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Abstract

Voltage-gated Ca^{2+} channels can be subdivided into two major classes. T-type Ca^{2+} channels are activated at low voltages and inactivate rapidly whereas L-, N-, P/ Q- and R-type Ca^{2+} channels open in response to large depolarization and are slowly or non-inactivating. We expressed a recombinant T-type Ca^{2+} channel α_1 -subunit in both HEK and CHO cells that had a threshold of activation of -70 mV, a peak current amplitude at -40 mV and inactivated during prolonged depolarizations, as determined by whole cell patch-clamp. Whole cell patch-clamp is the gold standard method for studying ion channel pharmacology but suffers from limited throughput. Therefore, we used IonWorks HT to develop a higher throughput electrophysiological assay for T-type Ca^{2+} channels. To optimize this Ca^{2+} current assay, we investigated the effects of cell lines, culture methods, recording buffer formulation, and assay materials on seal resistance, peak current amplitude, current stability, and pharmacology.

Comparison of IonWorks HT and Traditional Patch-clamp Recordings

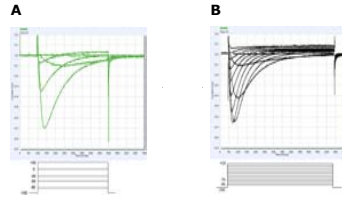


Fig 3. IonWorks HT recordings display the expected voltage dependence. **A.** Using the IonWorks HT, CHO cells expressing $\text{Ca}_v3.3$ were clamped at -100 mV and stepped sequentially to increasingly depolarized membrane potentials (-80 , -50 , -30 , 0 , $+30$ mV). Current traces for each step from a single cell were then manually overlaid for comparison. The lower traces represent the voltage commands. **B.** Using standard whole-cell patch-clamp, CHO cells expressing $\text{Ca}_v3.3$ were held at -100 mV and stepped sequentially to increasingly depolarized test potentials (-90 mV through $+20$ mV, in 10 mV increments). The lower traces represent the voltage commands.

Use of Voltage Ramp Protocol

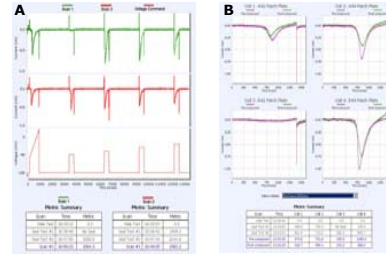


Fig 6. Voltage errors can affect $\text{Ca}_v3.3$. **A.** Voltage ramp (from -60 to $+30$ mV) followed by voltage steps (-60 , -50 , -40 , -30 mV) revealed variability in membrane potential where peak current is seen. **B.** Pairs of traces from 4 cells showing negative and positive ~ 5 mV shifts in V_{peak} between the pre- and post-compound periods. With voltage step protocol, this could be misinterpreted as a compound effect but peak detection using a voltage ramp minimizes the risk of such errors.

Glass-coated vs. Standard Polypropylene Compound Plates

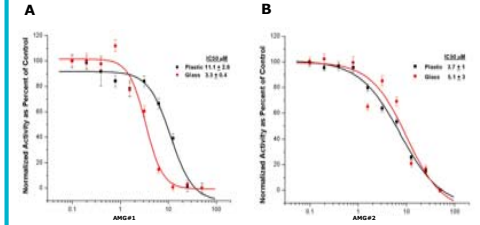


Fig 8. Glass-coated polypropylene compound plates may yield higher potency for a subset of compounds. **A.** Compound AMG#1 had a reproducible 3-4 fold decrease in IC_{50} when prepared in glass-coated polypropylene plates. **B.** Preparation of concentration-response dilutions in glass-coated polypropylene plates had no significant effect on IC_{50} values for compound AMG#2. Curves represent pooled and normalized data from 2 experiments with each datum point representing $N \leq 32$. Curves were plotted using OriginLab Origin and reported IC_{50} values were obtained using XLFit Software (IDBS).

IonWorks HT Electrophysiology

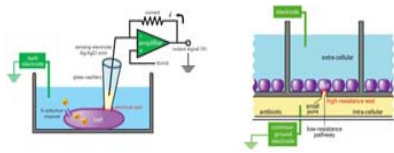


Fig 1. Conventional vs. planar patch-clamp recording. Conventional patch-clamp recording (left) and the planar patch-clamp configuration used on the IonWorks HT instrument (right). The configuration used on the instrument utilizes a common ground electrode in the lower (common) chamber. Voltage is controlled and ionic currents are measured by one of the 48 pins in the electronic head inserted into the upper compartment. The entire PatchPlate is read in 8 groups by the 48 electrodes in the electronic head. Electrical access to the interior of the cell is achieved by circulating a perforating agent (Amphotericin B) in the lower chamber.

Optimization of Conditions Yields $\text{G}\Omega$ Seals

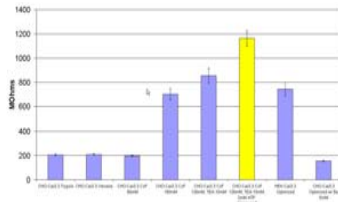


Fig 4. Careful attention to cell culture and cell preparation conditions in conjunction with custom solutions provided a significant increase in seal resistance and stability. Bars represent the median and s.e.m. for the seal resistance of those wells between 0.1 and 10 $\text{G}\Omega$ from a typical plate. Extracellular solution contained 2 mM Ca^{2+} as the charge carrier and intracellular solution contained CsF, TEA, HEPES, ATP. Amphotericin B buffer was prepared in this internal solution.

Comparison of Performance Metrics for Stable HEK-293 and CHO Cell Lines

Performance Parameter	CHO	HEK
Number Seals >100 $\text{M}\Omega$	339	248
Number Seals >1000 $\text{M}\Omega$	142	58
Mean Seal Resistance in $\text{M}\Omega$	1163	744
Percent $\text{G}\Omega$	42%	23%
Number of Currents >50 pA	255	212
Mean Current Amplitude pA	224 ± 168	626 ± 478
Overall Success Rate	66%	55%

Table 1. CHO cells provide higher seal resistances but lower mean current amplitudes than HEK-293 cells. Each column represents data from a single 384-well PatchPlate obtained with a voltage ramp protocol, optimized buffers and cell densities. Results were typical of those obtained with both cell lines over several weeks using these conditions.

Comparison of Rank Order and IC_{50} on IonWorks HT, Flash-luminometer and Patch-clamp

Compound	IonWorks HT	Flash-luminometer	Patch-clamp
AMG#1	3.3 ± 0.4 μM	2.5 ± 1.1 μM	2.3 ± 1.0 μM
AMG#2	5.1 ± 3.0 μM	2.1 ± 0.8 μM	n/a
AMG#3	4.6 ± 1.6 μM	1.8 ± 0.6 μM	0.8 ± 0.1 μM
Flunarizine	> 20 μM	$\sim 10 \pm 5$ μM	1.1 ± 0.2 μM
Mibefradil	> 20 μM	$\sim 10 \pm 3$ μM	$0.2-6.0$ μM^*

Table 2. IC_{50} values obtained with IonWorks HT are in accordance with historical values. When glass-coated compound plates are used, the IC_{50} values from IonWorks HT experiments match well with those obtained using flash luminometer and conventional patch-clamp. However, IC_{50} values for flunarizine and mibefradil could not be reliably determined on IonWorks HT. IonWorks HT values are the mean of at least two experiments on separate days. *Values are from published reports.

Calcium Channel Structure

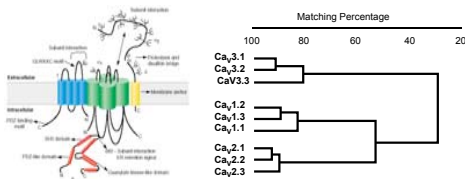


Fig 2. Biophysically and pharmacologically distinct Ca^{2+} currents arise from assembly of pore-forming α_1 -subunits with auxiliary subunits. While the α_1 -subunit contains most drug-binding sites, the $\alpha_2\delta$ -subunit represents a high-affinity site for the anti-epileptic, Gabapentin. However, the functional significance of this interaction is debated.

Comparison of Currents Recorded with Different Seal Resistances

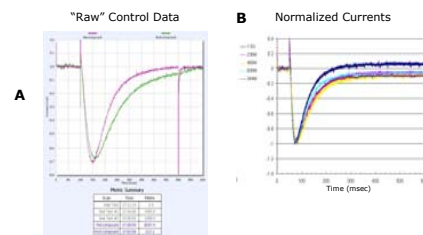


Fig 5. $\text{G}\Omega$ seals provide increased sensitivity and stability but are not required for reliable recordings. **A.** Current traces before and 25 minutes after buffer addition are shown. Even though seal resistance declined, current amplitude does not change. **B.** Representative traces from 5 cells (with different seal resistances from 230 $\text{M}\Omega$ to 1.5 $\text{G}\Omega$) on a single plate were normalized and overlaid to demonstrate consistent kinetics.

Comparison of Pharmacology in Stable CHO and HEK-293 Cell Lines

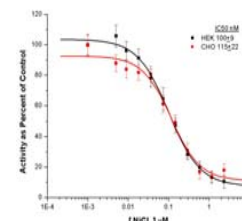


Fig 7. Block of $\text{Ca}_v3.3$ channels by NiCl_2 . Inhibition of $\text{Ca}_v3.3$ currents in both CHO and HEK-293 cells exhibited similar Ni^{2+} sensitivity. These curves represent data from 2 single plates and individual data points are means and s.e.m. Both plates were run on the same day using the same NiCl_2 source plate. Curves were plotted and IC_{50} values calculated using OriginLab Origin software.

Summary and Conclusions

CHO cells were found to yield more stable recordings but with lower mean current amplitudes than HEK-293 cells. Both cell lines worked well with average seal resistances per plate often exceeding 700 $\text{M}\Omega$ for recording periods of up to 70 minutes. With optimized buffers 20-30% of seals exceeded 1 $\text{G}\Omega$. In addition, voltage ramp protocols yielded IC_{50} values comparable to voltage step protocols. Moreover, peak Ca^{2+} currents measured using voltage ramps were less susceptible to voltage errors. A series of reference compounds was tested and found to yield IC_{50} values consistent with traditional patch-clamp and luminescence assays. In summary, an assay development approach incorporating electrophysiological expertise has allowed us to develop high-quality and high-throughput T-type Ca^{2+} channel assay that can be used for screening focused libraries of compounds and for confirming biological activity of molecules that are identified using other high-throughput screening platforms such as FLIPR or flash luminometer.