

## Biophysical modulation of hHCN2 by bPAC recorded on the SyncroPatch 384PE

The electrophysiology team at Nanion Technologies GmbH, Munich.

Cells engineered and kindly provided by Axxam S.p.A., Milan.



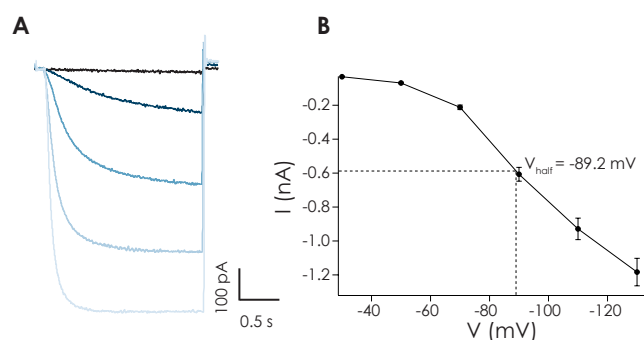
### Summary

Neuronal and cardiac rhythmicity is predominantly controlled by hyperpolarization activated cyclic nucleotide gated (HCN) channels. The HCN family comprises four members (HCN1-4) which are ubiquitously expressed in the central and peripheral nervous system<sup>1</sup>. Activated by hyperpolarization, HCN channels open slowly with no inactivation. Cyclic AMP (and other second messenger proteins) affects the activation properties independent of phosphorylation, modulating the voltage dependence of current activation and accelerating the kinetics of channel opening<sup>2</sup>. HCN mediates a  $\text{Na}^+/\text{K}^+$  conductance ( $I_h$ ) which contributes to the establishment of the resting membrane potential<sup>3</sup>. It is therefore not surprising that HCN channels play an important role in the regulation of neuronal firing and excitability as well as pacemaking. Disruption of HCN function slows down the heart rate and provides a potential target for the treatment of neuronal disorders such as epilepsy<sup>4</sup> and neuropathic pain<sup>5</sup>.

Here we present data collected on the SyncroPatch 384PE showing pharmacology and modulation of hHCN2 expressed in HEK cells. In addition, these cells heterologously express a light-sensitive bacterial phospho-adenylate cyclase (bPAC). We demonstrate two ways of triggering the cAMP pathway in order to modulate the HCN2 channel opening kinetics. First, we used the internal perfusion system of the SyncroPatch 384PE for direct application of cAMP to the intracellular environment. Second, we triggered the cAMP pathway by optical stimulation of bPAC. Further, we showed voltage dependent block of  $I_h$  with  $\text{Cs}^+$  and ZD7288. Ivabradine, a drug used for symptomatic management of stable heart related chest pain and heart failure blocked the channel with an  $\text{IC}_{50}$  of 0.1 mM in good agreement the literature<sup>6&7</sup>.

### Results

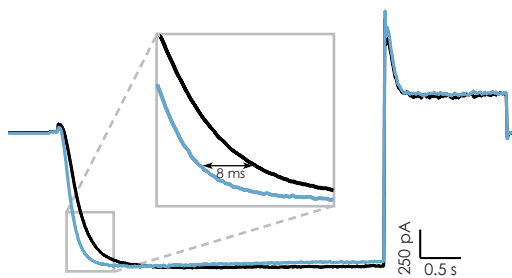
Currents mediated by hHCN2 were activated upon plasma membrane hyperpolarization Figure 1 shows current responses to decreasing voltage steps for an exemplar HEK cell expressing hHCN2 and the corresponding current-voltage plot for an average of 346 cells. The example trace shows typical slow activation upon hyperpolarization. The half maximal activation of HCN2 was  $-89.2$  mV.



**Figure 1: Activation of HCN2 expressed in HEK cells on the SyncroPatch 384PE by hyperpolarizing voltage steps. A** .Raw traces from an exemplar cell expressing hHCN2 recorded on the SyncroPatch 384PE. Shown are current responses to decreasing voltage steps from  $-30$  to  $-130$  mV. **B**. Current-voltage plot for an average of 346 cells. Shown are mean of steady-state current amplitudes  $\pm$  S.E.M.

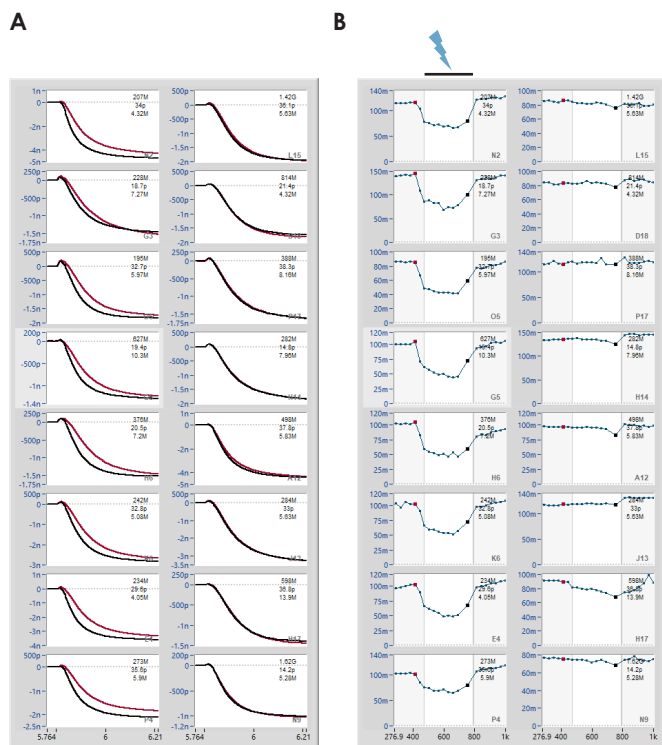
Application of internal cAMP did not further increase the current amplitude but modulated the current kinetics decreasing the time constant ( $\tau$ ) to reach the peak current by  $31\% \pm 1.1\%$  for an average for 204 cells.

# Application Note



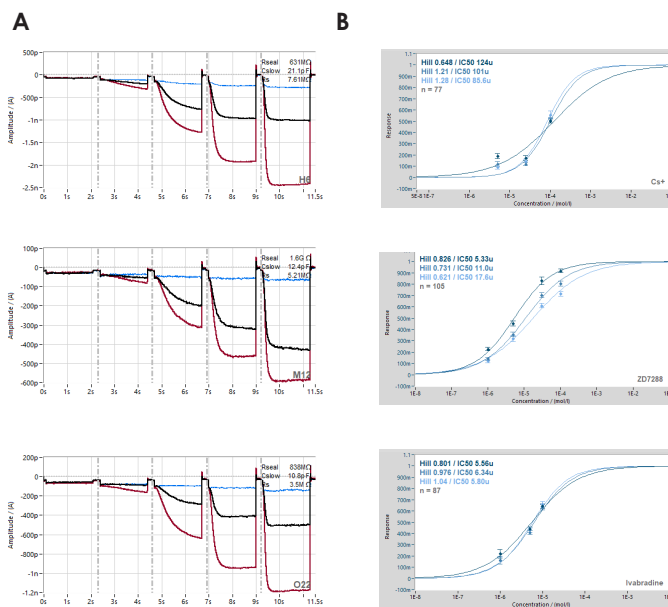
**Figure 2: Modulation of hHCN2 expressed in HEK cells on the SyncroPatch 384PE by cAMP.** Raw current traces from an example cell showing current activation by stepping the voltage from -30 mV to -130 mV (black trace) and current modulation by additional intracellular application of 2 mM cAMP (blue trace). The inset displays a higher resolution of current kinetics. This example cell shows a modulation of tau by 8 ms when cAMP was applied.

In these cells engineered by Axxam S.p.A the intracellular cAMP concentration can also be increased by light stimulation of bPAC. Figure 3 shows the reversible modulation of  $I_h$  current kinetics after blue light excitation ( $\lambda = 470 - 495 \text{ nm}$  for 0.5 Hz) decreasing tau of activation to a similar extent compared to the response after intracellular perfusion of cAMP.



**Figure 3: Modulation of  $I_h$  by light-induced cAMP production.** A. Raw current traces from 16 example cells showing the effect of blue light stimulation (8 cells on the left, black traces) and no light (red traces) and red and black traces (right). B. Online analysis plot showing tau versus time for the same cells shown in A.

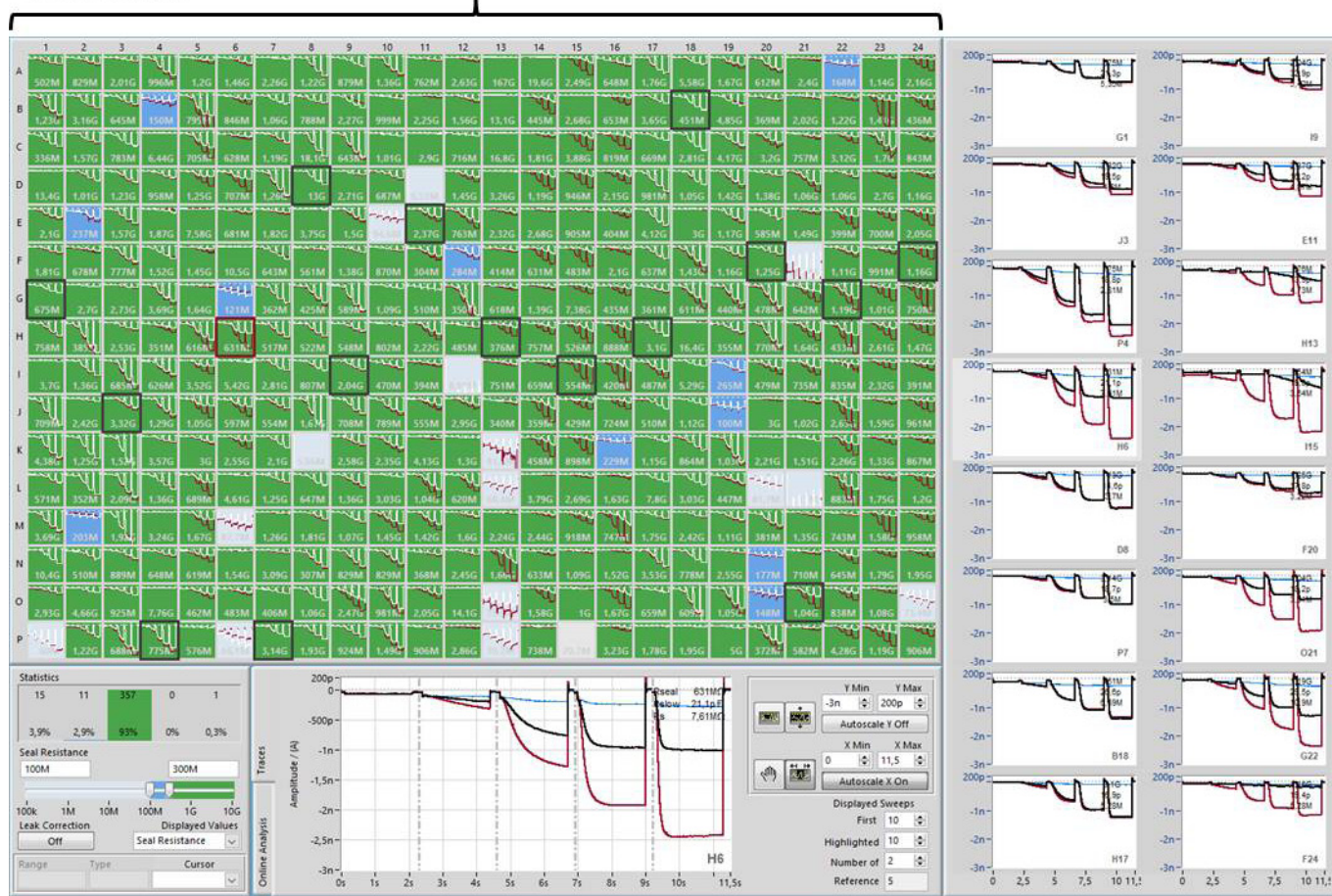
In order to study the current kinetics and the voltage dependent effect of compounds in parallel, voltage steps of increasing amplitude (-30 mV, -90 mV, -110 mV, -130 mV) were applied in one sweep. A single concentration of either  $\text{Cs}^+$ , ZD7288 or ivabradine was applied to each well and concentration response curves were calculated by combining the results across the plate (Figure 4). The  $\text{IC}_{50}$  of  $\text{Cs}^+$  and ZD7288 vary with different voltages ( $\text{Cs}^+_{-90} = 124 \mu\text{M}$ ,  $\text{Cs}^+_{-110} = 101 \mu\text{M}$ ,  $\text{Cs}^+_{-130} = 86 \mu\text{M}$  ( $n = 77$ ); ZD7288 $_{-90} = 5 \mu\text{M}$ , ZD7288 $_{-110} = 11 \mu\text{M}$ , ZD7288 $_{-130} = 17 \mu\text{M}$  ( $n = 105$ )) demonstrating a voltage dependent blocking effect of these compounds on the current. No such an effect was observed for ivabradine ( $\text{IC}_{50} \sim 6 \mu\text{M}$ ). For  $\text{Cs}^+$  and ZD7288, a cumulative concentration response curve was also performed (data not shown), resulting in comparable  $\text{IC}_{50}$ s as calculated from the single-point concentration response curves.



**Figure 4: Block of hHCN2 by  $\text{Cs}^+$ , ZD7288 and ivabradine.** A. Raw current traces (red traces) from example cells showing activation of  $I_h$  by decreasing voltage steps followed by block with single concentrations of  $\text{Cs}^+$ , ZD7288 or ivabradine (black traces in left panel) and full block with 2 mM  $\text{Cs}^+$  (blue traces). B. The concentration response curves were calculated across the plate. The data collected were normalized to the reference current and fitted with a standard Hill-equation. Average concentration response curves were calculated for the -90 mV, -110 mV and -130 mV voltage steps.

# Application Note

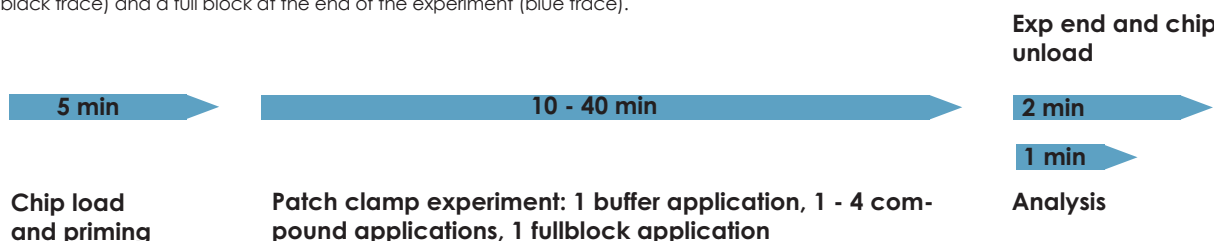
384 well color coded depictions of data traces eases the judgement of success rate



Highlighted raw traces of one cell showing effect of Cs<sup>+</sup> on I<sub>h</sub>

Highlighted raw traces of 16 cells showing effects of different compounds on I<sub>h</sub>

**Figure 5: Graphical user interface of the screening and data analysis software used on the SyncroPatch 384PE.** Screenshot depicts raw data traces of hHCN2-expressing HEK cells as recorded on one NPC-384 patch clamp chip. A single point concentration response curve for Cs<sup>+</sup>, ZD7288 and ivabradine was performed on individual wells. One control application of physiological solution was made followed by one concentration of compound on each well and increasing concentrations over the plate. Multi-hole chips were used where 4 holes were present per well. The data of the 384 well plate representation in the upper left part are color-coded for easy assessment of data. Depending on the seal resistance, pictures are green (R<sub>memb</sub> > 300MΩ), blue (R<sub>memb</sub> 100 - 300 MΩ) or light blue (<100 MΩ). One highlighted experiment is displayed at the bottom, 16 selected experiments are displayed on the right. Graphs show raw data traces of hHCN2 channels upon activation by hyperpolarizing voltage steps and application of buffer (red trace), followed by one compound addition (black trace) and a full block at the end of the experiment (blue trace).



**Figure 6: Timeline of an experiment on the SyncroPatch 384PE.** The completion of 1 experiment on the SyncroPatch 384 patch clamp chip (384 wells) for a 1 - 4 point concentration response curve on hHCN2-mediated currents took approximately 10-40 min.

# Application Note

Figure 5 shows a screenshot of the SyncroPatch 384 software during an experiment. A color-coded overview (based on seal resistance in this case) of all 384 wells gives the user a good impression of the success rate of the experiment. The user can easily toggle between raw traces and online analysis. In the example shown, raw traces are chosen and the graphs show control responses to physiological buffer (red traces), followed by block with Cs<sup>+</sup>, ZD7288 or ivabradine (black traces) and full block with 2 mM Cs<sup>+</sup> (blue traces). The experiment shows that time and state dependent compound effects can be simultaneously studied. The cells were stable for over 30 minutes and compound could be incubated for up to 8 minutes per concentration ensuring that full effect of the compound was reached. An individual well can be highlighted to monitor progression of the experiment and is shown enlarged at the bottom of the screen.

In conclusion, I<sub>h</sub> expressed in HEK cells can be recorded on the SyncroPatch 384PE with good success rates (89 % of wells were included in analysis of pharmacological experiments). The internal perfusion feature of

the SyncroPatch 384PE was used to modulate HCN2 using internal cAMP and alteration in the current kinetics could be monitored online. This continuous monitoring of analysis parameters such as tau is important for distinguishing compound effect from increase in leak or loss of seal and can be a benefit for assay development. Modulation of HCN2 by cAMP was also triggered by optical stimulation of bPAC, when using the light stimulation tool of the SyncroPatch 384PE. The HCN2 mediated current response was inhibited by Cs<sup>+</sup>, ZD7288 and ivabradine in a concentration-dependent manner as expected with an IC<sub>50</sub> within the range found in the literature<sup>6,7</sup>.

The SyncroPatch 384PE is a high throughput and highly reliable automated patch clamp device for recording hHCN2 mediated currents. User-friendly software, excellent success rates, single additions or multiple additions of compound to each well, perfusion of internal solution, light stimulation and easy analysis result in reliable high quality data at an increased throughput with an economical cost per data point.

## References

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## Methods

### Cells

HEK293 cells co-expressing hHCN2 and the Photo-activated Adenylyl Cyclase from *Beggiatoa* bacterium (bPAC) were engineered and kindly provided by Axxam S.p.A., Milan; (<https://axxam.com/>).

### Cell culture

Cells were cultured and harvested according to Nanion's standard cell culture protocol.

### Electrophysiology

Whole cell patch clamp recordings were conducted according to Nanion's standard procedure for the SyncroPatch 384PE using multi-hole (4 holes per well) chips. Perforated patch recordings were conducted. The internal solution was supplemented with 2 or 3 mM ATP and 2 mM cAMP, either present from the beginning or washed within the recording using the Internal Exchange function of the SyncroPatch 384PE. I<sub>h</sub> currents were evoked by stepping from V<sub>hold</sub> of -30 mV in -20 mV increments to -130 mV for 2 s. Blue light excitation (λ=470-495nm) was used to stimulate bPAC and hence to trigger the cellular cAMP pathway.

hHCN2-mediated currents were calculated as the difference of the inward current at the beginning and the end of the voltage step. A mono-exponential decay fit was used to determine the time constant (tau) of the current activation.