

Characterization of hCIC-1 on the SyncroPatch 384

The electrophysiology team at Nanion Technologies GmbH, Munich. Cells were kindly provided by Charles River.



Summary

The voltage-gated chloride channel gene (CIC) family is highly conserved and their members are present in both pro- and eukaryotes. In mammals, nine different CIC subtypes have been identified, which differ according to tissue distribution and subcellular location. CIC-1 is exclusively expressed in skeletal muscles¹.

CICs function as homodimers²⁻⁴, allowing chloride and other anions to be conducted through each single protopore. Gating of the single monomer is fast while the common gate to open and close the pores simultaneously is slow. The channel gating can be modulated by intracellular⁵ and extracellular chloride^{6,7}, as well as pH⁸.

CIC proteins mediate chloride flux across cellular membranes in most cell types and participate in maintenance of resting membrane potential. Plasma membrane chloride channels play an important role in reducing muscle excitability¹. CIC-1 contributes to membrane repolarization and stabilizes the membrane voltage in skeletal muscle. Experimental block of the chloride conductance mediated by CIC-1 facilitated muscle hyperexcitability, manifested as myotonia⁹.

Here we present data conducted on the SyncroPatch 384 showing characteristic biophysical properties and pharmacology of hCIC-1 expressed in CHO cells. We applied voltage protocols including various test potentials to study the voltage dependence of compounds. In order to investigate compound binding properties we used the internal perfusion system of the SyncroPatch 384 for direct application of anthracene-9-carboxylic acid (9-AC) to the intracellular environment. Moreover, we also investigated the effect of 9-AC and niflumic acid (NFA) when they are applied from the extracellular side.

Results

We first studied the properties of CIC-1 mediated currents in CHO cells. The current was isolated from K_v by using Cs^+ in the internal solution. Currents were obtained by application of a voltage step protocol (Fig. 1A) and showed characteristic fast activation and deactivation at hyperpolarization and saturation at positive potentials (Fig. 1B). To study the gating behaviour of CIC-1 deactivation curves were further analyzed. Half maximal activation (V_{half}) of the tail current (I_{tail}) was estimated to be -2.6 ± 1.6 mV ($n = 384$).

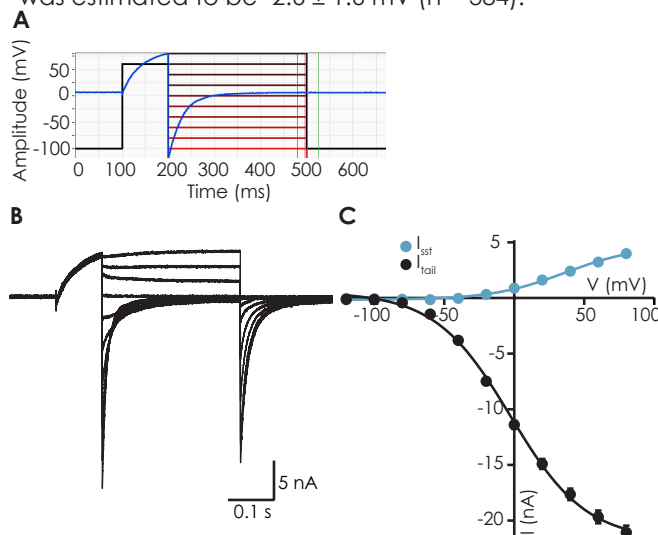


Figure 1: Voltage-dependent activation of hCIC-1. Currents were elicited by 300 ms voltage steps from 80 mV decreasing in 20 mV increments to -120 mV following a pre-pulse from a holding potential of -100 mV to 60 mV for 100 s, as illustrated in **A**. **B**. Example traces of currents obtained. The inward current typically deactivates with persisting hyperpolarization. Mean currents obtained at the end of the various voltage step (I_{sst}) and minimum current 1 ms after stepping the voltage back to -100 mV (I_{tail}) were further analyzed. **C**. Average current plotted against voltage of 384 cells. Blue indicates the average steady-state current of various test potential, black the average tail current. Data were fitted by a Boltzmann equation. Errors represent SD in all graphs.

Application Note

We used a low intracellular chloride concentration which explains the discrepancy of V_{half} compared to literature values¹⁰ as CIC gating has been shown to be strongly dependent on ionic strength.

Niflumic acid (NFA) and anthracene-9-carboxylic acid (9-AC) are common known pharmacological tools that are used to gain detailed insights into CIC-1 channel biophysics and to induce myotonia in animal models^{11,12}. Both compounds have been shown to affect CIC-1 mediated currents by acting from either the intracellular or extracellular side. For 9-AC, a hydrophobic binding pocket inside the CIC-1 channel has been proposed¹³, whereas for NFA, a non-steroidal anti-inflammatory drug, the binding site within the channel is still unknown.

In the experiment in Figure 2, different concentrations (10 μM , 30 μM , 60 μM) of 9-AC were perfused on the intracellular side of the cells, while currents were continuously recorded over time. The voltage protocol to activate CIC-1 mediated currents is given in Figure 2A. 9-AC_{in} blocks CIC-1 mediated currents at -125 mV in a concentration-dependent manner yielding an IC_{50} of $10.2 \pm 2.7 \mu\text{M}$ ($n = 320$).

In comparison, Figure 3 shows concentration response curves for 9-AC and NFA after extracellular application. The current at -125 mV was blocked by 75 % with 300 μM NFA and completely abolished after 60 μM 9-AC confirming the hypothesis that both compounds can easily permeate the plasma membrane to enter the inner side of the cell and reach their binding pockets¹⁵. NFA yielded an IC_{50} of $99.6 \pm 6.7 \mu\text{M}$ ($n = 184$), 9-AC an IC_{50} of $2.4 \pm 0.4 \mu\text{M}$ ($n = 174$). These values are consistent with those found in literature^{1,14}.

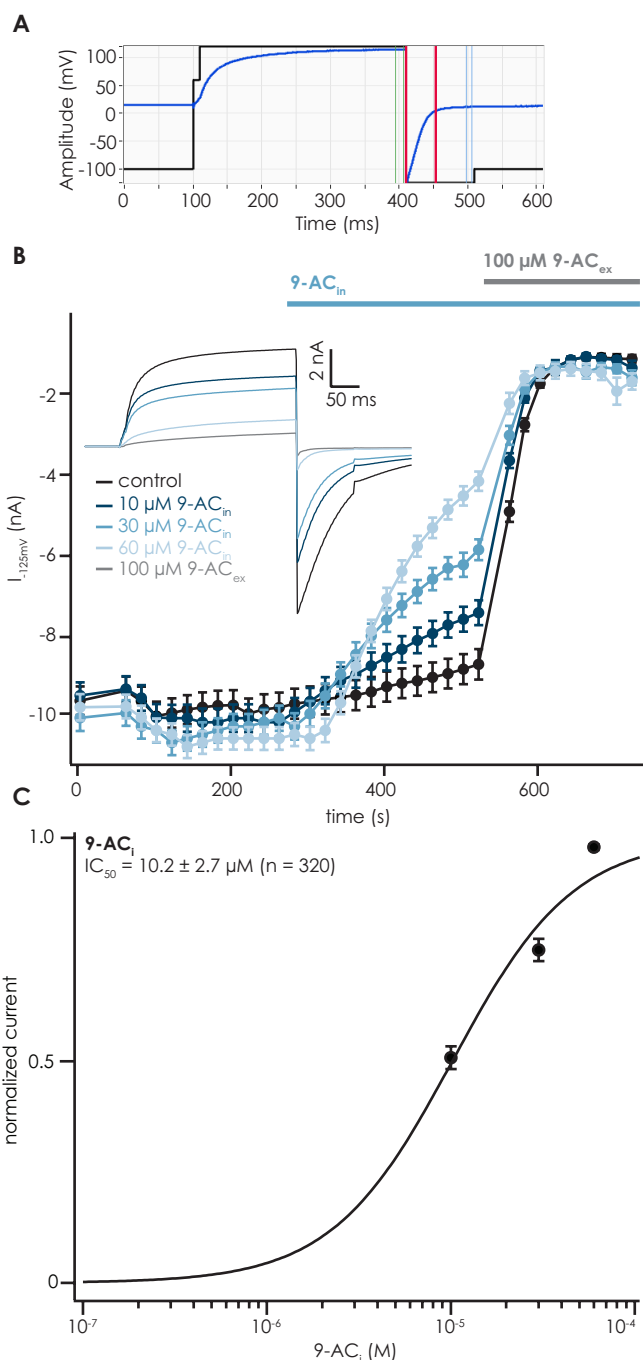


Figure 2. Intracellular perfusion of 9-AC blocks CIC-1 in a concentration-dependent manner. **A.** A triple-voltage step protocol was used to activate hCIC-1. From a holding potential of -100 mV, the first step was made to +60 mV for 10 ms, followed by a step to +100 mV for 300 ms and a third potential to -125 mV for 100 ms. **B.** Average traces from 335 cells expressing hCIC-1 activated by the voltage protocol shown in A. Time plot for an average of 82 (black), 82 (dark blue), 77 (blue) and 66 (light blue) cells receiving the indicated compound concentration from the intracellular side. Grey indicates full block by 100 μM extracellular 9-AC. Shown are mean of peak current recorded at -125 mV $\pm$ S.D. **C.** Concentration response curve was calculated across the plate. Intracellular application of 9-AC yielded an IC_{50} of $10.2 \pm 2.7 \mu\text{M}$ ($n = 320$).

Application Note

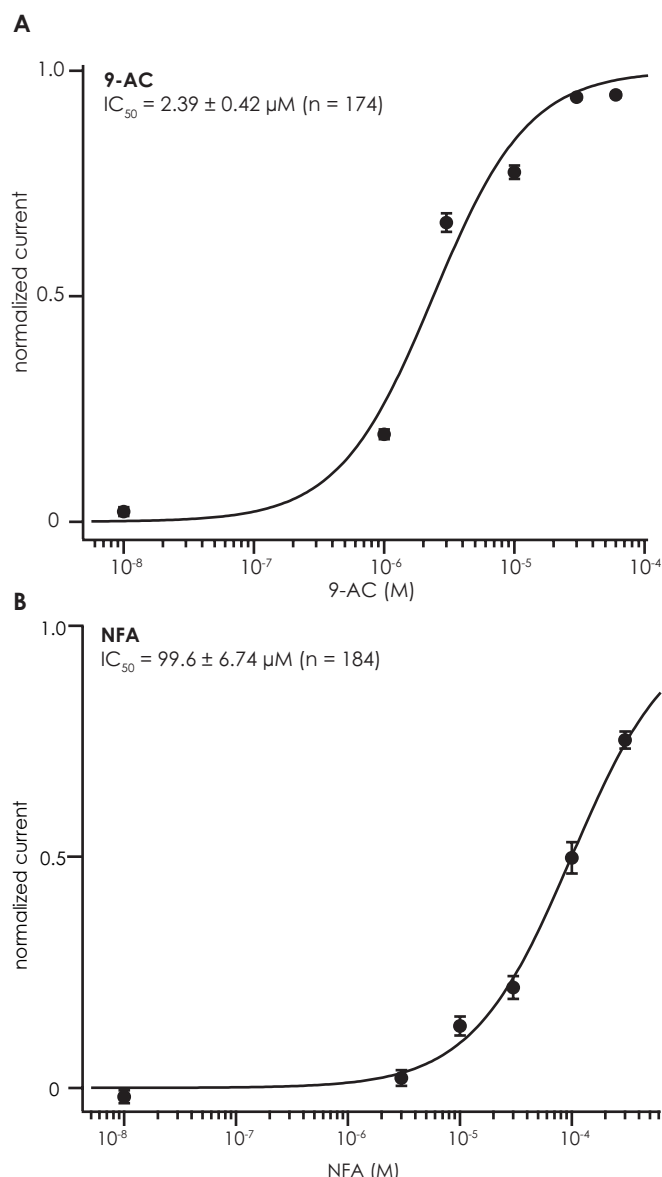


Figure 3: Extracellular application of NFA and 9-AC block CIC-1 mediated currents. The concentration response curves were calculated across the plate using a full block concentration (100 μM 9-AC) at the end of the experiment for normalization. **A.** The concentration response curve for 9-AC yielded an IC_{50} of $2.39 \pm 0.42 \mu M$ (n=174). **B.** The concentration response curve for NFA revealed an IC_{50} of $99.6 \pm 6.74 \mu M$ (n=184). Each compound was applied on one half of a single-hole NPC-384.

On the other hand, currents at +100 mV were less efficiently blocked by NFA: $IC_{50} = 389 \mu M$ and 9-AC: $IC_{50} = 7.1 \mu M$, confirming the voltage-dependent nature of the current block by these compounds¹⁶. Figure 4 shows the effect of 9-AC on the CIC-1 gating.

Steady-state current recorded at -125 mV was normalized to the maximum current amplitude of the same potential and plotted against the voltage step of the pre-pulse. Increasing concentrations of 9-AC shifted the V_{half} of deactivation to the right. Similar observations have been made for NFA. V_{half} values for respective 9-AC and NFA concentrations are summarized in Table 1.

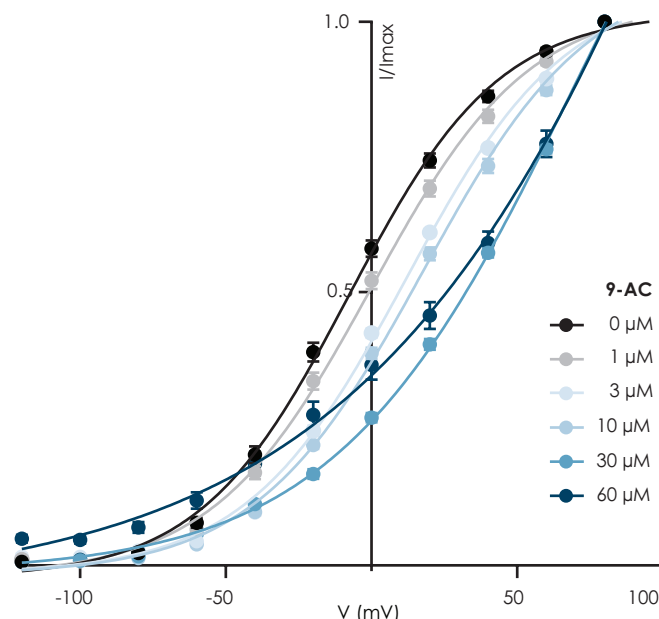


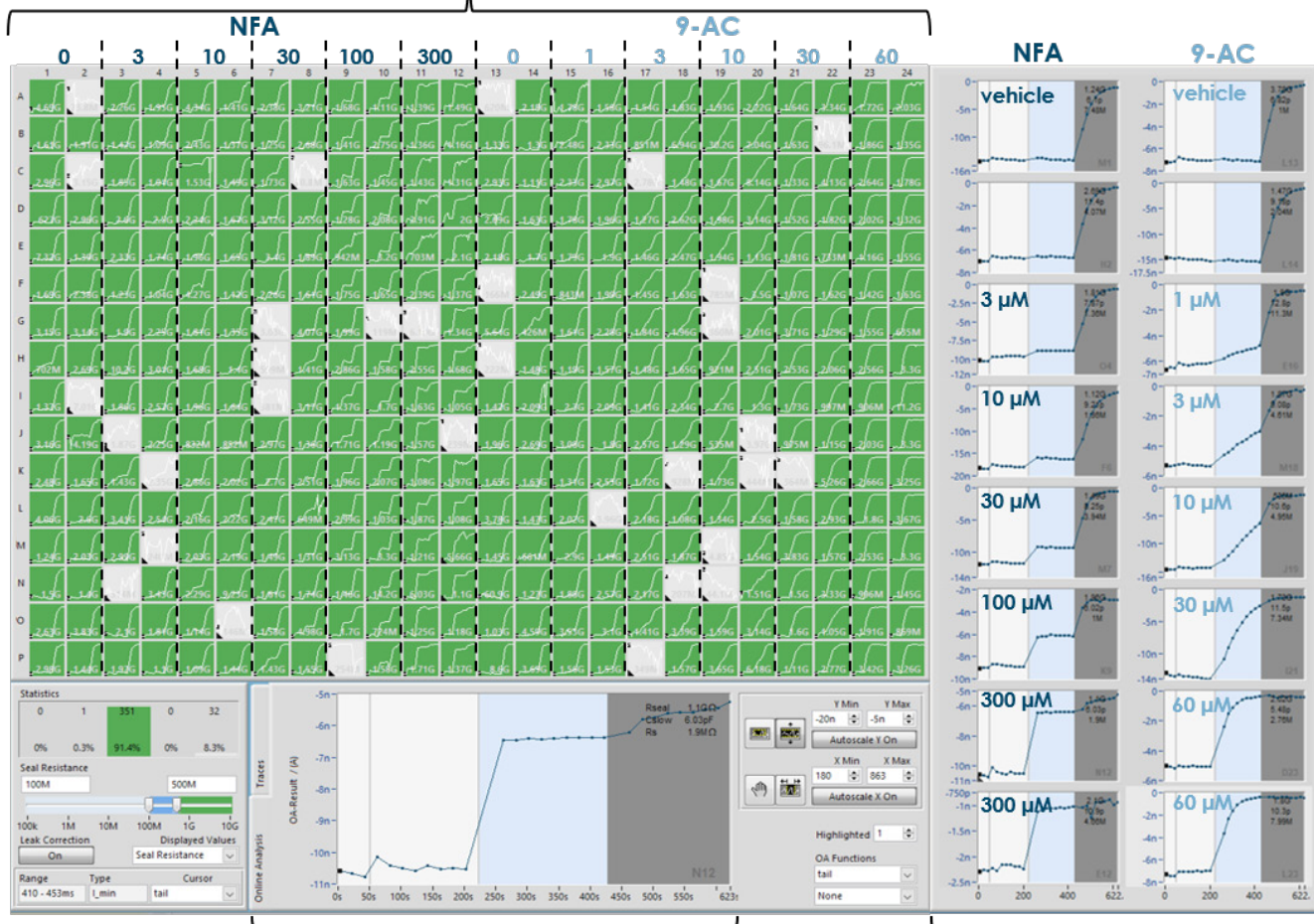
Figure 4: 9-AC shifts the V_{half} to depolarized potentials in a concentration dependent manner. Steady-state current at -125 mV normalized to the maximum current amplitude recorded at -125 mV was plotted against the various pre-pulses. For concentrations indicated, V_{half} was calculated by fitting the data points with a Boltzmann equation and summarized in Table 1.

Compound	Concentration (μM)	$V_{half} \pm SD$ (mV)	n
9-AC	0	-7.7 ± 1.5	31
	1	-0.3 ± 1.7	32
	3	11.8 ± 1.9	32
	10	17.6 ± 2.4	32
	30	70.6 ± 6.9	32
	60	174.3 ± 124	32
NFA	0	-6.5 ± 1.5	32
	3	-4.8 ± 1.4	32
	10	-1.5 ± 1.7	31
	30	13.5 ± 2.9	32
	100	20.7 ± 3.2	32
	300	32.6 ± 2.7	31

Table 1: Summary of V_{half} of deactivation for different concentrations of 9-AC and niflumic acid (NFA) recorded in one experiment. V_{half} values are presented as mean $\pm$ SD, n is the number of cells included for analysis. The experiment was done on one single-hole NPC384 chip.

Application Note

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



Highlighted time plot of 1 cell showing inhibition of peak current by NFA

Highlighted time plots of 16 cells showing inhibition of peak current by NFA or 9-AC

Figure 5: Graphical user interface of the screening and data analysis software used on the SyncroPatch 384. Screenshot of depiction of online analysis data of hCIC-1 expressing CHO cells as recorded on one NPC-384 (single-hole) patch clamp chip. Three hundred and eighty-four small color-coded pictures as seen in the upper left part display 384 recordings. One highlighted experiment is displayed at the bottom, 16 selected experiments are displayed on the right. Graphs show current amplitudes after vehicle addition (white region), followed by block by different concentrations of NFA or 9-AC (blue region; concentrations indicated) and finally full block with 100 μ M 9-AC (grey region).

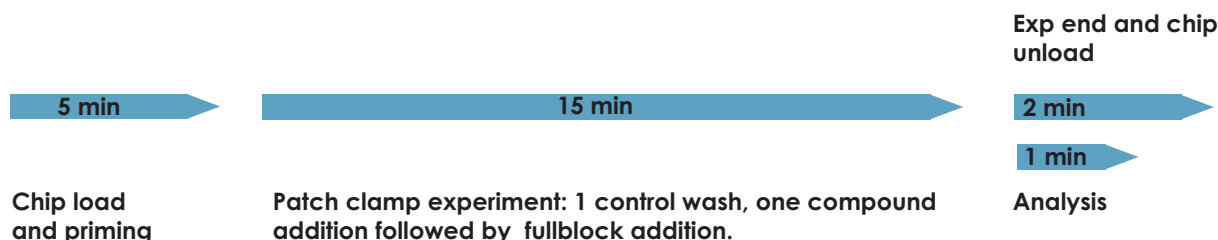


Figure 6: The completion of one experiment on the SyncroPatch 384 patch clamp chip (384 wells) for a single point concentration response curve including full block at the end of the experiment took approximately 23 minutes.

Application Note

Figure 5 shows a screenshot of the SyncroPatch 384 software during an experiment. The user can choose whether to visualize raw traces or online analysis. In this case, online analysis is chosen and the graphs represent current amplitude at hyperpolarization plotted against time. An individual well can be highlighted to monitor the progression of the experiment. In the Online Analysis view, the time points at which solution additions have been made are indicated by vertical lines, as well as different background colors. In this case, white shows activated hCIC-1 recorded in vehicle solution, blue shows inhibition by different concentrations of NFA or 9-AC and grey shows full block with 100 μ M 9-AC.

In conclusion, hCIC-1 expressed in CHO cells can be recorded on the SyncroPatch 384. The internal perfusion feature of the SyncroPatch 384 was used to block hCIC-1 using different concentrations of 9-AC and the typical slow decrease in the current could be stably monitored online for more than 20 minutes. This continuous monitoring of current amplitude is important for distinguishing compound

effect from increase in leak or loss of seal and can be a benefit for assay development. Voltage step protocols before and after intracellular application of 9-AC were applied to investigate the voltage dependence of the compound. The timeline of each experiment was about 23 minutes (start – end, Fig. 6) and included wash, block with internal perfusion of 9-AC, single compound concentration application and full block, all performed on each individual well, drastically reducing the consumable cost per data point to < \$0.60.

The SyncroPatch 384 is a high throughput and highly reliable automated patch clamp device for recording hCIC-1 mediated chloride currents. User-friendly software, excellent success rates, single additions or multiple additions of compound to each cell, perfusion of the internal solution 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

hCIC-1 expressing CHO were provided by Charles River.

Cell culture

Cells were cultured according to the manufacturer's recommendations and harvested according to Nanion's standard protocol using TrypLE.

Electrophysiology

Whole cell and perforated patch clamp recordings were performed according to Nanion's standard procedure for the SyncroPatch 384 using single hole chips. Currents were elicited by voltage steps of 300 ms from 60 mV to values between 80 mV and -120 mV in steps of -20 mV following a prepulse from a holding potential of -100 mV to 60 mV for 100 s. The intersweep interval was 20 s. Pharmacological experiments were performed every 20 s by continuous recording of the current elicited by a voltage step protocol starting from a Vhold of -100 mV, followed by a pre-pulse to +60 for 10 ms and a step to 120 mV for 300 ms, followed by a tail potential of -125 mV for 100 ms before going back to Vhold.