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HELICES D AND E OF HCN4 CHANNEL TUNE THE CAMP RESPONSIVENESS

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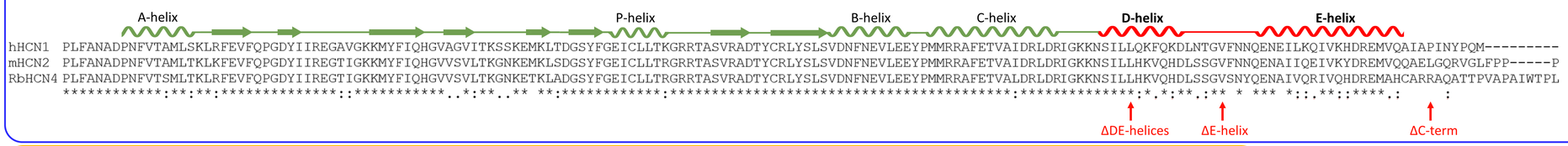
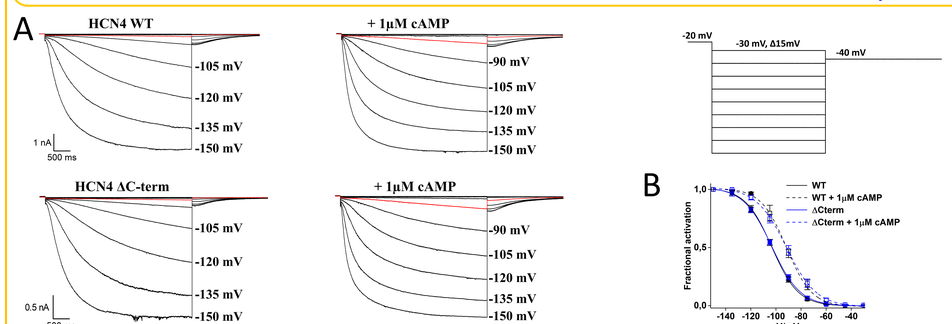
Introduction

Hyperpolarization-activated cyclic-nucleotide regulated (HCN) channels are non-selective cation channels controlling key physiological functions such as cardiac pacemaking and repetitive neuronal firing. They are activated by voltage and modulated by binding of cAMP to their cytoplasmic C-terminal region named cyclic nucleotide binding domain (CNBD)[1]. Altered cAMP responses caused either by single point mutations in the protein or by non-physiological levels of the cyclic nucleotide in the cell lead to pathological conditions both in the heart and in the nervous system[2,3]. The recently obtained cryo-EM structure of human HCN1 confirms previous NMR studies on cAMP-induced conformational changes and reveals the presence of two additional helices at the C-terminus of the CNBD which fold in the presence of cAMP[3]. Despite the CNBD regions of HCN1,HCN2 and HCN4 channels exhibit high sequence similarity, some worthy differences are observed within the D-E helices sequence, suggesting possible variations of their role in the different isoforms. We have previously shown, by ITC and patch clamp, that deletion of these helices (D and E) in HCN1 and in HCN2 decreases the affinity for cAMP Here we test the role of helices D and E in HCN4 in order to extend our study to the cardiac channel isoform. Measurements were performed using a new all-in-one integrated amplifier prototype, designed and developed by Elements SRL, the ePatch. The miniaturized ePatch amplifier is USB plug-n-play and integrates the analog front-end with multiple gains stage, the analog-to-digital converter and filters all into a single small headstage without any external bulky case.

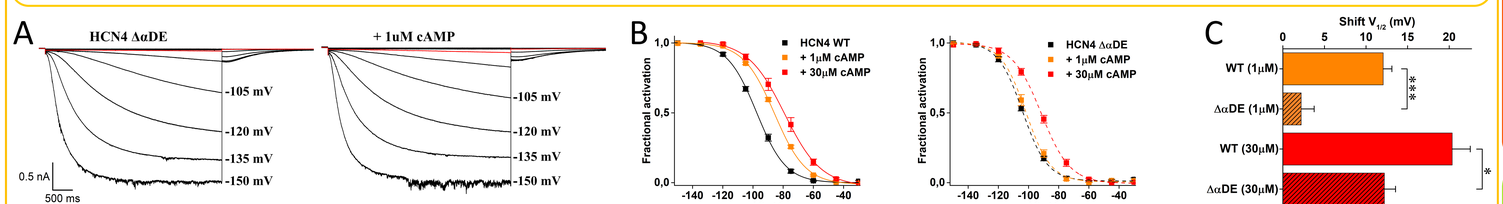


Deletion of the C-terminus downstream of DE helices does not affect cAMP response of HCN4 channel expressed in HEK293T cells



A, Representative current traces of RbHCN4 WT (top) and ΔC-term mutant (bottom) in control solution and in the presence of sub-saturating concentration (1μM) of cAMP in the pipette solution. The potential acquired at –75mV is highlighted in red. The applied voltage step protocol is shown (top, right). Currents were recorded in whole cell configuration, at R.T., 24h after transfection of HEK-293T cells with HCN4 WT and ΔC-term mutant cDNAs. **B,** Activation curves obtained from tail currents collected at -40mV. Fitting the Boltzmann equation to data yielded the following values of half activation voltage ($V_{1/2}$) and inverse slope factor (s). HCN4 WT (black continue line), $V_{1/2}$ = -103.3 ± 1.5 mV, s= 10.5 ± 0.8 mV, N=5; HCN4 WT + 1μM cAMP (black dashed line), $V_{1/2}$ = -91.6 ± 1.1 mV, s= 10 ± 1 mV, N=3; HCN4 ΔC-term (blue continue line), $V_{1/2}$ = -103.2 ± 1.2 mV, s= 11.3 ± 0.9, N=5; HCN4 ΔC-term + 1μM cAMP (blue dashed line), $V_{1/2}$ = -91.7 ± 1,2 mV, s= 11.9 ± 0.9. mV, N=4. All data were acquired at 5 kHz (SR) using ePatch saved in .abf format and analyzed offline.

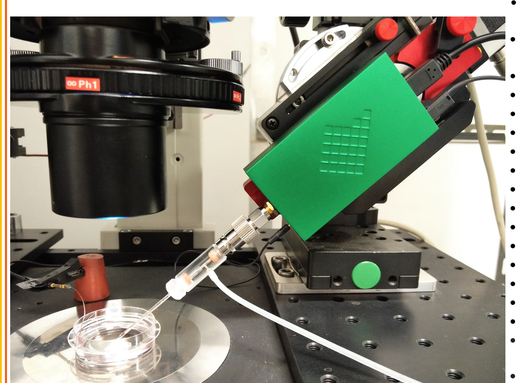
Deletion of the C-terminus upstream of the DE helices decreases cAMP response of HCN4 channel expressed in HEK293T cells



A, Representative current traces of HCN4 ΔαDE mutant in control solution and in the presence of subsaturating concentration (1μM) of cAMP in the pipette solution. Experimental condition and acquisition protocol are the same described in the upper panel. In red is highlighted the potential recorded at –75mV. **B,** Activation curves obtained from tail currents collected at –40mV of WT and mutant in control solution and in presence of sub-saturating (1μM) and saturating (30μM) concentration of cAMP in pipette solution. Fitting the Boltzmann equation to data yielded the following values of half activation voltage ($V_{1/2}$) and inverse slope factor (s). Left, HCN4 WT (black continue line), $V_{1/2}$ = -97.9 ± 0.8 mV, s= 9 ± 0.9 mV, N=10; HCN4 WT + 1μM cAMP (orange continue line), $V_{1/2}$ = -85.8 ± 0.6 mV, s= 10.3 ± 1.1 mV, N=5; HCN4 WT + 30μM cAMP (red continue line), $V_{1/2}$ = -79.5 ± 2 mV, s= 11.8 ± 1.6 mV, N=3; HCN4 ΔαDE (black dashed line), $V_{1/2}$ = -103.9 ± 0.7 mV, s= 8.3 ± 0.6, N=6; HCN4 ΔαDE + 1μM cAMP (orange dashed line), $V_{1/2}$ = -101.7 ± 1,4 mV, s= 8.2 ± 0.7 mV, N=5; HCN4 ΔαDE + 30μM cAMP (red dashed line), $V_{1/2}$ = -91.7 ± 1.1 mV, s= 9.2 ± 0.9 mV, N=5. **C,** Mean shift induced by 1μM or 30 μM cAMP on HCN4 WT and ΔαDE mutant calculated from activation curves shown on the left. Shift values are as follow: HCN4 WT + 1μM cAMP shift $V_{1/2}$= 12 ± 1 mV; HCN4 ΔαDE + 1μM cAMP shift $V_{1/2}$= 2.2 ± 1.5 mV; HCN4 WT + 30μM cAMP shift $V_{1/2}$= 20.3 ± 2 mV; HCN4 ΔαDE + 30μM cAMP shift $V_{1/2}$= 12.2 ± 1.3 mV. ***,P<0.001; *<0,05 using t-student test. All data were acquired at 5 kHz (SR) using ePatch amplifier, saved in .abf format and analyzed offline.

ePatch details

The miniaturized **ePatch** amplifier integrates in a small headstage the low-noise amplifier, the pulse generator and the digitizer into only 42 x 18 x 78 mm and it is connected directly to the USB port of a laptop without the need of any other external bulky digitizer. The ePatch amplifier is controlled by the EDR3 EPISODIC software interface, developed and released by Elements, that enables an easy control of the amplifier and displays data in real time, both in the gap-free or episodic modalities. ePatch features follow:



- Open input (RMS) noise: 115 fA rms @ 1kHz - 460 fA rms @ 10 kHz - 3.6 pA rms @ 100 kHz
- Current ranges: ±200pA (Gain 2.25GΩ), ±2nA (Gain 225MΩ), ±20nA (Gain 22.5MΩ), ±200nA (Gain 2.25MΩ)
- Max sampling rate: 200 kS/s
- Parametric voltage protocols for pulse generator in the range of ± 500 mV
- C-fast - C-slow - R-series - P/N compensations
- C fast compensation range: 0 - 11 pF
- C Slow compensation ranges: C in 0 - 250 pF, τ in 0 - 3300μs
- R series correction ranges: R in 0 - gain, τ in 0 - 1000μs
- R series correction ranges: τ in 0 - 1000μs
- Automatic electrode voltage offset fine compensation
- Continuous C-membrane and R-seal estimation
- Programmable digital I/O
- Zap pulse
- USB powered
- Dovetail or rod bar mounting
- Compatible with standard pipette holders (BNC or SMA connectors)
- Software real-time data analysis (I/V graph for channel conductance and reversal potential, FFT, etc.)
- Real time membrane resistance, access resistance and capacitance monitoring software tool (R and C values can be automatically saved for every sweep)
- data outputs saved in different formats: .dat (e.g. Matlab™), .abf (e.g. pClamp™)

Conclusions and future perspectives

Our results show that D and E helices of HCN4 channels are involved in the control of cAMP affinity suggesting that this regulatory mechanism is conserved among the three most represented isoforms (HCN1, HCN2, HCN4). Further patch clamp experiments will be performed on HCN4 ΔαE mutant in order to clarify the individual role of D and E helices. Notably, a deletion of DE helices studied in 2002 by another research group showed an alteration of the cAMP response in HCN4 expressed in CHO cells[5]. Our recordings demonstrate the high-quality data acquired by the ePatch, which are comparable to those acquired by state-of-the-art amplifiers in terms of signal to noise ration and data content.

References

[1] R. B. Robinson and S. A. Siegelbaum 2003, *Annu. Rev. Physiol.* 65:453 [2] M. Baruscotti et al 2017, *Eur. Heart J.*38:280 [3] E. C. Emery et al 2012, *Trends Pharmacol. Sci.* 33:456 [4] C. Lee and R. Mackinnon 2017, *Cell* 168:111 [5] Liao et al 2002, *J Gen. Physiol.*, 140(5):557-66

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