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High-Throughput Screening for Ion Channel Modulators

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ABSTRACT

Ion channels present a group of targets for major clinical indications, which have been difficult to address due to the lack of suitable rapid but biologically significant methodologies. To address the need for increased throughput in primary screening, the authors have set up a Beckman/Sagian core system to fully automate functional fluorescence-based assays that measure ion channel function. They apply voltage-sensitive fluorescent probes, and the activity of channels is monitored using Aurora's Voltage/Ion Probe Reader (VIPR). The system provides a platform for fully automated high-throughput screening as well as pharmacological characterization of ion channel modulators. The application of voltage-sensitive fluorescence dyes coupled with fluorescence resonance energy transfer is the basis of robust assays, which can be adapted to the study of a variety of ion channels to screen for both inhibitors and activators of voltage-gated and other ion channels.

INTRODUCTION

Ion channels regulate the movement of ions across biological membranes and play a crucial role in maintaining and modulating cellular function. Regulating ion channel function has proven to be a successful approach in drug therapy, and currently, approximately 15% of the world's 100 top-selling drugs are targeted at ion channels.[1] Therapeutic indications for these drugs include cardiac arrhythmias, hypertension, and a number of neurological disorders (for reviews, see Clare et al.,[2] Curran,[3] and Weinreich and Jentsch[4]). Efforts in genomics have identified a large number of previously unknown ion channels with possible new indications that, given good accessibility of this target class, predict a good potential for new drugs.

Despite this attractive setting, high-throughput screening for ion channel modulators has proven to be difficult and time-consuming, especially for voltage-gated ion channels. Patch clamping remains the "gold standard," and although a number of companies are attempting this technique (e.g., Sophion Biosciences, Ballerup, DK; CeNeS, Cambridge, UK), electrophysiological techniques are still restricted to screening a relatively low number of samples per day. New technologies in this area include the direct coupling of ion channels to semiconductors on silicon chips but are at present only used in academic research.[5] The initially developed radioligand binding assays require previous knowledge of binding sites, and because other sites can be allosterically coupled, potentially valuable leads are missed.[6]

Functional assays are ideally suited to find modulators of ion channel function, but the radioactive efflux assays initially developed require high amounts of radiotracer to load the cells. The conversion of the ^{86}Rubidium-efflux assay, routinely applied to study potassium channels to a nonradioactive format using atomic absorption spectoscropy as readout,[7] has removed the need for radioactive tracers but is limited in throughput. A generic approach to measure ion channel function is the use of microphysiometry,[8] in which the change of the extracellular pH in response to any change in cells can be followed. Although readers are commercially available (Molecular Devices) and the assay is applicable to a broad range of targets, the throughput is limited due to the length of measurements and the large number of cells required.

The advance of fluorescent assays has significantly enhanced the portfolio of suitable assay technologies. Activity of calcium channels can be measured using calcium-sensitive dyes—for example, Fluo-3 on systems such as the Fluorescent Imaging Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, CA).[9] Indirect measuring of membrane potential can be achieved with the FLIPR using oxonol dyes from the DiBac series (bis-barbituric acid oxonols), which show a change in distribution on changes in membrane potential that can be followed by whole-cell imag-

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ing.[10] The potential for further miniaturization of this technology has been shown using a Lab-on-a-chip approach to measure membrane potential.[11] However, as the translocation of these dyes in response to changes in membrane potential is slow and many ion channels are rapidly inactivated or desensitized after opening, it may be necessary to keep channels in an open state by pharmacological modification.

The development of improved membrane-potential sensors based on the fluorescent resonance energy transfer between voltage-sensing oxonol dyes and voltage-independent donor fluorophors,[12,13] as well as the introduction of a kinetic reader to follow these events in real time (Aurora Biosciences, San Diego, CA), has provided the basis of a new set of generic assays for ion channel function providing increased throughput (for review, see Gonzalez et al.[14]). Building on this technology, we have integrated the Voltage/Ion Probe Reader (VIPR) (Aurora Biosciences) into a Beckman/Sagian core system (Beckman/Sagian, Indianapolis, IN) to provide a platform for automated screening for ion channel modulators. This is a first step to meet the increased throughput demand for ion channel screening at Pfizer UK.

MATERIALS AND METHODS

VIPR assay

Cells were harvested using cell dissociation buffer (Sigma) and plated at 5 to 7 × 10^4 cells/well into black, clear-bottom 96-well plates (Corning CoStar, Corning, NY) using a MultiDrop (Labsystems, Helsinki, Finland) 24 h prior to the experiment, and then they were loaded into the CO_2 incubator on the linear track. Low (4.5 mM) K^+ buffer was used for incubations, and high (164.5 mM) K^+buffer was used as a stimulus for depolarization. Stock concentrations of the dyes, CC2-DMPE and $DiSBAC2_{(2)}$, were prepared in neat dimethyl sulfoxide (DMSO) and stored at –20 °C. Working concentrations of the FRET dye pair are dependent on the assay but typically are in the region of 5 µM (with 0.1 mg/ml Pluronic F127) for CC2-DMPE and 5 µM (with 0.5 mM ESS-CY4) for $DiSBAC2_{(2)}$. In addition, the incubation times stated are typical but have to be optimized for every cell line. Compounds were prepared in white 96-well plates (Matrix, Hudson, NH), and the final compound concentration in the assay was 10 µM and 0.25% DMSO.

For dye loading in high-throughput screening (HTS), cells were washed twice with 250 µl low K^+ buffer; CC2-DMPE was added with the MultiDrop, and plates were incubated for 45 min. Excess dye was removed by washing the cells twice with 250 µl low K^+ buffer followed by addition of compounds (in low K^+ buffer) and $DiSBAC2_{(2)}$/ESS-Cy4 by a Multimek 96-well pipettor. After a 45-min incubation, the cell plates were loaded onto the VIPR, which was set to column-reading mode. Every column was initially read for 5 s to establish the baseline before high K^+ buffer was added to the cells (1:1 v/v to lead to a final $[K^+]$ of 85 mM) to evoke depolarization, and the signal was monitored for another 20 s at 1 Hz. Within-HTS values for $R_{initial}$ and R_{final} were derived by averaging the VIPR response at preset time intervals (typically for 5 s) before and after depolarization.

Data analysis

Assay performance was judged by calculating the Z' value,[15] using the high and low controls present within each 96-well plate. Screening data were analyzed using Pfizer proprietary software, and IC_{50} experiments were fitted with a nonlinear 4-parameter fitting routine using Grafit 4.01 (Erithacus Software, Horley, UK).

Patch clamping

Cells (2 × 10^5 per dish) were plated out onto glass coverslips (3.5 mm diameter) and incubated at 37 °C in a 5% CO_2 incubator for 1 h before use. Whole-cell membrane currents were recorded in voltage-clamp mode using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA) and low-pass filter (8-pole Bessel, –80 dB response at 1 kHz). To reduce errors in membrane potential with large currents, we employed series resistance compensation, usually up to 80% (τ_c = 100 µs). Data acquisition and voltage protocols were controlled using pCLAMP software (version 8.0, Axon Instruments) and stored on an IBM-compatible computer. Microelectrodes had resistances of 1 to 3 MΩ when filled with pipette solution. Currents were evoked by a repetitive 500 ms test pulse applied from a resting potential of –60mV to +40mV at 20-s intervals. At least 10 control readings were obtained prior to the application of a test compound. The response to a known inhibitor was determined to define the maximally inhibitable current of the channel under investigation. For data analysis, peak currents were measured from each test pulse. Data for the same compound at different concentrations (on separate cells) were fitted to a sigmoidal curve to determine an IC_{50} concentration.

RESULTS AND DISCUSSION

FRET-based measurement of ion channel function

Figure 1 illustrates the theory of the FRET-based assay to measure ion channel activity. The general assay principle developed by Aurora Bioscience (San Diego, CA) requires the cells to be preloaded with 2 fluorescent dyes.[14] The 1st dye, a coumarin-linked phospholipid (CC2-DMPE), inserts into the outer leaflet of the cell membrane, remains stationary, and acts as an energy donor. The 2nd dye, which is the real voltage sensor, is a negatively charged oxonol dye—$DiSBAC2_{(2)}$—that changes its distribution across the membrane according to the membrane potential. In the example (Fig. 1a) with a negative resting membrane potential, the dye is in close proximity to the donor dye, and energy transfer can take place. A change in the membrane potential with depolarization redistributes the oxonol dye, and due to the increased distance between donor and acceptor, energy transfer is less efficient (Fig. 1b). These changes are followed in real time by measuring the fluorescence at 460 nm (blue) and 570 nm (red) simultaneously; the ratio of signals from both channels is calculated and plotted over time (Fig. 1c).

As the VIPR is measuring the changes in fluorescence over time, the initial data output is a fluorescence intensity plot over time for all 96 wells. These raw data are background subtracted

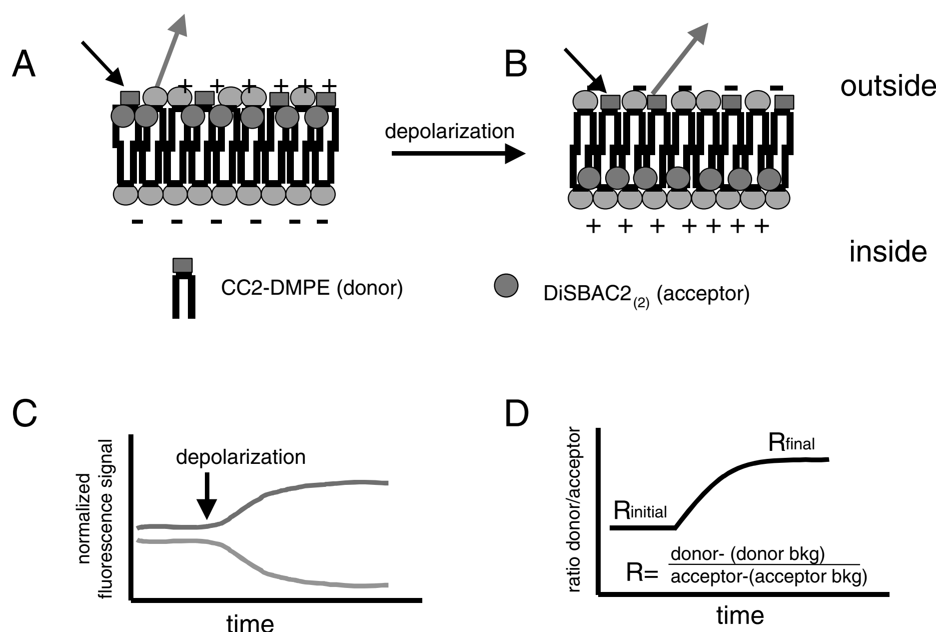

FIG. 1. Theory of FRET-based detection of ion channel activity and data analysis. (**A**) Negative membrane potential. Donor and acceptor are in close proximity, and energy transfer takes place. (**B**) Redistribution of the acceptor and change in the efficiency of energy transfer after depolarization. (**C**) Fluorescence signal measured in the red and blue channel during depolarization. (**D**) Transformation of kinetic data into single-point ratio values for further analysis.

to account for nonspecific fluorescence. The background fluorescence was measured in a plate without cells, and background plates were interspersed into the screening run (2 plates per robotic run, i.e., at a ratio of 1:30-1:50) to account for potential changes in signals over time. The average signal from all wells in one row was used to background subtract the signal for the corresponding row in the assay plate. Using the initial ratio of the 2 emission wavelengths ($R_{initial}$, before depolarization) and the ratio at a preset time after depolarization (R_{final}), the data were normalized ($R_{final}/R_{initial}$), and a single value for each well was derived from the kinetic data (Fig. 1d). The calculated single ratio values were further transformed into percent inhibition or activation relative to the high and low controls of each plate. Although the assay window (i.e., the difference between maximum and minimum signal) is relatively small in VIPR assays when compared to other assay types, the low standard deviations achieved provide acceptable statistical quality. In contrast to assays relying solely on fluorescence intensity measurements, variability in cell number and differences in dye uptake from plate to plate have less influence on assay performance as a ratio of intensities is used for analysis. Figure 2 illustrates primary dose-response data as a readout from the VIPR and the direct transformation of these background-corrected ratio values into a concentration response curve.

Assay development

In addition to pharmacological characterization of compounds, VIPR can be used for high-throughput screening. Assay development and pharmacological characterization of the cell lines used in the HTS are performed on a stand-alone version of the VIPR using similar equipment as present on the linear track system. This setup allows the rapid conversion of the assay into a format that is compatible with full automation of the assay procedure. Initial experiments for assay development focus on the optimal dye concentrations, loading times, and cell density to maximize the assay window, and the DMSO tolerance of the cells is established (depending on the assay, up to 2% DMSO is tolerated [data not shown]).

For pharmacological characterization of the cell lines and assay, compounds known to affect the channel under investigation are tested and a rank order of potency established. Figure 3 shows a correlation between IC_{50} values derived from patch clamping and VIPR analysis. For the compounds tested in this example, both methods generated IC_{50} values in the same order of magnitude. Recent reports, showing large differences in IC_{50} values as well as the minimal detection doses for a set of standard compounds in different assay formats for the hERG potassium channel, have again highlighted the importance of this part of the assay validation.[16]

Another aspect of assay development is the reproducibility of results from experiment to experiment, not only for the high-throughput screening but also for further characterization of compounds and to establish SAR within lead optimization programs. In cell-based assays, differences in cell viability and seeding density can influence both the screening results and follow-on hit characterization. As exemplified in Figure 4, a standard compound used for assay validation of the VIPR assay shows good interexperimental reproducibility. Over a number of experiments, we derived an average IC_{50} of 96 nM (standard deviation ± 34 nM; $n = 17$); importantly, the IC_{50} value was within the preset quality limit of 3-fold variation in 16 out of the 17 experiments underlining the high quality of this assay.

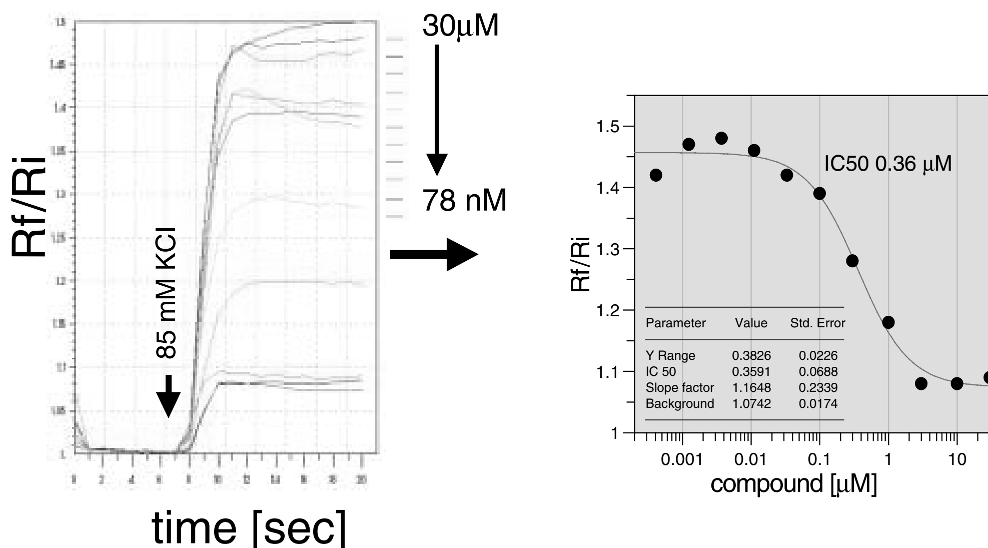

FIG. 2. Dose-response measurements on the Voltage/Ion Probe Reader (VIPR). Pharmacological profiling of compounds or hits from the high-throughput screening using the VIPR technology. In this example, the ratio values are directly translated into dose-response curves using a 4-parameter fitting routine.

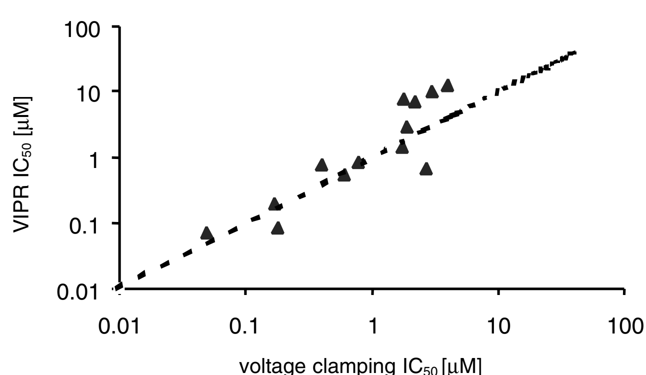

FIG. 3. Correlation of Voltage/Ion Probe Reader (VIPR) and patch clamping–derived IC_{50} values. IC_{50} values derived from electrophysiological experiments were plotted against the corresponding values from VIPR assays. The dotted line highlights the ideal correlation (i.e., the perfect match of VIPR and the patch clamp–derived IC_{50} value). The R^2 value for this set of compounds was 0.8. For the compounds tested, the rank order of potency was maintained in both assays.

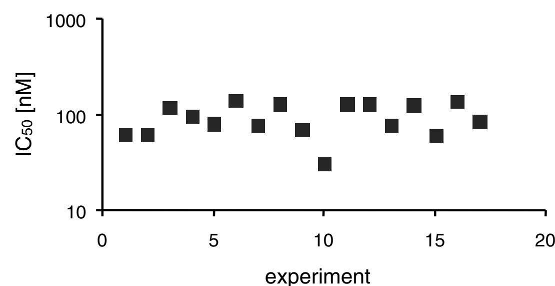

FIG. 4. Reproducibility of Voltage/Ion Probe Reader (VIPR)–based IC_{50} determination. IC_{50}s were determined on 17 separate days of assay development, including 2 batches of cells over a period of 2 months.

The results of these experiments demonstrate that the VIPR assay developed for this target had sufficient sensitivity to identify ion channel modulators in high-throughput screening as well as the ability to rank compounds by their potency for the follow-up of hits derived from the screening campaign.

System layout

The Beckman/Sagian linear track used for high-throughput screening is dedicated to run ion channel assays, and a minimal set of equipment has been integrated, with the VIPR being the only detection system. Apart from the VIPR, all components of the system are Beckman "core components" simplifying the initial design and building of the linear track system. For compound plate and cell plate storage, a hotel and CO_2 incubator with 130-plate capacity are incorporated. A Multimek 96 tip pipettor is used for compound handling; dyes are added with a MultiDrop 8-channel dispenser. A washer (Biotek El_x405), de-lidding station, and barcode reader complete this setup. The first generation of VIPR can handle 96-well plates, and a column of 8 wells is read simultaneously. Liquid handling in the form of a Hamilton ML2200 8-channel dispenser is integrated into the VIPR, and this is used to add the stimulus to the wells after an initial reading has established the baseline of the signal. Although a very simple, theoretically robust layout, the system had initial reliability problems. Most of these problems could be traced to the de-lidding station and were solved in conjunction with the manufacturer. With respect to assay prosecution, the nonhomogeneous nature of the VIPR assay technique requires washing steps within the dye-loading procedure and is the cause for some variation in the assay performance. Blocking of washer tips, as well as the residual volume left in the wells after washing, is a common occurrence and cannot be easily addressed. Careful quality control and maintenance of the washer are the only solutions to this

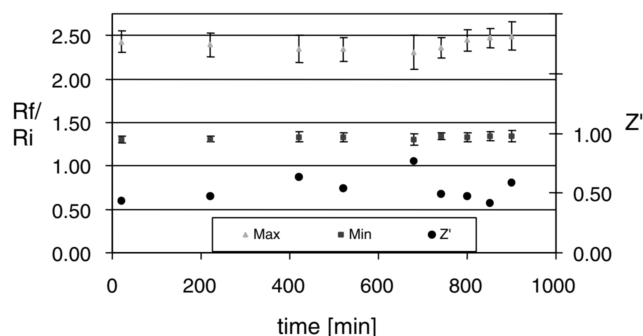

FIG. 5. Assay stability over time. Shown are maximum and minimum values as well as Z' prime values from quadrants of plates with stimulated or unstimulated cell from a 15-h robotic run.

problem, as attempts to avoid the washing of cells and simplify the dye-loading procedure have not been successful for the targets within our portfolio.

During assay development and screening, the use of HEPA filters and the precleaning of plates with compressed air have greatly reduced dust-related fluorescent emissions, particularly in the "blue channel" at 460 nm, that interfere with the VIPR readout. As company safety guidelines required a complete enclosure of the linear track robot, the environmental control provided by the enclosure reduced the problem of dust in automated high-throughput screening.

High-throughput screening

When refining an assay into a high-throughput format, several quality checks are made to assess the suitability of the assay for automation. Within our screening group, there is a standardized set of experiments performed before screening starts. These include tests to establish the stability of reagents and signal on the robot for the duration of robotic runs, as well as the inclusion of standards as IC_{50} curves on separate days to validate the assay in automation mode. Figure 5 shows the analysis of quality control plates for a 15-h robotic run. Maximum and minimum values and the resulting assay window are stable, and the calculation of Z' values from quadrants of the plates (stimulated and unstimulated) showed no time-dependent deterioration in assay performance. After these tests, a subset of compounds randomly selected from the compound collection is used to assess assay performance under screening conditions in at least 2 independent experiments (data not shown).

The analysis of exemplified raw screening data in Figure 6a shows a stable baseline and clearly defined active compounds. The maximum controls are reliably differentiated and a stable assay window maintained.

The quality of automated assays is routinely judged by statistical measures such as the Z' factor.[15] This calculation takes the assay window (i.e., the distance between the mean of minimum and maximum signals as well as the error on the mean of these values) into account. Figure 6b shows a typical example of the screening data obtained. Plotted are the Z' factor against the plate number for 4 subsequent robotic runs with a total of 270 plates. Z'

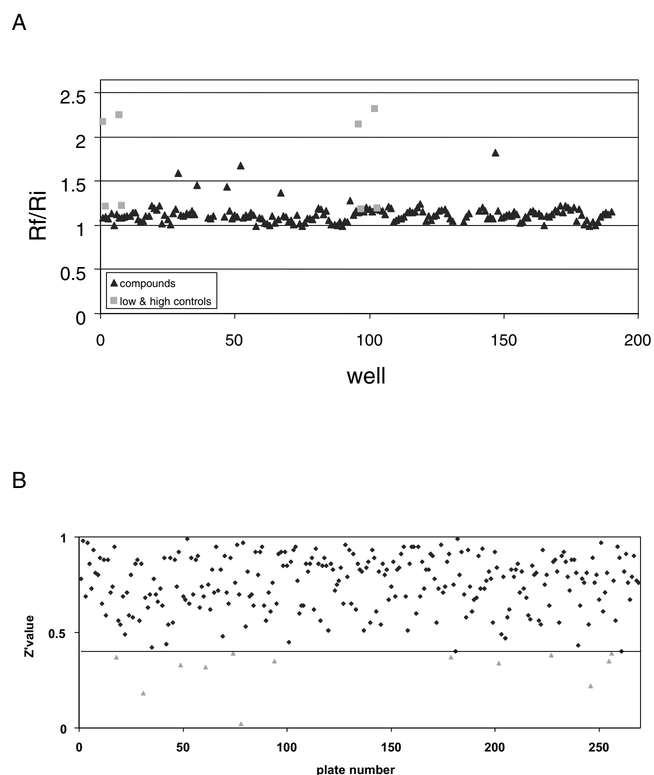

FIG. 6. Analysis of screening data. (**A**) Exemplified raw data from Voltage/Ion Probe Reader (VIPR)–based high-throughput screening. Raw ratio data from assay plates highlighted are the values for the high and low controls from the corresponding plate. (**B**) Analysis of Z' prime values derived from primary screening on the automated VIPR system. Plotted are the Z' prime values calculated from the high and low controls on each plate of successive HTS runs. Highlighted are all plates that fall short of the 0.4 quality criteria, respectively.

factors of > 0.4 are deemed acceptable for this type of cell-based assay within our assay development and HTS groups. On this basis, > 90% of plates screened can be used for further analysis, with a repeat rate of less than 10%, which is in the same range as found with biochemical assays in our experience.

An analysis of the frequency distribution of responses after screening > 240,000 compounds (Fig. 7) against one of our ion channel targets showed that a normal distribution centered on a response rate of 2%, which is in agreement with the prediction that most compounds should be inactive. In the HTS shown in Figure 7, the cutoff value for selecting "active" compounds was set as low as 20% activity. This guaranteed the assay to be as inclusive as possible of potential ion channel modulators while producing a number of false positives, as indicated by the confirmation rate, which is a direct consequence of the low cutoff close to the noise produced within the assay. This is acceptable as the VIPR is used as a filter to reduce the number of compounds for characterization in more complex, information-rich secondary assays. From 246,215 compounds screened in this example, 3573 compounds (1.45%) were active in the primary screening

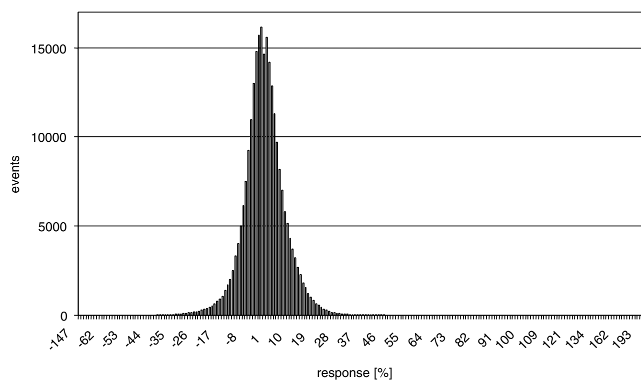

FIG. 7. Frequency distribution of inhibition values. Plotted is the frequency distribution for all inhibition values obtained in the primary high-throughput screening.

and 257 (0.1%) were confirmed as active in a 2nd round of screening (primary screening and confirmation on VIPR). After chemistry assessment, compounds were selected for further characterization.

CONCLUSIONS

The integration of the VIPR into a Beckman/Sagian linear track robot has allowed the full automation of screening for ion channel modulators and provided an increase in throughput to up to 6000 to 8000 compounds per day compared to the magnitude lower throughput of patch clamping and manual rubidium uptake assays previously applied. This is a robust system that allows for functional HTS of ion channel targets. Even with this increase in throughput, it takes considerable time to screen the whole compound collection and necessitate continued support from the groups providing cells. The relatively large assay volumes in 96-well plates translate into large amounts of the dyes, which constitute the most expensive item in this setup. The introduction of VIPRII, which is capable of handling 384-well plates with an accompanying reduction in cell number and assay volume, enables increased throughput and decreased cost per data point. Thus, at the moment, we consider VIPR the only platform adaptable for a wide range of ion channels while guaranteeing the required throughput, although we look forward to evaluation of the automated patch clamp in the near future.

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