Eur Biophys J (2009) 38:273–278
DOI 10.1007/s00249-008-0356-2

REVIEW

High throughput functional screening of an ion channel library for drug safety and efficacy

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Received: 22 May 2008 / Revised: 17 June 2008 / Accepted: 17 June 2008 / Published online: 2 August 2008

Abstract Development of a large, representative library of ion channel-expressing cell lines is described. Validation on a full range of automated patch clamp and fluorescence illumination platforms is ongoing. Library "books" can be mixed and matched into channel panels according to tissue, therapeutic area and ion channel family. Unexpected results using a cardiac channel panel show that this panel may serve as a biomarker for cardiac risk assessment.

Keywords Ion channel library · Automated patch clamp · Automated fluorescence · High throughput screening · Cardiac channel panel™

Introduction

Ion channels are primary targets for therapeutic areas such as hypertension, cardiac arrhythmias, pain, epilepsy, anxiety, insomnia, stroke, inflammation, diabetes, incontinence, irritable bowel syndrome, asthma and chronic obstructive pulmonary airway disease. Ion channels are secondary targets for safety and toxicology studies including assessment of QT risk, syncope, convulsions, nervousness, weakness, ataxia, dizziness, diarrhea, constipation, and dyspnea. It is not surprising that ion channels are important targets for both therapeutics and side effects. Efficacy and safety studies of ion channels have been hampered because rapid, cost-effective, accurate functional screens of a broad range of ion channels have not been available. These obstacles have now been removed with the development of a screening platform to interrogate a representative library of ion channel-expressing cell lines using automated electrophysiological instruments. Automated patch clamp and automated fluorescence measurement of membrane potential, intracellular calcium and potassium flux are powerful methods for analyzing ion channel functions. In this paper, we describe development and validation of a large representative ion channel library. We use the hERG "book" as an example of the process. Members of the library can be arranged into channel panels according to tissue, therapeutic area or ion channel family. We give examples in which unexpected mechanisms of efficacy or safety were demonstrated by screening a cardiac channel panel assembled from the library.

EBSA Satellite Meeting: Ion channels, Leeds, July 2007.

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Methods

Development of the ion channel library

Construction of ion channel cell lines proceeds in five phases. Phase 1 is cloning and sequencing of the channel cDNAs required to create the cell lines and is done by RT-PCR (reverse transcriptase-PCR) from human RNAs. The endpoint of this phase is preparation of linearized plasmid DNA for transfection. Phase 2 involves transfection of mammalian cell lines with cDNAs, phase 3 sorting, and phase 4 selection of individual subclones expressing the ion channel(s) of interest. Phase 5 includes validation and optimization of individual subclones using both manual and automated patch-clamp electrophysiology and automated fluorescence measurement of membrane potential, intracellular calcium or potassium. The validation of each cell line includes Western blot analysis and an electrophysiological profile on a representative set of automated instruments

presently used for library screens, i.e., PatchXpress®, IonWorks® Quattro™, QPatch, and FLIPR$^{Tetra®}$. Optimization is an ongoing process to maximize efficiency of the screening process for each cell line and each instrument.

Phase 1: cloning of ion channel cDNAs

Human RNAs purchased from commercial sources serve as the template for RT-PCR for ion channel cDNAs. Sequencing of full-length cDNAs prior to transfection ensures a match between our clones and the appropriate human GenBank sequences.

Phases 2–4: transfection and isolation of mammalian cell lines overexpressing ion channels

The parent cell lines for transfection were HEK293 and CHO cells. High transfection efficiencies were achieved with nucleofection kits from Amaxa (Lodish et al. 2000). The ion channel cDNA(s) were co-transfected with a cDNA encoding GFP (Lodish et al. 2000). We used the FACSAria™ flow cytometer in a two step process to select clones that were stably expressing ion channels. Following transfection, a population of green fluorescent cells was collected on the cell sorter. After growth for 1–2 weeks in selection antibiotic(s) to enrich for stably transfected cells, individual cells were sorted on the FACSAria™ and collected into single wells of 96-well plates. Growth and scale-up of clones derived from individual cells was followed by functional clone selection on a 384-well IonWorks® Quattro™.

Phase 5: validation of ion channel library clones

Each cell line validation consists of the cDNA sequence corresponding to the appropriate GenBank accession number, overexpressed protein shown by Western blot analysis and electrophysiology characterized by the current–voltage relationship behavior and pharmacology. We use hERG as an example of how validation is performed on each ion channel-expressing cell line.

Solutions

The physiological solution (HB-PS) used for whole cell recordings was (composition in mM): NaC1, 137; KCI, 4; $CaC1_2$, 1.8; $MgCl_2$, 1; HEPES, 10; Glucose, 10; pH adjusted to 7.4 with NaOH. Pipette solution for whole cell recordings was (composition in mM): potassium aspartate, 130; $MgC1_2$, 5; EGTA, 5; ATP, 4; HEPES, 10; pH adjusted to 7.2 with KOH. Pipette solution was prepared in batches, aliquoted, stored frozen, and a fresh aliquot thawed each day.

Validation methods

Manual patch clamp

Cells in dishes were transferred to the recording chamber and superfused with HB-PS solution. Patch pipettes were made from glass capillary tubing using a P-97 micropipette puller (Sutter Instruments, CA). A commercial patch clamp amplifier was used for whole cell recordings. Before digitization, current records were low-pass filtered at one-fifth of the sampling frequency.

Acceptance criteria

1) Initial seal resistance greater than 1 GΩ.
2) Access resistance ≤5 MΩ.
3) Stable leakage current <100 pA at −80 mV HP or less than 10% of the amplitude of the peak test pulse current.
4) Voltage error (test pulse current amplitude x access resistance) ≤5 mV.
5) Normal test pulse current waveform (hERG peak tail current amplitude > prepulse current amplitude).
6) Rundown of test pulse amplitude <2% per minute.

Automated patch clamp

To collect cells from the culture dishes, cells were washed twice with Hank's Balanced Salt Solution, treated with trypsin and resuspended in the culture media (1–1.5 × 10^6 cells in 20 ml). For recovery after treatment with trypsin, cells in suspension were stored 1–3 h in a tissue culture incubator at 37°C in a humidified 95% air, 5% CO_2 atmosphere. For use in the PatchXpress® system, the cells were washed one time with HB-PS and re-suspended in 150 µl of HB-PS.

Acceptance criteria

1) Membrane resistance (R_m) ≥200 MΩ.
2) Access resistance (R_a) ≤8 MΩ (after 60% compensation).
3) Peak hERG tail current ≥200 pA.
4) Leak current ≤25% of the peak current.
5) Run-down of peak current ≤2.5% per min.

hERG concentration-response using manual patch clamp

Three to six concentrations at or around the IC_{50} determined by automated patch clamp were applied to cells expressing hERG. Test article application was limited to the time necessary to reach steady-state block, but no longer than 12 min. Each concentration had an $n \geq 2$ where n = number of observations. Onset and steady-state block of hERG current due to test article was measured using a pulse pattern with fixed amplitudes (conditioning prepulse, + 40 mV for 2 s; test

pulse, −40 mV for 2 s) repeated at 10 s intervals, from a holding potential of −80 mV. Peak tail current were measured during the step to −40 mV.

hERG concentration-response using automated patch clamp

At least four concentrations of each test article were applied to cells expressing hERG. Test article application was 12 min. Each concentration had an $n \geq 3$ where $n \geq$ number of observations. Onset and steady-state block of hERG current due to test article were measured using a pulse pattern with fixed amplitudes (conditioning prepulse, +40 mV for 2 s; test pulse, −40 mV for 2 s) repeated at 10-s intervals, from a holding potential of −80 mV. Peak tail current was measured during the step to −40 mV.

Data analysis

Data were stored on the ChanTest computer network (and backed-up nightly) for off-line analysis. Data acquisition and analyses were performed using the suite of pCLAMP (ver. 82 and 9.0) programs (Axon Instruments, CA). Steady state is defined by the limiting constant rate of change with time and was accepted as <1% change for three pulses. The steady state before and after test article application was used to calculate the percentage of current inhibited at each concentration. Concentration-response data were fit to an equation of the following form: $\%\text{Block} = \{1 - 1/\{1 + ([\text{Test}]/\text{IC}_{50})^{N}\}\} \times 100$ where [Test] is the concentration of test article, IC_{50} is the concentration of test article producing half-maximal inhibition, N is the Hill coefficient, and % Block is the percentage of hERG current inhibited at each concentration of test article. Nonlinear least squares fits were made to a cooperative binding model and solved with the Solver add-in for Excel 2000 (Microsoft, WA). The IC_{50} was calculated assuming a cooperative binding relationship between test article and channel.

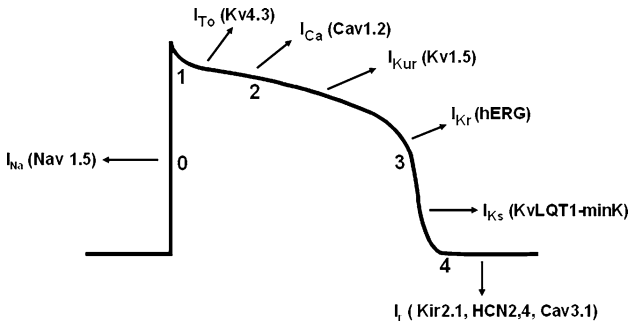

Fig. 1 Cardiac action potential. A schematic of the cardiac action potential labeled with the names of the contributing ion currents and channels

Development of a cardiac channel panel for profiling compound effects on repolarization

The major current determinants of the cardiac action potential and its duration are shown in Fig. 1.

The human cardiac channel panel consists of hERG, KvLQT1/mink, Kv4.3, Kv1.5, Kir2.1, Kir2.1/SUR2A, Nav1.5, Cav1.2, Cav3.1, HCN4 and HCN2. Each member of this panel has been validated for screening using manual and automated patch clamp by procedures similar to those described for hERG (Table 1). IC_{50} s were measured at ChanTest.

Table 1 Human cardiac channel panel

Class	Channel	Function	Positive control	*FASTPatch*® IC_{50} (μM)	Manual patch IC_{50} (μM)
Voltage-gated sodium	Nav1.5	I_{Na}, action potential initiation and conduction	Lidocaine	289	296
Voltage-gated potassium	hERG1	I_{Kr}, action potential phase 3 repolarization	E-4031	0.015	0.019
	KvLQT1/mink	I_{Ks}, action potential phase 3 repolarization	Chromanol 293	13.4	10.7
	Kv4.3	I_{To}, action potential phase 1 reloparization	Flecainide	41	35
	Kv1.5	I_{Kur}, atrial repolarization	4-Aminopyridine	281	264
Inward-rectifier potassium	Kir2.1	Resting potential, action potential phase 4 repolarization	$BaCl_2$	3	2.7
	Kir3.1/Kir3.4	Muscarinic receptor-coupled potassium channel responsible for vagal control of heart rate	Tertiapin-Q	0.017	0.004
Hyperpolarization-gated	HCN4	I_h, hyperolarization-activated S-A pacemaker potential	Zatebradine	1.4	1.96
Voltage-gated calcium	Cav1.2/α_2-δ,β_2 (L)	$I_{ca,L}$, action potential phase 2 depolarization	Nifedipine	0.01	0.02
	Cav3.2 (T)	$I_{ca,T}$, S-A pacemaking, atrial conduction	$NiCl_2$	3.7	2

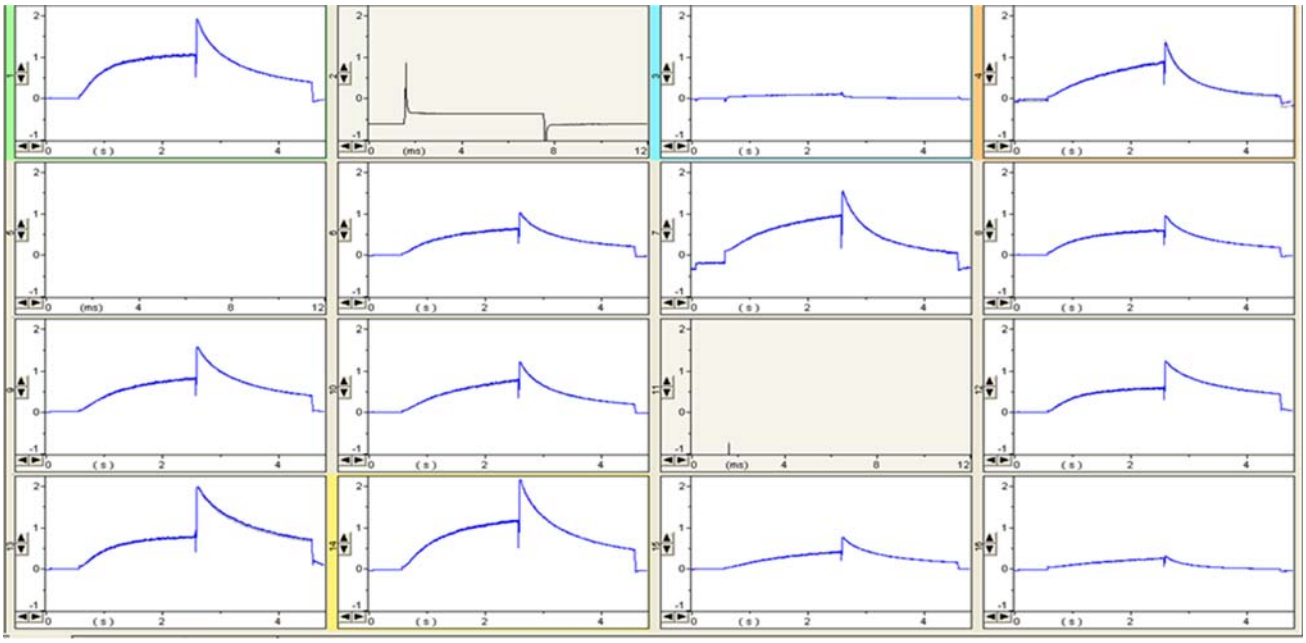

Fig. 2 Screen shot of hERG currents from a 16-well PatchXpress® chip. hERG currents in the 16 wells of PatchXpress® using the voltage protocol described in Sect. "Methods". 11/16 wells have acceptable hERG currents by the acceptance criteria that were listed. Time in seconds, current in nA

Results and discussion

Validation of hERG for automated patch clamping

Figure 2 is a screen shot of hERG currents recorded from a *FASTPatch*® seal chip. The cDNA sequence was identical to GenBank accession #AF363636. Western blot analysis verified hERG overexpression in the cell line. Functional validation showed the characteristic biophysical properties of hERG, namely rapid onset of voltage-dependent activation and its rapid removal and slow deactivation during the tail currents.

The conventional manual patch clamp (MPC) method is considered the "gold standard" for measurement of hERG potassium channel inhibition (Kirsch et al., 2004). Figure 3 is a comparison between manual and automated patch clamp hERG currents and E-4031 block. Table 2 compares the IC_{50} values between manual and automated patch clamp methods for a range of drugs known to block hERG.

The cardiac channel panel: a biomarker for cardiac risk assessment

hERG achieved prominence in cardiac risk assessment as a result of the adverse reports of sudden cardiac death associated with the second generation, non-sedating H1 antihistamine Seldane (terfenadine) (Brown and Rampe 2000). The sedative effect of first generation antihistamines was responsible for a number of dangerous accidents and Seldane's safety from sedation, as well as its efficacy, propelled the drug into blockbuster status. ADRs were infrequent: there were ~83 reported for ~200 million patients and there were five cases of Torsades des Pointes (TdP) per million patient months of treatment. At 60 mg b.i.d., QTc was increased a mere 6 ms (Morganroth et al. 1993). Nevertheless, the drug's widespread usage, the link with concomitant CYP3A4 antibiotic substrate and the emergence of safer, second-generation antihistamines lead to its withdrawal from the market. At about the same time, Roy et al. 1996, using molecular genetics and electrophysiology, showed that hERG was the molecular target of terfenadine. hERG block was the only robust, preclinical signal for the delayed depolarization responsible for QT prolongation and TdP and led to the ascendance of the hERG assay in preclinical cardiac risk assessment.

However, a hERG signal does not necessitate delayed repolarization and an APD or QT signal does not necessitate TdP (Table 3). Both types of discordance, hERG block without repolarization (Type 1) and repolarization delay without hERG block (Type 2) are readily understood when the cardiac channel panel is used as a biomarker for preclinical cardiac risk assessment.

The cardiac channel panel in Table 3 shows that E-4031 is a pure hERG blocker. The effects of E-4031 are concordant; the drug delays cardiac repolarization and produces TdP. The table also shows the classical Type 1 discordance of verapamil due to approximately block of both hERG and the L-type calcium channel.

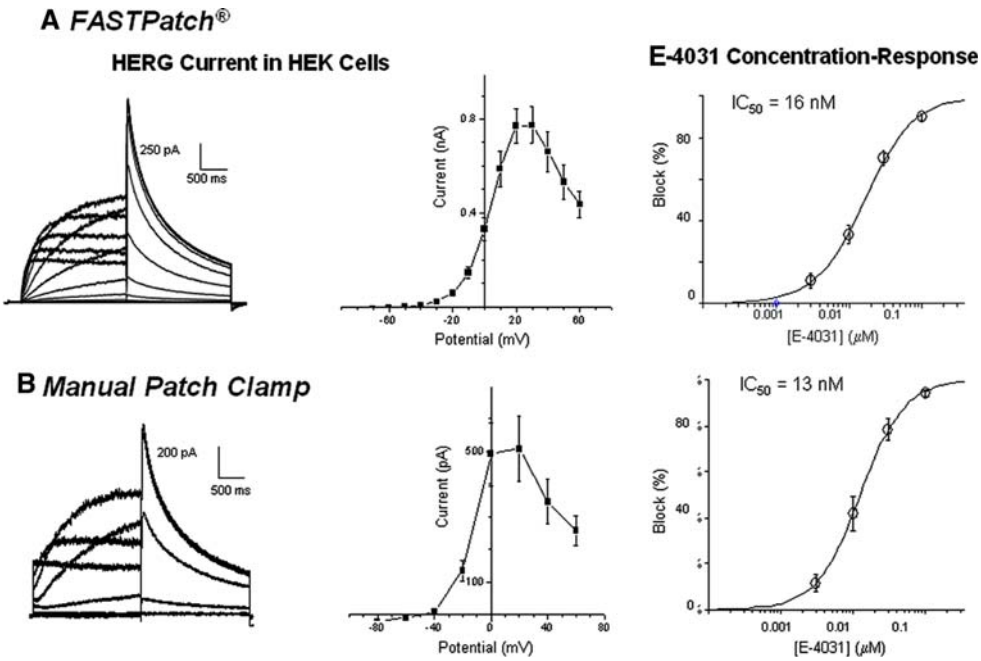

Fig. 3 hERG current–voltage relationship using the voltage protocol described in Sect. "Methods". The *FASTPatch*® and manPatch records are similar as is the plot of the I–V plot. The IC_{50} values for block by E-4031 are similar

Table 2 IC_{50} values obtained by manual and automated patch clamp

Test article	IC_{50} (μM)	
	Manual patch clamp	Automated patch clamp
Cisapride	0.0392	0.0202
E-4031	0.0185	0.0154
Verapamil	0.3788	0.3122
Diphenhydramine	3.6000	3.7000
Terfenadine	0.0243	0.0178
Astemizole	0.0013	0.0037
Pimozide	0.0009	0.0013
Ketanserin	0.1387	0.0991

CT-1 and alfuzosin produced unexpected effects on the cardiac channel panel. CT-1 is a potent hERG blocker but affects other cardiac channels as well notably Nav1.5 and Cavs1.2 and 3.2. This drug does not delay repolarization and is not torsadogenic (Lacerda et al. 2005), probably because block of inward calcium and sodium currents compensates for block of outward hERG potassium current. Moreover, block of sodium and calcium currents was strongly use-dependent. This property may also contribute to the absence of TdP. A second drug, alfuzosin (Uroxatral), is an alpha-adrenergic blocker used for benign prostatic hypertrophy. The drug carries a precautionary label due to QT prolongation. However, the effect is not due to hERG block but rather to increased sodium current (Lacerda et al. 2008). Although QT is prolonged, conduction velocity is increased and the increased wave length of the propagated action potential may account for the absence of adverse cardiac events with use of this drug.

Patch clamp is a powerful tool for measuring ion channel function. Done manually, however, patch clamp is labor-intensive, slow and expensive. Automation of patch clamp current measurements increases throughput between 15-

Table 3 Map of the cardiac channel panel. Effects of E-4031, verapamil, CT-1 and alfuzosin on eight cardiac channels

	Cav3.2	Cav1.2	Nav1.5	HCN4	Kv1.5	Kv4.3	KvLQT1/minK	hERG
E-4031*	**4.6**	**4.0**	**5.3**	**11.1**	**3.2**	**5.7**	**46.3**	**99.3**
Verapamil		**65.4**						**97.8**
CT-1	**83.2**	**92.8**	**62.9**	**46.4**		**59.5**		**98.7**
Alfuzosin**	**9.9**	**0.5**	**5.0**	**8.4**		**2.9**		**39.3**

20% < block <50%
50% < block
5% increase

Data presented as mean of the block (n = 2–3), 10 μM, 0.1 Hz; *FASTPatch*®

* 1 μM

** Lacerda et al. 2008

and 45-fold over manual measurements with little loss of accuracy. Higher throughput of thousands of data points/day (dps/d) can be attained with an acceptable decrease in accuracy. As a result, compound screening of a cardiac channel panel can be done in 1–2 days at a reasonable cost.

With the high throughputs of automated fluorescence measurement (10,000 dps/d) and subsequent follow-up with automated patch clamp, cardiac risk assessment can be evaluated in early discovery, thereby reducing the attrition rate that is the bane of drug development.

Conclusions

Automated patch clamp screening of a library of ion channels is a fast, cost-effective way to evaluate safety and efficacy during drug development.

Acknowledgments Interactions with electrophysiologists, molecular biologists and cellular biologists at ChanTest is gratefully acknowledged. Special thanks to Tony Lacerda, Yuri Kuryshev, Barb Wible and Glenn Kirsch.

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