

## Application Report

# Qube Opto 384 - Modulation of an intracellular messenger pathway by combining automated patch clamp and optical stimulation

Optical stimulation in combination with high throughput electrophysiology was used for an evaluation of the phosphorylation-induced changes in HCN2 kinetics.

### Summary

In a stable HCN2/bPAC-HEK293 cell line, optical stimulation changed the HCN2 ion channel kinetics and resulted in:

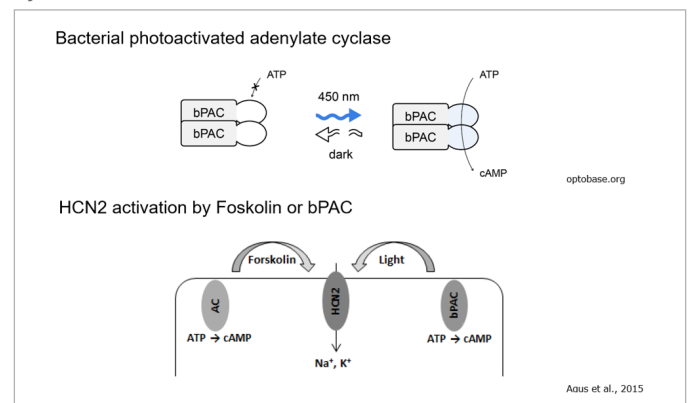
- Larger HCN2 mediated currents
- Faster HCN2 activation kinetics
- A depolarized shift in the voltage dependence of activation

### Introduction

Nature has evolved a wide range of light-activated proteins, and in the fast-developing field of optogenetics, these have been converted into tools for studying protein function, membrane currents, and signaling pathway activation. Where automated patch clamp allows precise voltage and current control, and fast chemical modulation from the outside, activation of light activated proteins, using the optogenetics feature, provides a new method to modulate the cell intracellularly.

Here, we employed the light-activated bacterial enzyme, bPAC, to modulate protein phosphorylation through cAMP production, and evaluated the effect on the kinetics of the HCN2 ion channel (Fig. 1).

### Optical modulation of intracellular pathways. HCN activation by forskolin



**Fig. 1:** bPAC activation results in cAMP production which modulates HCN2 channel kinetics, similar to forskolin mediated HCN2 modulation.

### bPAC adenylyl cyclase

bPAC is a photoactivated adenylyl cyclase from *Beggiatoa*, a sulfide-oxidizing bacterium<sup>1,2</sup>. bPAC has an absorption maximum at 441 nm, and when activated by light, the enzyme converts ATP into cAMP, thereby increasing the intracellular cAMP level (Fig. 1)<sup>1,3</sup>. This leads to activation of a myriad of cellular reactions, and intracellular signaling pathways<sup>3,4</sup>. Protein kinase A (PKA) is one of the cellular proteins that is regulated by cAMP levels. When cAMP binds to PKA, it becomes enzymatically active and phosphorylates target proteins.

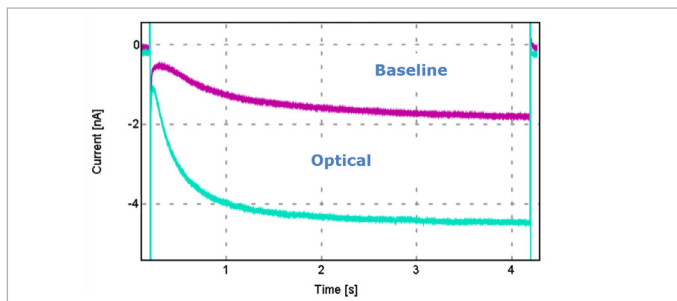
### HCN2 ion channels

Hyperpolarisation-activated cyclic nucleotide modulated ion channels (HCN) are, to a high degree, regulated by PKA<sup>5,6</sup>. It is a family of ion channels best known for their fundamental pacemaker role in the heart. Modulation of cardiac activity by cAMP is attributed mainly to HCN4, the principal isoform expressed in the SA node, whereas HCN2 has a supporting role<sup>7,8</sup>. HCN2 channels are also present in neuronal tissues, and are potential targets for therapeutic intervention in neuropathic pain<sup>5,9</sup>.

## Results

### Optical stimulation of bPAC changes the HCN2 activation kinetics

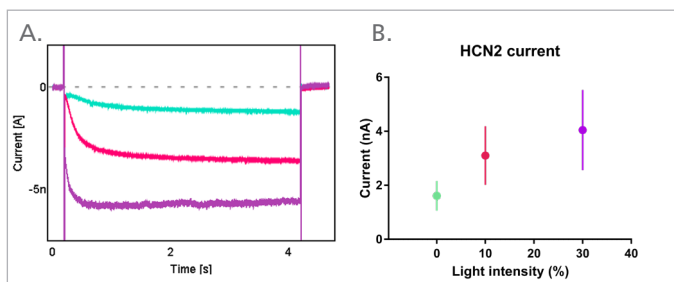
To evaluate the bPAC mediated modulation of the HCN2 ion channel kinetics, we activated HCN2 by stepping the membrane voltage from -30 mV to -90 mV for four seconds (Fig. 6, Methods). The optical stimulation did indeed both change current size, and activation kinetics (Fig. 2). The change in current size, and the activation kinetics were evaluated both before onset of the optical stimulation protocol and five minutes into the optical stimulation. Five minutes of stimulation resulted in a 95% ( $\pm 37\%$ ,  $n=349$ ) increase in max current from  $(1.6 \pm 0.5)$  nA to  $(3.1 \pm 0.3)$  nA, and the activation time constant ( $\tau$ ) shifted from  $(903 \pm 32)$  ms to  $(357 \pm 14)$  ms upon optical stimulation of bPAC (average  $\pm$  SD,  $n=349$ , Fig. 2).



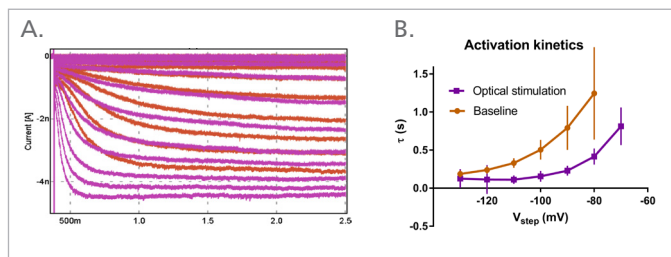
**Fig. 2:** Typical HCN2 response to a voltage step from -30 mV to -90 mV for four seconds (Fig. 6, Methods), before (purple) and five minutes into the optical stimulation (green). The maximum current increased ( $95 \pm 37\%$ ), and the average  $\tau$  value shifted from  $(903 \pm 32)$  ms to  $(357 \pm 14)$  ms. Light intensity: 10%. Stated values are the average  $\pm$  SD of  $n=349$  experiments.

### Optical stimulation and IV relationship

To further characterize how the bPAC-mediated increase in cAMP changed HCN2 activation kinetics, both the voltage and the light dependence was investigated. Increasing the light intensity from 0% to 30% resulted in a significant ( $>2$  nA) increase in HCN2 mediated current (fig. 3). In response to both increasing voltage steps, and to optical stimulation of bPAC, the HCN2-mediated current amplitude increased, and  $\tau$  decreased (Fig. 4A). Quantification of  $\tau$  (Fig. 4B) demonstrates that the effect of optical stimulation on the activation time increases when the voltage is stepped to more hyperpolarized potentials.



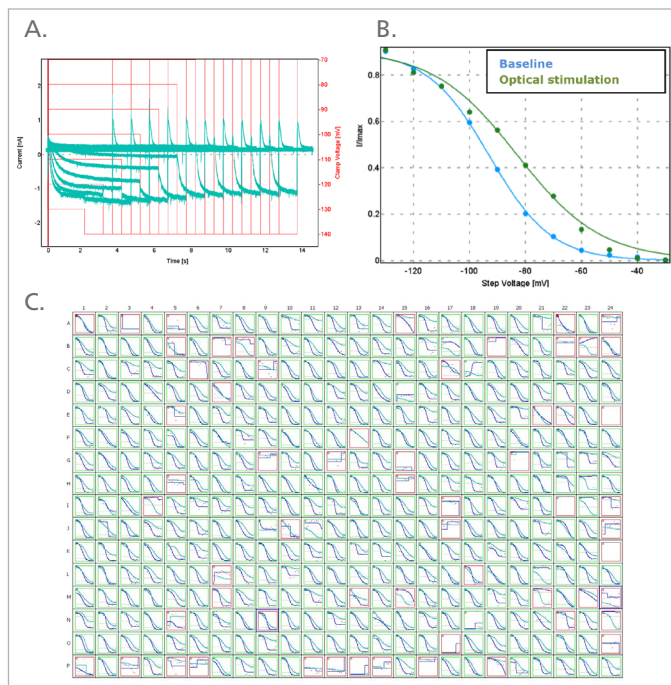
**Fig. 3:** A) Typical HCN2 response to increasing light intensities, 0 % (green), 10 % (red) or 30 % (purple), after at least 5 minutes of optical stimulation. B) Average stable current from a 200 ms interval at the end of the voltage step, showing the effect of optical stimulation on current size. Data points are average  $\pm$  SD of  $n=348$  experiments.



**Fig. 4:** A) Typical HCN2 response to increasing voltage steps before (red), and after (purple) five minutes of optical stimulation. B) The effect of optical stimulation on the activation kinetics. Only time constants with an average lower than 2 s were included in the analysis. Data points are average  $\pm$  SD of  $n=219$  experiments.

### Optical stimulation shifts the voltage dependence of activation

The normalized current-voltage relationship was evaluated ( $I/I_{max}$ , Fig. 5) to demonstrate how optical stimulation, through cAMP elevation, mediates a shift in HCN2 voltage dependence. For this purpose, each voltage step of an IV-protocol was followed by a 1.5 s step to -140 mV to reveal the maximum current. At low hyperpolarized steps, up to 12 seconds was needed for full HCN2 opening, but as large HCN2-mediated currents can be cytotoxic over a longer period, the voltage steps decrease in duration with increasing hyperpolarization (for protocol and calculation see Fig. 7 in the method section). A Boltzmann fit to the  $I/I_{max}$  values revealed a shift of  $(8.7 \pm 3.1)$  mV in  $V_{1/2}$  from  $(-90.7 \pm 5.8)$  mV to  $(-81.5 \pm 4.9)$  mV upon optical stimulation (Fig. 5, 10% light intensity,  $n=324$ ).



**Fig. 5:** A) Typical HCN2 current in response to an IV-protocol without optical stimulation, where the duration decreases with increasing hyperpolarization voltage-steps (see methods for details). B) A Boltzmann fit to the normalized current recorded before and after optical stimulation. C) Experiment plate view displaying the resulting Boltzmann fits in a 384 well plate. The Boltzmann fit to the  $I/I_{max}$  values revealed an  $(8.7 \pm 3.1)$  mV depolarized shift in  $V_{1/2}$  upon optical stimulation, ( $n=324$ , Average  $\pm$  SD).

## Methods

### Cells and ringer solutions

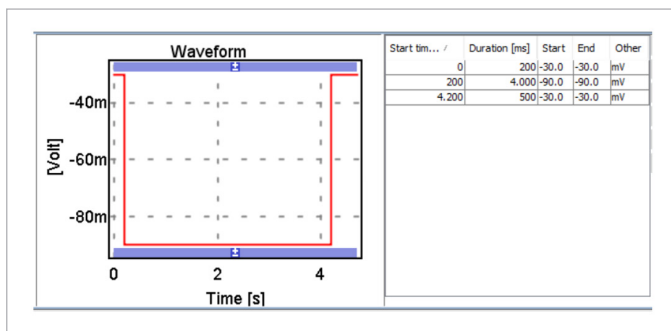
Cell line: HCN2/bPAC-HEK293 cell line kindly provided by Axxam SpA, Milan, Italy

Intracellular saline: Sophion F-free IC

Extracellular saline: Sophion EC

### Voltage and light protocols

Cells were clamped at -30 mV and the excitation wavelength was 475 nm. After a baseline period, light was switched on for 500 ms every two seconds. After five minutes, the light was switched off to ensure reversibility.



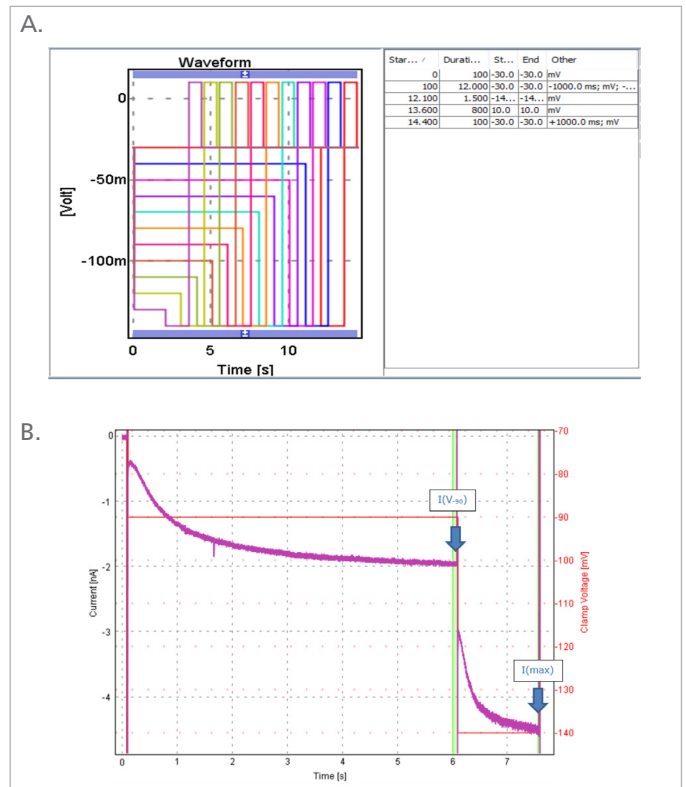
**Fig. 6:** Voltage protocol with a four seconds voltage step from -30 mV to -90 mV for HCN2 activation.

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**Fig. 7:** A) The step protocol employed to investigate the impact of optical stimulation on HCN2 activation. The current is elicited by decreasing membrane voltages (from -30 mV to -130 mV) in steps. Up to 12-second long voltage steps were needed at low hyperpolarisations to reach a stable current. However at large hyperpolarizing steps, the large current is cytotoxic, hence the length of the voltage step was shortened as the step voltage applied became more negative (spanning from 12 s at -30 mV to 2 s at -130 mV). Following the voltage step, a 1.5 s step to -140 mV was used to find the max current. B) A typical HCN2 response to the -90 mV step in the voltage protocol displayed in Fig. 7A to demonstrate how  $I_{(V-90)}$  is determined. Two cursor intervals (200 ms, green) are inserted for measuring the average current at the individual voltage steps (here  $I_{(V-90)}$ ), and from a -140 mV hyperpolarizing step ( $I_{(max)}$ ).

### Author:

Kim Boddum, Application scientist, Sophion Bioscience A/S  
Jean-Francois Rolland, Head Electrophysiology, Axxam SpA