SOPHION BIOSCIENCE

Application Report

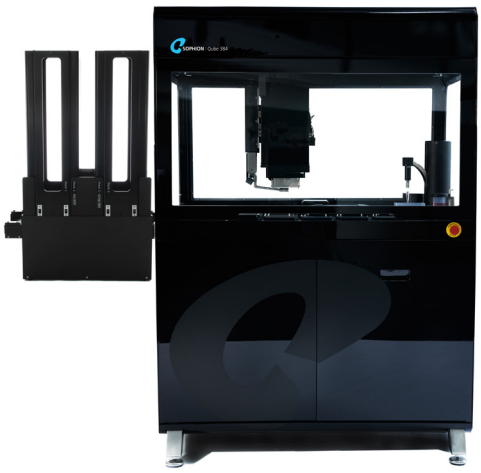
$Ca_V1.2$ on Qube 384

Reliable and reproducible experiments in high throughput studies with high success rates

Summary

For a long time, screening assays on $Ca_V1.2$ have been challenging due to $Ca_V1.2$ cell lines exhibiting declining current levels in the course of the experiment. Here we report a robust assay with high success rates and reliable pharmacology.

- Success rates of 91% for Ca^{2+} currents and 98% for Ba^{2+} were achieved
- Stable currents with as low as 0.6 ± 1.8% rundown per minute
- Reference pharmacology in accordance with literature values
- Use-dependent and independent mode of action distinguished with different voltage protocols



Introduction

The voltage-sensitive L-type Ca^{2+}-channel (LTCC) $Ca_V1.2$ is a crucial component for controlling intracellular activity and thereby essential in the cardiovascular and neuronal system. It is widely expressed in vascular smooth muscle tissue and the heart muscle[1-3]. The opening of the channels leads to an increase of intracellular calcium, which acts as second messenger and thereby affects a variety of cellular processes[4] including heart muscle contraction. $Ca_V1.2$ is therefore an important target in e.g. safety pharmacology screening. The channels are known to require a large depolarization for their activation and once activated they display a long-lasting current flow, which typically can be blocked by low micromolar concentrations of e.g. dihydropyridines, phenylalkylamines and benzothiazepines[5,6]. In these studies, currents from HEK-h$Ca_V1.2$ were recorded on the high-throughput platform Qube 384 in both single-hole and multi-hole mode. Success rates, IV characteristics and the pharmacological effects of three different compounds were determined.

Results and discussion

The following sets of experiments were carried out:

1) Calcium currents and rundown analysis on multi-hole QChips
2) Calcium currents recorded on single-hole QChips
3) Current-voltage relationship
4) Pharmacology of $Ca_V1.2$ channels
5) Barium currents recorded on multi-hole QChips

Calcium currents and including rundown analysis

Cells were clamped to -100 mV and calcium currents were evoked by application of a depolarization step to +20 mV for 200 ms (Fig.1). The time between sweep start was 15 seconds. 60 sweeps of single stimulation were followed by 25 stimulation trains, containing 5 voltage steps at 2.5 Hz each (Fig.2).

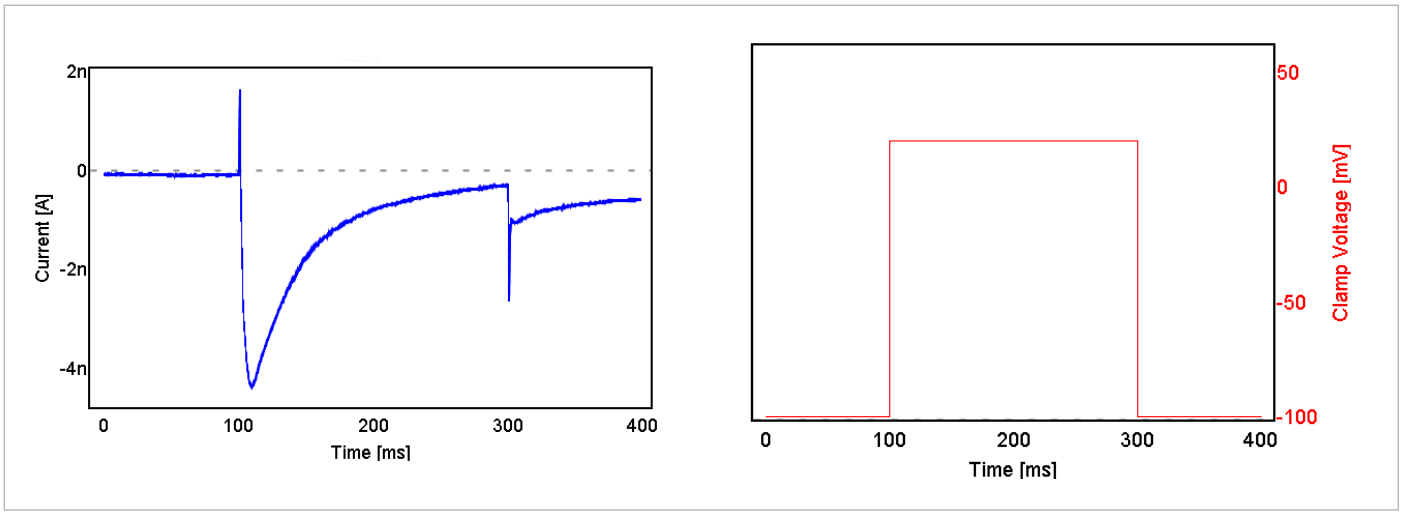


Fig. 1: Original recording of a calcium current (left) following a depolarization from -100 mv to +20 mV (right) in $Ca_V1.2$-expressing cells.

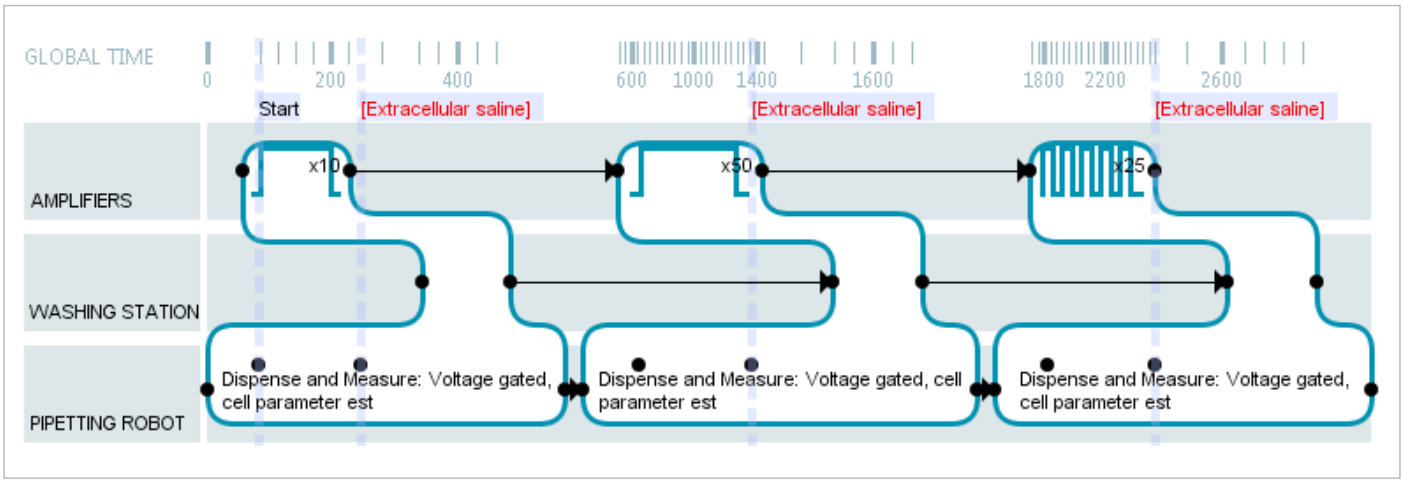


Fig. 2: Example of an experiment setup for $Ca_V1.2$ rundown measurements in the Sophion Viewpoint software. The first block contains 10 single depolarizations and is followed by 50 depolarizations (second block). Lastly, 25 train stimulations are applied to the $Ca_V1.2$-expressing cells.

The criteria for a successful recording on a multi-hole QChip were:

- I_{peak} > 500 pA on average in the first 10 sweeps
- $R_{membrane}$ > 100 MΩ per cell on average in the first 10 sweeps
- C_{total} > 60 pF on average in the first 10 sweeps

In order to maintain high success rates, it is useful to keep a low passage number for this cell line. A plate-view of Ca^{2+}-currents is shown in Fig. 3 and the success rates are compared with Ba^{2+}-currents (see later) in Table 1.

A typical concern in $Ca_V1.2$ assays is the rundown phenomenon under continuous stimulation. The currents evoked by the first stimulation were compared with those of the 50th stimulation (the 2nd block of Fig.2), where each sweep was separated by 15 seconds. The overall rundown when comparing currents at peak 1 and peak 50 was as low as 0.6 ± 1.8% per minute.

Table 1: Success rates dependent on charge carrier and after quality criteria mentioned in the text.

Charge carrier	Ca^{2+}	Ba^{2+}
Success rate - multi-hole	91%	98%
Success rate - single-hole	72%	

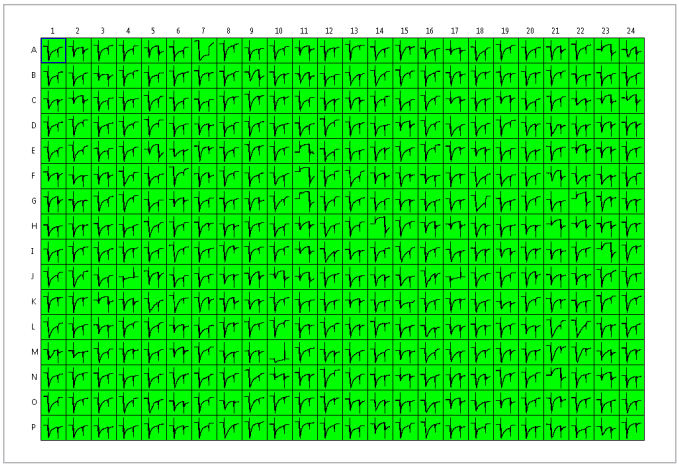


Fig. 3: Plate overview of calcium currents through $Ca_V1.2$ (multi-hole).

Besides the single pulse protocol, another 25 train stimulations of 5 pulses at 2.5 Hz, with each train separated by 30 seconds, were also executed in the same experiment (third block, Fig.2). This resulted in an inactivation of 3 ± 1% per minute between the current of the first and the 25th train (Fig.4). Within the first train, the last current peak showed 3.2 ± 0.6% inactivation per minute in comparison to the first evoked current.

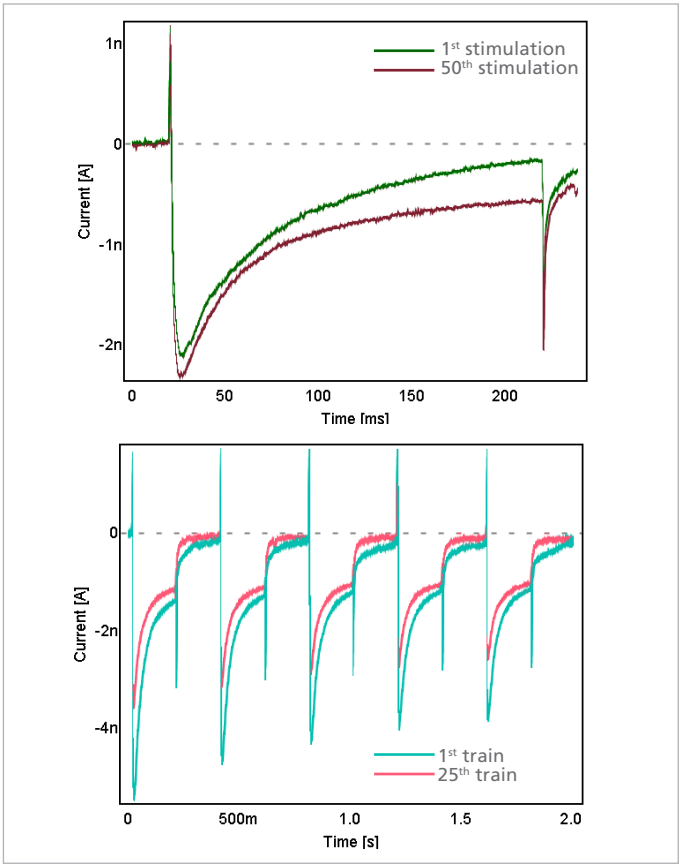


Fig. 4: Top panel shows example traces of 1st (brown) vs 50th (dark green) stimulation and lower panel shows example trace of 1st (green) vs 25th (red) train stimulation. On average, rundown in the single stimulation protocol was as low as 0.6 ± 1.8% per minute.

Calcium currents recorded on single-hole QChips

Cells were clamped to -100 mV and calcium currents were evoked by application of a depolarization step to +20 mV for 200 ms (Fig.5). The time between sweep start was 15 seconds.

For measurements on a single-hole QChip the following success criteria were used:

- I_{peak} > 100 pA on average in the first 10 sweeps
- $R_{membrane}$ > 100 MΩ on average in the first 10 sweeps
- C_{total} > 6 pF on average in the first 10 sweeps

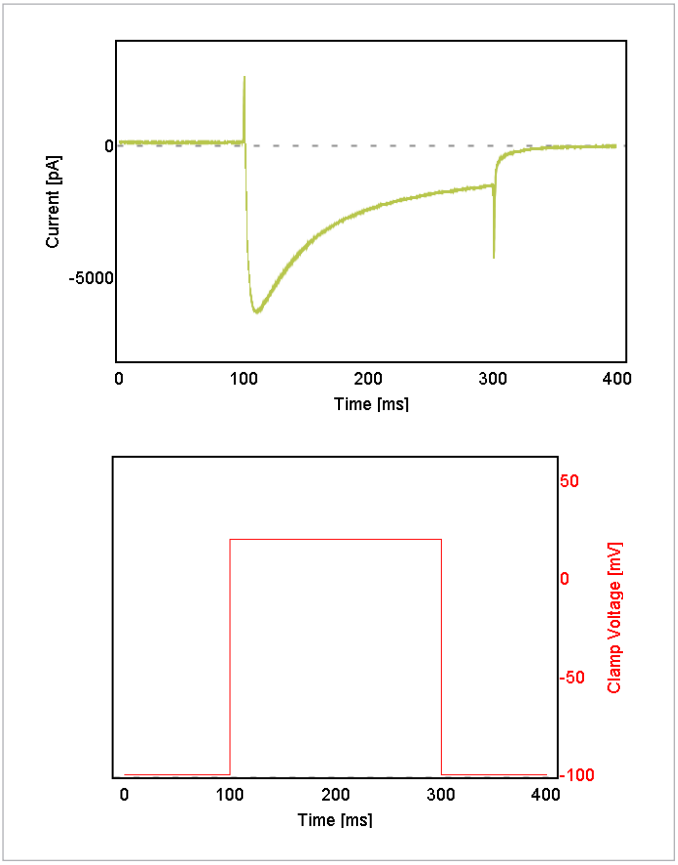


Fig. 5: Example of a calcium current through $Ca_V1.2$ (top) following a depolarization from -100 to +20 mV (bottom) on a single-hole QChip.

Current-voltage relationship

Calcium currents were evoked by applying a depolarization step protocol from -100 mV to +60 mV for 200 ms in increments of 10 mV. The time in between sweeps was 15 seconds. The resulting peak currents were normalized to each cell's peak current at +20 mV and plotted as a function of the stimulating potential (Fig.6).

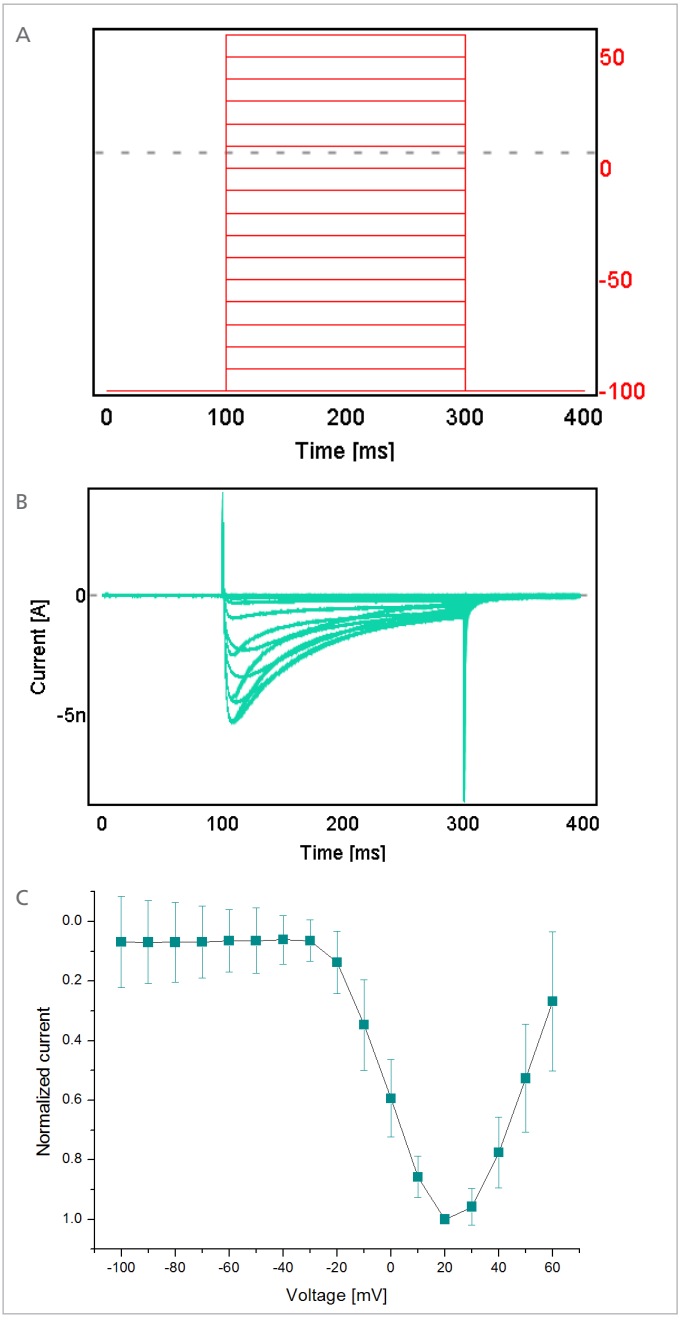


Fig. 6: A) Step protocol in increments of 10 mV ranging from -100 mV to +60 mV. B) Current response from $Ca_V1.2$ channels. C) Analysis of the peak current as a function of stimulating potential. All current values are normalized to the current at +20 mV depolarization. Values are mean ± SD.

Pharmacology of $Ca_V1.2$ channels

Cells were clamped to -100 mV and calcium currents were evoked by application of a depolarization step to +20 mV for 200 ms (see Fig.1). After 10 activation sweeps, 10 train stimuli of 5 pulses at 2.5 Hz each were executed, the first set without, the second one with compound (Fig.7).

Nicardipine, diltiazem and verapamil were applied in 7 different concentrations (non-cumulative): The highest concentration of the compounds was 30 µM for nicardipine, 100 µM for diltiazem and 50 µM for verapamil. Each lower concentration was a serial 3-fold dilution.

The current amplitude of the last depolarization step of the train in the liquid phase [Compound1] was normalized to the last depolarization step of the train in the liquid phase [Second extracellular saline]. To carve out potential use-dependent mode of action, the first depolarization step of the stimulation train in the compound period [Compound 1 (Fig.7)] was normalized to the first stimulation step in the saline period [second Extracellular saline (Fig.7)]. This revealed that verapamil had no effect, diltiazem exhibited a slight block at its highest concentration, whereas nicardipine still provided a sigmoidal concentration dependency. The Hill fits to these concentration dependent effects are shown in Fig.8 and results are compared in Table 2. These results confirm the strong use-dependency of verapamil and diltiazem whereas nicardipine only exhibits a weak separation between the two modes of action.

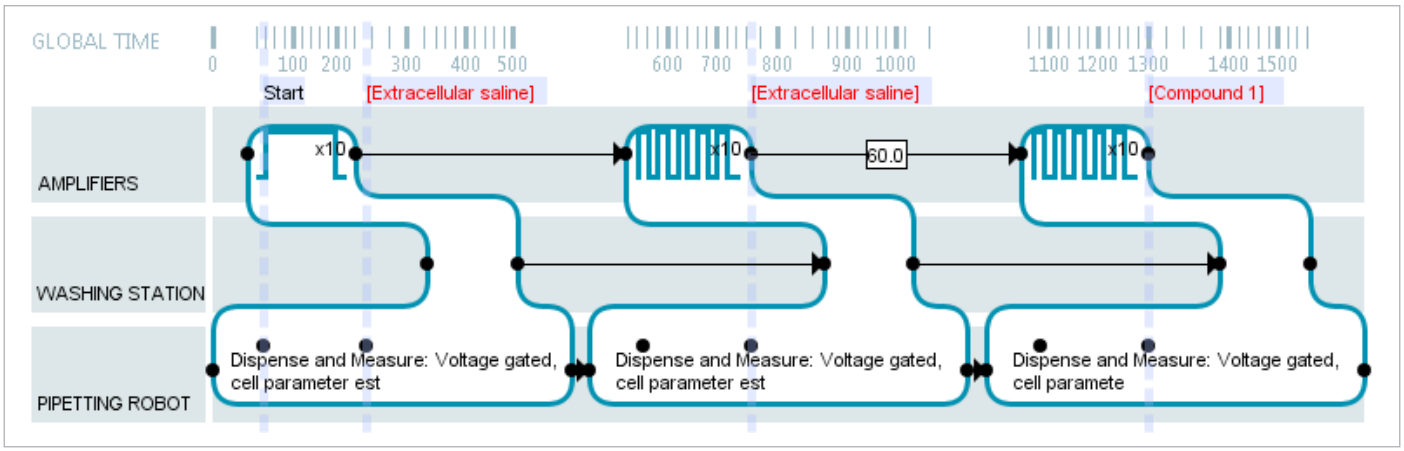


Fig. 7: Experiment setup for $Ca_V1.2$ pharmacology measurements

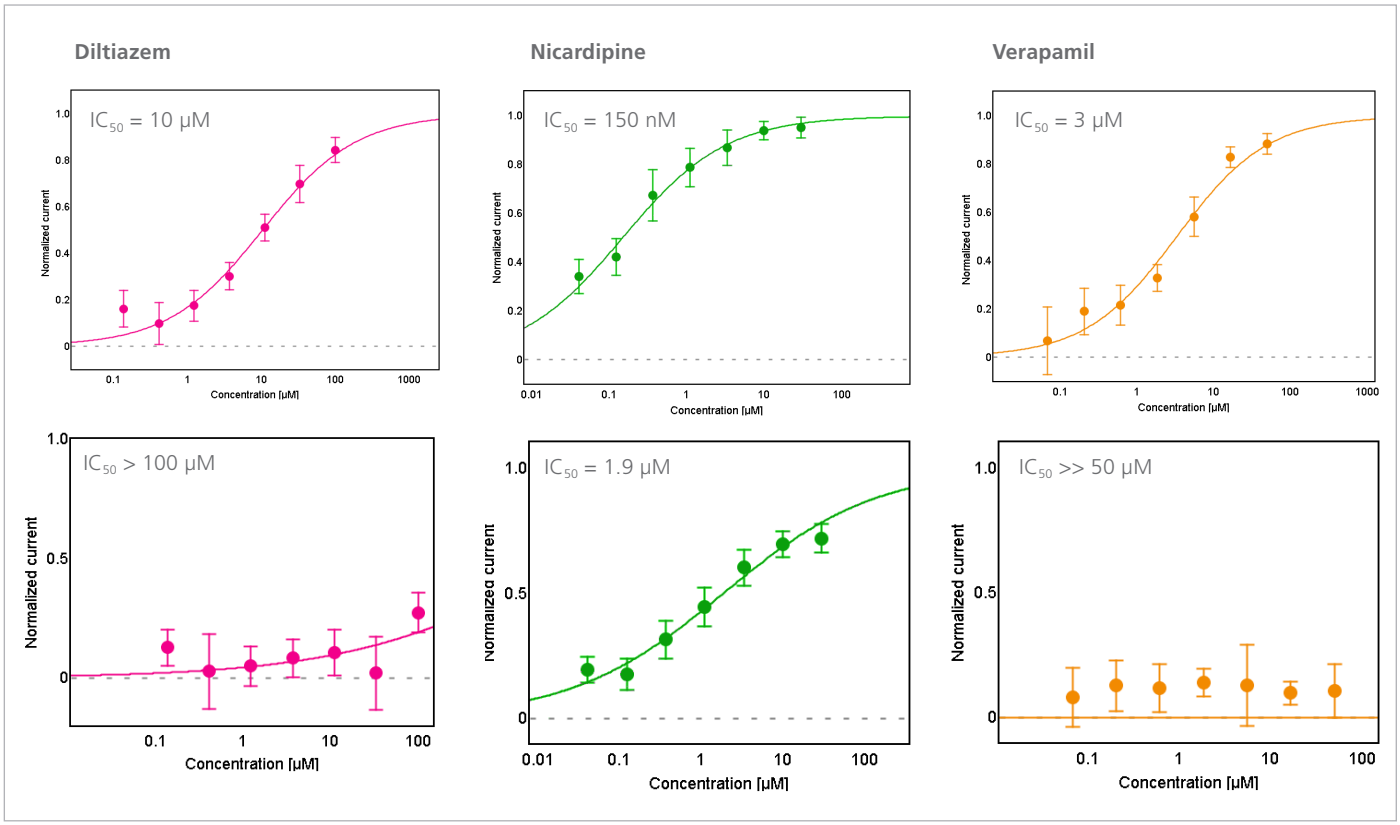


Fig. 8: Use-dependent and use-independent mode of action. Upper panel shows Hill-fit of compound effect on the last step in the train normalized to the last step in the train in the baseline period. The lower panel shows the compound effect on the first step in the train normalized to the first step in the train in the baseline period. The specific compounds are noted on the graph and the data point values are mean ± SD.

Table 2: Mode of action effect of three reference compounds

Compound	Mode	Qube 384	Literature
Dilitazem	IC_{50} (last/last)	10 µM	33 µM[7]
	IC_{50} (first/first)	> 100 µM	
Nicardipine	IC_{50} (last/last)	150 nM	60 nM[8]
	IC_{50} (first/first)	1.9 µM	
Verapamil	IC_{50} (last/last)	3 µM	2 µM[9]
	IC_{50} (first/first)	>> 50 µM	

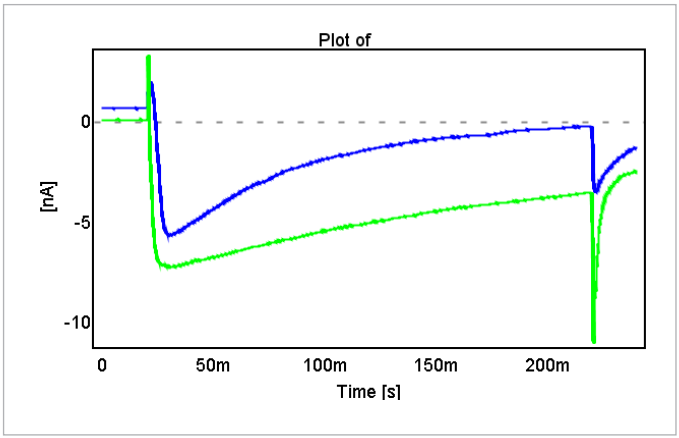


Fig. 9: Original traces of a barium current (10mM Ba^{2+}) in green vs. a calcium current (10mM Ca^{2+}) in blue through $Ca_V1.2$ on the same Qchip.

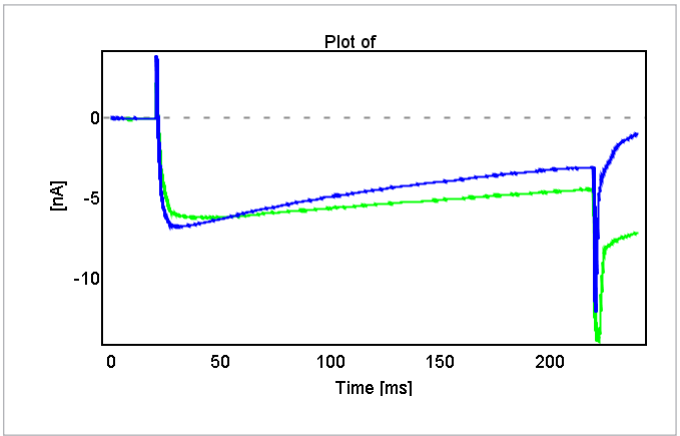


Fig. 10: Original traces of barium currents on the same cell in the first (green) vs the 30th stimulation (blue).

Barium currents recorded on multi-hole QChips

Barium currents were evoked by application of a depolarization step from -100 to +20 mV for 200 ms (see Fig.1). The time between sweep start was 15 seconds. 50 sweeps of single stimulations were followed by 10 sweeps stimulation in the presence of 10 µM nifedipine. Barium currents were evoked with a success rate of 98% (according to multi-hole success criteria) and the average current amplitude was 6.7 nA with typical kinetics (Fig.9). The application of 10 µM nifedipine led to a complete block of current (not shown). Continuous depolarizations over 13 minutes did not significantly alter the amplitude of barium currents (Fig.10).

Conclusion

Calcium and barium currents through $Ca_V1.2$ were recorded on the high-throughput platform Qube 384 in both single-hole and multi-hole mode. The current levels were stable over time and high success rates were achieved with cells at low passage number. The current-voltage relationship and pharmacology experiments on the Qube were in accordance with literature values and highly reproducible. Consequently, Qube is an ideal platform for running $Ca_V1.2$ experiments for screening as well as characterization.

Materials and methods

Cells

Experiments in this study were performed on HEK-hCa$_V$1.2 cells, which were kindly provided by B'SYS. The cells express the human CACNA1C, CACNB2 and CACNA2D genes after induction with tetracycline.

Cell culture

HEK-hCa$_V$1.2 cells were cultured and harvested according to Sophion standard procedures. However, a few parameters were adjusted to take cell-specific properties into account. When sub-culturing for mother and experiment flasks, the following cell-densities were used:

1. Add 6x10^4 cells/cm^2 for sub-culturing/experiments after 24 hours.
2. Add 4x10^4 cells/cm^2 for sub-culturing/experiments after 48 hours.
3. Add 3x10^4 cells/cm^2 for sub-culturing/experiments after 72 hours.
4. Add 1.6x10^4 cells/cm^2 for sub-culturing/experiments after 96 hours.

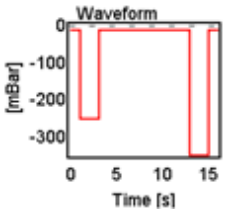
The rate of cell growth for this cell line can vary and cell numbers might therefore have to be adjusted to the individual rate of cell division. Furthermore, it is recommendable to thaw fresh cells every 4-6 weeks.

Cell and induction media were used according to the B'SYS HEK hCa$_V$1.2 specification sheet. The cells were harvested in 5 ml SFM and immediately transferred to the Qube where the cells were prepared for experiments using the automatic cell preparation unit.

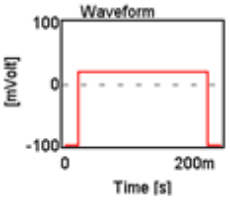
Experimental setup

For worktable, cell preparation and clean-up, Qube default protocols were used.

Whole-cell protocol:
A two second suction pulse from -10 mbar to -250 mbar was followed by 10 seconds at -10 mbar and thereafter a two second suction pulse from -10 mbar to -350 mbar was applied. For more parameters, see Fig.11.



Voltage protocol: Cells were held at -100 mV holding potential and were depolarized for 200 ms to +20 mV. For the IV relationship studies, a voltage step protocol up to +60 mV was used.



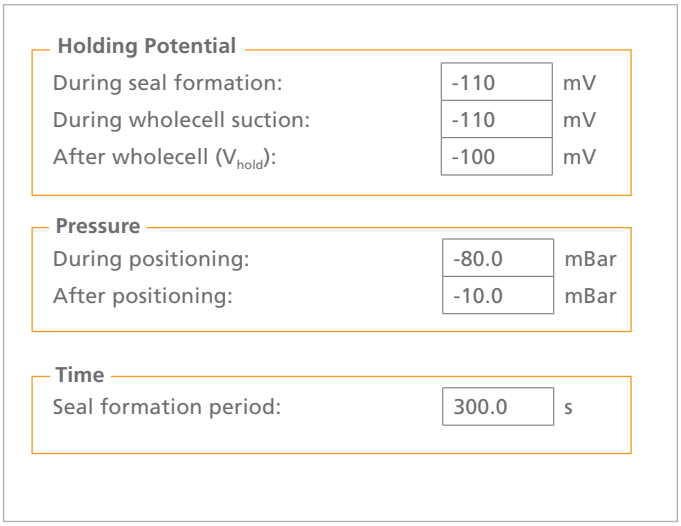


Fig. 11: Detailed parameters of the whole-cell protocol.

References:

1. Sinnegger-Brauns MJ, Hetzenauer A, Huber IG et al. 2004. Isoform-specific regulation of mood behavior and pancreatic β cell and cardiovascular function by L-type Ca^{2+} channels. J Clin Invest; 113:1430-9
2. Moosmang S, Schulla V, Welling A et al. 2003. Dominant role of smooth muscle L-type calcium channel $Ca_V1.2$ for blood pressure regulation. EMBO J; 22:6027-34
3. Zhang J, Berra-Romani R, Sinnegger-Brauns MJ et al. 2007. Role of $Ca_V1.2$ L-type Ca^{2+} channels in vascular tone: effects of nifedipine and Mg^{2+}. Am J Physiol Heart Circ Physiol; 292:H415-25
4. Tsien RW, Tsien RY 1990. Calcium channels, stores, and oscillations. Annu Rev Cell Biol; 6:715–760
5. Reuter H 1983. Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature; 301: 569–574
6. Reuter H 1979. Properties of two inward membrane currents in the heart. Annu Rev Physiol; 41: 413–424
7. Catterall WA, Perez-Reyes E, Snutch TP et al. 2005. International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. Pharmacol Rev; 57(4):411-25
8. Lin M, Aladejebi O, Hockerman GH 2011. Distinct properties of amlodipine and nicardipine block of the voltage-dependent Ca^{2+} channels $Ca_V1.2$ and $Ca_V2.1$ and the mutant channels $Ca_V1.2$/dihydropyridine insensitive and $Ca_V2.1$/dihydropyridine sensitive. Eur J Pharmacol; 16;670(1):105-13
9. Kuryshev YA, Brown AM, Emir Duzic E et al. 2014. Evaluating State Dependence and Subtype Selectivity of Calcium Channel Modulators in Automated Electrophysiology Assays. Assay Drug Dev Technol; 12(2): 110–119

Acknowledgement:

Cells kindly provided by B'SYS

Author:

Melanie Schupp, Application scientist

Sophion Bioscience A/S, Baltorpvej 154, 2750 Ballerup, Denmark
Phone: +45 4460 8800 Fax: +45 4460 8899, E-mail: info@sophion.com

sophion.com