subunit that can associate with auxiliary $\beta$ subunits. Nine $\alpha$ subunits, named $Na_{1.1}$ through $Na_{1.9}$, and 3 $\beta$ subunits, named $\beta_1$, $\beta_2$, and $\beta_3$, of NaChs have been cloned and expressed. Several additional atypical sodium channel-like proteins have been cloned but still await functional expression. The $\alpha$ subunits are large (~260 kD) membrane-spanning proteins composed of 4 segments that each contain 6 transmembrane regions. When expressed in mammalian cells, recombinant $\alpha$ subunits form functional channels with appropriate ion permeation, voltage sensing, and inactivation properties. The $\beta$ subunits play modulatory roles in channel gating and regulation of expression levels. The various $\alpha$ and $\beta$ subunits have distinct spatial and temporal patterns of expression throughout the nervous system and nonneuronal tissues. $Na_{1.2}$ (rat brain type IIA) represents a highly tetrodotoxin (TTX)–sensitive NaCh that is broadly expressed in neurons.

State-dependent block of NaChs is an important mechanism of action of many anticonvulsants, local anesthetics, and antiarrhythmics, which generally exhibit greater affinity for the open or inactivated states of the channel than for the resting state (Fig. 1) and, as such, are considered use-dependent blockers. Specific examples include the anticonvulsants phenytoin, carbamazepine, and lamotrigine, as well as the antihistamine/local anesthesia drugs diphenhydramine, hydroxyzine, and chlorpheniramine. These drugs inhibit both resting and active states of the channel. In contrast, some anticonvulsants exhibit greater affinity for the open state, such as lamotrigine, gabapentin, pregabalin, and diphenylhydantoin. These drugs are considered use-dependent blockers. This review focuses on the development of state-dependent assays for NaCh inhibitors using the FLIPR $V_m$ dye assay.
considered use dependent. It exhibits high affinity for the inactivated state of the channel and can cause cardiac toxicity with overdose. At these high concentrations, it blocks human cardiac NaChs in a state-dependent manner. It exhibits high affinity for the inactivated state of the channel and low affinity for the resting state. Recovery from inactivated state block is relatively slow and may mechanistically contribute to the observed cardiotoxic side effects.

Taking advantage of the electrogenericity of NaChs, we have developed a homogeneous, fluorescence-based, functional high-throughput assay of NaCh activity using the Molecular Devices FLIPR Membrane Potential (V_m) assay kit in stable rNa,1.2-HEK cells. Changes in fluorescence using the fluorescence imaging plate reader (FLIPR) V_m indicator dye linearly correlate with direct electrophysiology (EP) measurements of membrane potential in current clamped cells. Thus, rNa,1.2-HEK cells are loaded with the fluorescent V_m indicator dye, and a change in cellular membrane potential in response to the NaCh inactivation inhibitor, veratridine, is read on a FLIPR. A similar FLIPR V_m dye assay format has been used previously for pharmacological profiling of rNa,1.2, hNa,1.5, rNa,1.8, and native TTX-sensitive NaChs in rat cerebellar granule neurons. However, a comprehensive analysis of how the FLIPR data relate to resting or inactivated state block, as well as the assay’s utility to profile small molecules with a large dynamic range of potencies, particularly those with high potencies (IC50 values ≤ 400 nM), was not presented. Thus, to address some of these outstanding mechanistic and dynamic range issues and to extend the utility of this assay to selectivity screening for unknown NaCh blockers, we describe in this study the development of a rNa,1.2-HEK cell-based assay, show detailed comparison of the FLIPR-based pharmacological profiles compared to EP K and K values for a large collection of compounds with several different chemotypes over a wide range of potencies, and present the results from a screen of the Library of Pharmacologically Active Compounds (LOPAC, Sigma). Our data suggest that this FLIPR assay correlates better with K values than with K values across a wide range of compound potencies and varying chemotypes. These data also show that the assay can be used as a counterscreen to identify compounds that exhibit secondary pharmacology at NaChs to avoid potential side effects.

MATERIALS AND METHODS

Materials

Cell culture and molecular biology reagents were purchased from Invitrogen Corporation (Carlsbad, CA) unless otherwise noted. Proprietary compounds from the Purdue Pharma internal library were synthesized to > 97% purity using standard protocols or as referenced. All other chemicals and reagents were purchased from Sigma (St. Louis, MO).

Cloning and stable rNa,1.2-HEK cell line development

The plasmid pCMV-RIIA, which encodes rNa,1.2 under control of the cytomegalovirus (CMV) promoter, was obtained from Dr. Alan Goldin, University of California, Irvine. The construct also contained the neomycin gene for selection of stable cell lines with the neomycin analog G418. HEK-293 cells were plated at a density of 3 × 10^5 cells per well in a 6-well plate and transfected the following day with pCMV-RIIA DNA using Transfast reagent (Promega Inc., Madison, WI). After 3 weeks of selection in 400 μg/mL G418, resistant colonies were isolated, expanded, and plated in 35-mm dishes coated with poly-D-lysine for EP analysis. Several clones expressing TTX-sensitive inward currents were identified. The clone that expressed the largest amplitude currents was expanded and further EP analyses performed as described below.
Cells were cultured using standard techniques as described previously.19

Whole-cell patch-clamp recordings

For electrophysiology, cells were plated onto 35-mm Petri dishes (precoated with poly-D-lysine) at a density of 2 to 4 × 10^4 cells dish^-1 on the day of reseeding from confluent cultures. Cells were suitable for recording 3 to 4 days after plating. Whole-cell voltage-clamp recordings were made using conventional patch-clamp techniques20 at room temperature (22-24 °C). Currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Union City, CA) and were leak-subtracted (P/4 or by the built-in analog circuitry), low-pass filtered (3 kHz, 8-pole Bessel), digitized (20- to 50-μsec intervals), and stored using Digidata 1200 B interface and Pclamp8/Clampex software (Axon Instruments). The external solution for rNa1.2-HEK cells contained (in mM) the following: 150 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 D-glucose, 5 HEPES, pH 7.4 (NaOH). The patch-clamp pipettes were pulled from thick-walled borosilicate glass (WPI, Sarasota, FL). The internal solution contained (in mM) the following: 130 CsF, 20 NaCl, 1 CaCl₂, 2 MgCl₂, 10 EGTA, 10 HEPES, pH 7.4 (CsOH); osmolality was set at ~10 mmol kg^-1 lower than that for the external solution. The pipette resistances ranged from 1.5 to 3 MΩ. Residual series access resistance was in the range of 0.5 to 1.2 MΩ after partial (75%-80%) cancellation using built-in amplifier circuitry. Drug stock solutions and intervening intervals of wash were applied through a linear array of flow pipes (Drummond Microcaps, 2-mL, 64-mm length), producing full solution exchange within a few hundred msec. Drug stock solutions of 10 to 30 mM were prepared in DMSO and then were diluted into working external saline solutions on each day of experimentation. For all drugs, the concentration of DMSO in most experiments did not exceed 0.1% by volume, which had no measurable effect on NaCh current (I_{Na}).

Electrophysiology protocols

Sodium currents in rNa1.2-HEK cells are half-maximally activated by depolarization to −18.2 ± 0.3 mV (n = 13) with a steepness of 5.7 ± 0.3 mV and reach maximum at −0 mV.21 With 100-msec depolarizing conditioning pre-pulses, half-maximal inactivation occurs at −53.9 ± 1.7 mV with the slope of 6.4 ± 0.1 mV per e-fold change in membrane potential (n = 17).21 The channels recover from inactivation following a double-exponential time course: 80% to 85% of channels recovered within the first 2-5 msec; the remaining 15% to 20% of channels recovered more slowly, with a time constant of ≥ 200 msec. Most compounds tested by these methods significantly retard the recovery of rNa1.2 channels from inactivation.21,22 Based on this knowledge, we designed simplified protocols to assess inhibition of resting and inactivated states of the channels for this study. To quantify the affinity toward resting state, cells were held at −110 mV and pulsed to 0 mV every 10 sec to cause maximal Na⁺ current. At such a negative voltage, there was virtually no resting inactivation (< 1%). Steady-state currents were measured after ≥ 2 min in control or drug-containing solutions. The magnitude of inhibition was measured at high drug concentration, usually 3 μM. From the fractional response (FR) at this antagonist concentration, [antagonist], the estimate for the apparent dissociation constant for resting state, K_R, was obtained using the following equation: \[ K_R = \frac{FR/(1 – FR)}{[antagonist]} \] (assuming 1:1 binding stoichiometry). To measure the affinity toward the inactivated state, a double pulse protocol was used. From a holding voltage of −110 mV, cells were depolarized by a conditioning step to −20 mV for 3 sec. This step caused complete inactivation of NaChs and was long enough to ensure steady-state binding to inactivated channels, even for the slowest compounds. This step was followed by a short (5-msec) hyperpolarizing gap back to −110 mV, which was sufficient to permit > 80% recovery from fast inactivation in control. Then, a 5-msec test pulse to 0 mV was applied to measure the proportion of channels available for activation. In the presence of a drug, only unbound channels have a chance to recover from inactivation at a normal pace because repriming from inactivation is significantly retarded by the drug binding. Thus, the current caused by the test pulse reported the proportion of liganded (and blocked) channels. For some compounds presented in this study, a single concentration was empirically chosen that caused ~50% inhibition.23 From the FR at this concentration, the estimate for K_R was obtained using the following equation: \[ K_R = \frac{FR/(1 – FR)}{[antagonist]} \] (assuming 1:1 binding). For the remainder of the compounds, partial concentration-inhibition curves were generated by applying increasing antagonist concentrations in a cumulative manner. In this case, individual data sets were fitted by a sigmoid equation, \[ 1/(1 + ([antagonist]/K)_{dop}) \], using Origin 5.0 (Northampton, MA). Data are described as mean ± standard error of the mean (n = number of cells). The rNa1.2 state-dependent inhibitor, 4-(4-fluoro-phenoxo) benzaldehyde semicarbazone (PBSC; V102862),21,23 was chosen as a positive control in all measurements.

V_m measurements in rNa1.2-HEK cells on FLIPR—96- and 384-well formats

For the 96-well format, cells were seeded into poly-D-lysine precoated black-well, clear-bottom 96-well Biocoat plates (Beckton Dickinson, Franklin Lakes, NJ) at 75,000 cells well^-1 in 100 μL growth media 24 h prior to assay. For the 384-well format, cells were seeded into poly-D-lysine precoated black-well, clear-bottom 384-well Biocoat plates (Beckton Dickinson) at 50,000 cells well^-1 in 20 μL growth media 24 h prior to assay. On the day of assay, cells were washed twice with 100 or 50 μL (96- or 384-well format, respectively) of assay buffer (138 mM NaCl, 2.68 mM KCl, 0.49 mM MgCl₂, 8.9 mM Na₂HPO₄, 0.9 mM CaCl₂, 0.33 mM sodium pyruvate, 1.47 mM KH₂PO₄, 5.56 mM glucose, pH 7.4). Cells were then loaded for 0.5 to 1 h at 37 °C, 5% CO₂ with 50 or 20 μL well^-1 (96- and 384-well format, respectively) FLIPR V_m dye dissolved in the same buffer. After loading, cells were placed...
onto the stage of a FLIPR or FLIPR. The FLIPR protocol monitored baseline fluorescence for 15 sec, added 50 or 20 µL of 4× antagonist dilution (96- or 384-well, respectively), monitored fluorescence for 5 min, added 100 or 40 µL of 2× veratridine (96- or 384-well, respectively), and read the fluorescence for an additional 5 min. The final DMSO concentration was held constant at 0.8%. Fluorescence values were obtained using the “Max-Min” statistic of the FLIPR software from the time of compound addition to the end of the experiment.

Data analysis

The concentration of veratridine yielding 50% of its maximal effect (EC50 value) or the concentration of antagonist yielding 50% of its maximal inhibition (IC50 value) was calculated from theoretical curves generated using nonlinear regression curve fitting analysis in GraphPad Prism, version 3.02 (San Diego, CA). Linear regression analysis and corresponding statistical significance were carried out using GraphPad Prism 3.02.

RESULTS

Membrane potential (Vm) dye fluorescence responses to varying concentrations of veratridine were measured in rNav1.2-HEK cells in the 96-well format on a FLIPR plate reader (Fig. 2). Veratridine-induced increases in fluorescence were reproducible and concentration dependent. The average signal-to-background (S/B) ratio, as determined by fluorescence change (ΔF) after 25 µM veratridine divided by ΔF after buffer only, was 20.4 ± 1.2 (n = 16 plates). The average EC50 value for veratridine was 22.2 ± 3.2 µM (n = 3), consistent with previously reported values.

A variety of NaCh reference antagonists, as previously characterized by EP or the Na+ influx assay, were tested for their ability to inhibit the response to 25 µM veratridine. Inhibition was dependent on compound concentration, except for lidocaine, which exhibited no inhibitory activity up to 66 µM (Fig. 3). Rank order of potency for compounds that exhibited inhibition was as follows: TTX >> flunarizine > pimozide > lamotrigine > carbamazepine. Respectively, average IC50 values were 0.0042 ± 0.0006 µM (n = 16), 0.154 ± 0.024 µM (n = 16), 0.744 ± 0.155 µM (n = 8), 19.5 ± 4 µM (n = 8), and 144 ± 30.7 µM (n = 8), consistent with the literature. Lack of lidocaine inhibition at the concentrations tested also is consistent with a previously reported veratridine-based Vm assay but is inconsistent with both literature and in-house EP Ki values (~10 µM; data not shown).

To validate the FLIPR Vm dye assay with respect to traditional values derived by EP, antagonists active in the FLIPR assay were characterized by EP for state-dependent block of whole-cell currents in rNav1.2-HEK cells. In a representative experiment, very little block was observed with 3 µM of compounds G or E (Purdue Pharma L.P. proprietary compounds) at a negative holding potential of −110 mV (Fig. 4A; Table 1), suggesting low affinity of these compounds to the resting state of rNav1.2 channels. However, in a double pulse protocol where a depolarizing prepulse to −20 mV is used to drive all the channels into the inactivated state, the percent inhibition increased dramatically (Fig. 4B), suggesting higher affinity inhibition of inactivated channels as compared to resting block. Specific control and compound current traces, indicated by arrows in panels A and B, also reveal a greater degree of compound blocking activity in the inactivated state than in the resting state (Fig. 4C-D). Under inactivated state conditions, current amplitudes overall are appropriately lower. This is due to a slow inactivation process that occurs during the depolarizing prepulse that results in a lower number of channels available for opening.
State-Dependent Sodium Channel Membrane Potential Assay

FIG. 3. Representative fluorescence imaging plate reader (FLIPR) Vm traces and corresponding concentration-inhibition curves showing the effects of various NaCh blockers on the Vm response to veratridine. (A) A representative 96-well plate of rNav1.2-HEK cell responses to 25 µM veratridine in the presence of varying concentrations of the indicated antagonists is illustrated. The top halves of columns 1 and 12 are the positive control responses to 25 µM veratridine in the absence of antagonist. The bottom halves of columns 1 and 12 are the negative control wells demonstrating blocked responses to 25 µM veratridine in the presence of 2 µM tetrodotoxin (TTX). Columns 2 to 11 contain duplicates of varying concentrations of the 4 antagonists indicated. Due to large differences in potency, the highest concentrations in column 2 varied for each inhibitor as follows: flunarizine, 16.7 µM; lamotrigine, 500 µM; carbamazepine, 500 µM; and TTX, 1.7 µM. All antagonists were serially diluted in 3-fold increments to column 11. (B) Representative plate formatted similarly to that described in panel A, demonstrating antagonist inhibition of the response to veratridine. Again, the highest concentrations in column 2 varied for each inhibitor as follows: flunarizine, 16.7 µM; pimozide, 16.7 µM; lidocaine, 66 µM; TTX, 1.7 µM. (C) Representative antagonist concentration-inhibition curves for Vm dye responses to veratridine. Data points represent the mean ± SEM of 4 wells from 2 plates for flunarizine and TTX. Data points represent the mean ± SD of 2 wells from 1 plate for all other compounds. Curves are representative of 8 to 16 similar determinations.

Compounds such as these, with profiles of greater block under inactivated state conditions than in the resting state, are considered state-dependent inhibitors.

Validation with respect to EP was conducted for a set of 21 compounds representing several different structural classes, including known NaCh blockers,22,23 as well as compounds whose NaCh activity was previously uncharacterized.25,26 FLIPR IC50 values were compared to EP K, (inactivated state block) or EP K, (resting state block) values. Where available, FLIPR data were compared to published whole-cell voltage clamp Ki and K values. In some cases, literature data were not available; as such, whole-cell voltage clamp K, and K, values were directly measured. All values are presented in Table 1. The Vm dye assay IC50 values matched EP K, values within 0.1- to 1.5-fold. In contrast, Vm dye assay IC50 values less reliably matched EP K, values, with 10- to 760-fold discrepancies. These data were plotted as a linear regression analysis to assess the correlation between FLIPR Vm dye assay IC50 values and EP K, or K, values (Fig. 5). The analyses showed that FLIPR Vm dye data significantly correlated with EP K, values. Conversely, this comparison was not statistically significant when FLIPR values were compared to EP K, values.

Upon validation, configuration of the FLIPR Vm dye assay for compound screening was assessed in 96- and 384-well formats. Representative mock screening plates are shown in Figure 6. Mock plates received 0.8% DMSO in assay buffer (no compounds), followed by addition of 25 µM veratridine. Responses to veratridine were consistent throughout all positive control wells in 96- and 384-well formats. Inclusion of TTX (2 µM) blocked the responses completely in the negative control wells. Average Z' factors for 96- and 384-well plates were 0.51 ± 0.02 (n = 35) and 0.36 ± 0.05 (n = 6), respectively.

Using the FLIPR Vm dye 96-well formatted screening assay, we screened LOPAC to determine the proportion of known, biologically active molecules in this library that exhibit nonspecific activity at rNav1.2. The screening concentration was 1 µM. The average Z' factor was 0.49 ± 0.04 (n = 16), and the average S/B was 23.6 ± 1.2 (n = 16). Hit cutoff was set to 50% inhibition of the response to 25 µM veratridine. Of the 16 LOPAC plates, 99 active wells were identified and cherry-picked for single-point confirmation at 1 µM. Of these, 10 wells confirmed (0.8% confirmed hit rate). The purified, crystalline form of these 10 LOPAC hits was ordered from Sigma and retested in concentration-inhibition format. Nine of the 10 reconfirmed.

The identities and IC50 profiles for the confirmed hits from the LOPAC are shown in Table 2. One compound, flunarizine, is a reported NaCh inhibitor with submicromolar potency.18,27,28 The additional 8 compounds have other reported pharmacological activities, including dopamine receptor inhibition, serotonin receptor agonism or uptake inhibition, and diacylglycerol kinase inhibition. For these compounds, moderate- to high-potency NaCh inhibition is a previously uncharacterized activity. Nonetheless, NaCh inhibition obtained using the FLIPR Vm dye assay for these compounds was confirmed in the EP K, assay (Table 2). These data were analyzed by linear regression to assess the correlation between the FLIPR Vm assay IC50 values and EP K, values in this newly discovered sodium channel inhibitor data set. The correlation between the 2 assays for these LOPAC hits was significant, with r² = 0.76, p < 0.01. This level of correlation matches very closely the value achieved with the 21 known sodium channel blocker compound correlation described earlier.
Sodium channels are critically involved in the generation of action potentials in neurons, cardiac tissue, and other excitable cells.\(^2\) Deregulation of neuronal NaCh function can underlie diseases of the nervous system hyperexcitability such as chronic neuropathic pain, head trauma, epilepsy, and stroke.\(^2\) Similarly, deregulation of neuronal NaChs.\(^33-36\) In neurons, this secondary pharmacology mechanisms have been shown to promiscuously inhibit cardiac NaChs.\(^29,30\) Through the abnormal activation of these channels in the heart can lead to long QT syndrome,\(^29,30\) which increases the risk of fatal ventricular arrhythmias. Deregulation in skeletal muscle can lead to periodic paralysis.\(^31,32\) Compound therapeutics designed to pharmacologically target other mechanisms have been shown to promiscuously inhibit cardiac and neuronal NaChs.\(^33-36\) In neurons, this secondary pharmacology may underlie some additional therapeutic effects, such as the antihyperalgesic efficacy of some antidepressants.\(^36\) In the heart, it may underlie toxic side effects, such as cardiotoxicity, that is observed with some antidepressants and psychotropic agents.\(^33,35\) Thus, NaChs represent key targets for alleviating debilitating disease, as well as key mechanisms for deleterious side effects of some compound therapeutics.

Conventional methods for assaying NaCh activity include radiolabeled toxin-binding assays,\(^37,38\) radioative ion influx assays,\(^39,40\) EP patch-clamp techniques,\(^20,41\) and cellular toxicity assays.\(^42\) Radiolabeled toxin-binding assays involve measuring the compound inhibition of specific \(^{[3]}\text{H}\)batrachotoxinin A 20-alpha-benzoate, \(^{[3]}\text{H}\)saxitoxin, or other NaCh-selective radiolabeled toxin binding to NaChs. The advantage of these assays is amenabley to high throughput, especially if designed in the scintillation proximity assay (SPA) format. A drawback, however, is that these assays do not have a functional endpoint; thus, detection is restricted to compounds that interact directly, or allosterically, with the specific toxin-binding site being measured. In addition, these assays generate significant quantities of costly radioactive waste.

Radioactive ion influx assays involve measuring compound inhibition of \(^{[22]}\text{Na}^+\) or \(^{[14]}\text{C}\)guanidinium flux across the cell membrane.\(^39,40\) Sufficient S/B is achieved in the presence of a NaCh inactivation inhibitor, such as veratridine.\(^43\) The advantage of these assays is that they measure functional NaCh activity and thus are less limited by the compound mechanism of interaction. Assay throughput is significantly higher than traditional EP approaches. Nonetheless, multiple wash steps are required, necessitating a non-homogeneous assay format. In addition, they produce large quantities of radioactive waste. The incubation time is relatively long,
commonly 1 h, introducing the potential to measure interaction with processes downstream of the channel. And finally, they require the use of a chemical modifier of channel inactivation, introducing the risk of 1) missing compounds that interact with channel conformations that occur in the absence of the inactivation inhibitor and/or 2) detecting compounds that interfere directly with the chemical modifier and that would not otherwise affect NaCh activity under more natural activation conditions.

Electrophysiological patch-clamp assays measure the amount of current necessary to clamp the cell membrane at a given V_m after depolarization-induced channel activation. To characterize the compound mechanism of interaction or state dependence, many variations on the basic assay protocol are commonly used.

These include varying the frequency, length, and magnitude of the depolarizing stimulus; the presence or absence of prepulse depolarization; and the length and magnitude of prepulse depolarization. In this regard, EP offers the advantages of a functional, nonradioactive readout with exquisite time resolution and mechanistic detail. However, traditional EP has inherent limitations, including low throughput (1 cell assayed at a time), a requirement for highly skilled technical personnel with specific expertise, and specialized equipment incompatible with standard laboratory robotics. Although recent advances in higher throughput EP such as IonWorks or PatchXpress (both Molecular Devices Inc.) can theoretically reduce the magnitude of these limitations, these technologies are still relatively expensive, not yet integrated as standard...
screening laboratory equipment, and not yet empirically proven to deliver the same quality of information regarding compound affinity and mechanism of action as manual patch-clamp techniques can deliver.

Cellular toxicity assays involve measuring cell death after induction of intracellular Na⁺ ion accumulation in the presence of a NaCh inactivation inhibitor, such as veratridine, and a Na⁺/K⁺ ATPase inhibitor to prevent Na⁺ ion homeostasis. The advantage of these assays is that they are functional and high throughput. The disadvantages include a long reaction incubation time of about 24 h, the use of a chemical modifier of channel inactivation, and the use of a Na⁺ ion extrusion pump inhibitor. Such complexity in chemical modifications to the cellular system may introduce artifacts that are modified by the test compounds but that are not relevant to native channel physiology.

Thus, we have described and characterized in detail a fluorescence-based FLIPR V_{m} assay approach to measure NaCh activity. The assay can be performed in either 96- or 384-well assay formats; is homogeneous, fluorescence based, and kinetic; and uses relatively short reaction times of approximately 5 min. Screening factors were moderate to good in both microtiter formats, although the 96-well format was clearly better. Variability in positive and negative controls in the 384-well format led to a marginally satisfactory average Z' factor. Most likely, this could be improved with more assay optimization, such as better mixing upon agonist addition in the FLIPR; more extensive cell density optimization;

![FIG. 5.](image_url) Correlation of fluorescence imaging plate reader (FLIPR) V_{m} assay IC₅₀ values and patch-clamp Kᵢ/Kᵦ values. (A) Significant correlation is seen between the FLIPR IC₅₀ and patch-clamp Kᵢ values for a set of 21 compounds of varying chemotypes. (B) Lack of a significant correlation is seen when analyzing a 17-compound subset of those from panel A, chosen based on availability of values measured for both FLIPR IC₅₀ and patch-clamp Kᵢ values. Data points represent the mean IC₅₀, Kᵢ, or Kᵦ values presented for the compounds indicated in Table 1.

![FIG. 6.](image_url) Representative fluorescence imaging plate reader (FLIPR) trace responses to veratridine in 96- and 384-well screening mode format. (A) A representative 96-well plate of rNa, 1.2-HEK cell responses to 25 µM veratridine is illustrated. Rows E to H of columns 1 and 12 are negative control wells representing 2 µM tetrodotoxin (TTX) inhibition of the response to 25 µM veratridine. All other wells are the response to 25 µM veratridine in the absence of inhibitors. (B) A representative 384-well plate of rNa, 1.2-HEK cell responses to 25 µM veratridine is illustrated. Rows I to P of columns 1, 12, 13, and 24 are 2 µM TTX inhibition of the response to 25 µM veratridine. All other wells are the response to 25 µM veratridine in the absence of inhibitors. In both formats, the responses were consistent and reproducible across wells and across independent plates. The 96- and 384-well plates are representative of 35 and 6 plates, respectively.
and/or reisolation of a more robust rNa 1.2-HEK cell line with greater S/B. The assay reported appropriate pharmacological profiles as compared to EP-determined \( K_v \) values. Although a similar FLIPR \( V_m \) assay of NaChs has been reported previously,\(^1\) our data have substantially extended the meaning and characterization of the data generated by this type of assay. We have shown that over a chemically diverse set of small molecule compounds with potent \( K_v \) values, in addition to TTX, the FLIPR \( V_m \) data closely match that of EP. Moreover, the data significantly correlated with EP \( K_v \), but not EP \( K_r \), across a large dynamic range of compound potencies (ranging from single nanomolar to hundreds of micromolar \( K_i \) values), suggesting that the assay most accurately approximates open or inactivated state block of the channel but not resting state block.

Several issues with the FLIPR \( V_m \) assay remain outstanding and confer some limitations. Similar to the radiolabeled ion influx assays and the cellular toxicity assays, the FLIPR \( V_m \) assay relies on the use of veratridine, a chemical modifier of channel inactivation, and in this respect suffers the same drawbacks already discussed. Furthermore, veratridine modifies channel inactivation by slowing it down significantly and stabilizing the channel in the open state. Thus, theoretically, this assay by definition does not measure resting state block or \( K_r \), as we have observed through lack of correlation between FLIPR IC\(_{50}\) and EP \( K_r \) values. But more important, this assay also might not be measuring inactivated state block. Interestingly, however, the data do statistically correlate with inactivated state block (\( K_i \)). Therefore, it is possible that the assay measures open state block of the channel. This idea is supported by our observed lack of lidocaine inhibition at concentrations up to 66 \( \mu M \), which has also been previously reported in a similar assay\(^1\) (IC\(_{50}\) value: 190 \( \mu M \)). In contrast, the EP \( K_f \) for lidocaine is \(-10 \mu M \), and the EP \( K_r \) is > 100 \( \mu M \).\(^3\) Mechanistically, lidocaine has little to no affinity for the resting or open state of the NaCh. Its use-dependent inhibition is purely derived from binding to the inactivated, but not open, state.\(^6\) Comparison of our FLIPR \( V_m \) assay data to EP \( K_i \) values suggests that such a compound is relatively rare and that most use-dependent compounds bind with similar affinity to open and inactivated states but with substantially lower affinity to the resting state. A substantial number of compounds with this type of mechanistic profile have been confirmed using EP. Such compounds include bupivacaine, imipramine, carbamazepine, and diphenhydramine.\(^3,13\) Thus, based on this collective evidence, an assay that measures open state block theoretically should approximate EP \( K_v \) values relatively well. Nonetheless, under natural conditions, the open state of the NaCh is extremely transient (1-2 msec); most compounds are thought not to bind this rapidly. Therefore, this assay risks reporting information that is not highly relevant to a natural physiological state.

Alternatively, correlation between the FLIPR \( V_m \) assay with EP \( K_v \) values may be mechanistically related to the conformational state of the channel.\(^7\) If chemical activation of NaChs by veratridine results in a conformational state similar but not identical to a state induced by a depolarization protocol that drives the channel into the inactivated state, then compounds may exhibit similar affinities, as the receptor target is generally the same. Such a model could also account for an occasional outlier, such as lidocaine, which lacks inhibition in the FLIPR assay, because such compounds may only bind if the exact conformational state they require is strictly achieved. To fully delineate the exact theoretical model that underlies the FLIPR \( V_m \) assay, further investigation into the mechanistic basis for this assay is warranted.

In screening the LOPAC library, we have shown that a significant number of biologically active compounds that were designed to interact with other targets exhibit high- to moderate-potency NaCh inhibition. A similar result was seen for the set of proprietary Purdue compounds presented in Table 1, which were designed in the context of discovery programs unrelated to NaChs. These data demonstrate the relevance and importance of including a relatively easy and high-throughput functional assay of NaChs as a counterscreen assay for discovery programs aimed at identifying compounds active at other targets.

The FLIPR \( V_m \) assay, with its various caveats taken into account, does provide a convenient, high-throughput, and useful

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**Table 2.** \( V_m \) Assay IC\(_{50}\) and EP \( K_i \) Values of 9 Confirmed LOPAC Hits

<table>
<thead>
<tr>
<th>Compound</th>
<th>FLIPR IC(_{50}) (nM)</th>
<th>EP ( K_i ) (nM)</th>
<th>Primary Pharmacology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flunarizine</td>
<td>124 ± 8</td>
<td>58 ± 9</td>
<td>Ca(^{2+})/Na(^+) channel antagonist</td>
</tr>
<tr>
<td>Butaclamol</td>
<td>733 ± 194</td>
<td>1610 ± 282</td>
<td>Dopamine receptor antagonist</td>
</tr>
<tr>
<td>Chlorophenyl piperazine</td>
<td>1688 ± 202</td>
<td>2470 ± 619</td>
<td>5-HT, receptor agonist</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>1054 ± 72</td>
<td>384 ± 18</td>
<td>5-HT uptake inhibitor</td>
</tr>
<tr>
<td>R 59949</td>
<td>5343 ± 684</td>
<td>NA</td>
<td>Diallylglycerol kinase inhibitor II</td>
</tr>
<tr>
<td>s-Fluoxetine</td>
<td>1045 ± 266</td>
<td>1941 ± 232</td>
<td>5-HT uptake inhibitor</td>
</tr>
<tr>
<td>GBR-12935</td>
<td>257 ± 9</td>
<td>34 ± 4</td>
<td>Dopamine uptake inhibitor</td>
</tr>
<tr>
<td>ST-148 maleate</td>
<td>514 ± 39</td>
<td>573 ± 126</td>
<td>D2 dopamine receptor antagonist</td>
</tr>
<tr>
<td>RBI-257 maleate</td>
<td>714 ± 73</td>
<td>641 ± 87</td>
<td>D4 dopamine receptor ligand</td>
</tr>
</tbody>
</table>

\( \text{FLIPR IC}_{50,50} \) were determined in a 96-well format. Values represent mean ± SEM (\( n = 3 \)). FLIPR, fluorescence imaging plate reader; EP, electrophysiology; LOPAC, Library of Pharmacologically Active Compounds; NA, data not available.
method to screen for NaCh inhibitor compounds, with statistically significant correlation to use-dependent block. The assay can be used to prioritize compounds for potency at NaChs or for selectivity counterscreening against these channels. Subsequently, later-stage compounds of highest interest would still benefit from detailed mechanistic analysis by EP.

REFERENCES

State-Dependent Sodium Channel Membrane Potential Assay


Address reprint requests to:
Elfrida R. Benjamin
Purdue Pharma L.P.
6 Cedarbrook Drive
Cranbury, NJ 08512
E-mail: Elfrida.Benjamin@pharma.com