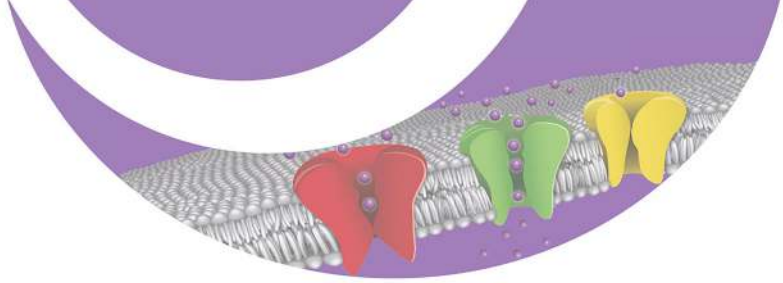
metrion biosciences



Application Report

ASIC1a ligand-gated ion channel assay

Rapidly activating and desensitising ligand-gated ion channel receptors can provide a technical challenge on automated patch clamp electrophysiology platforms. This can affect their biophysics, pharmacology and assay reliability. We present data on an optimised and validated acid-activated receptor assay on the QPatch that is stable enough for drug discovery screening.

Summary

- Metrion has developed a robust and cost-effective ASIC1a ion channel assay on the QPatch48 gigaseal quality automated patch clamp platform.
- The assay was pharmacologically validated using a toolbox of reference compounds.
- The validated assay is capable of supporting medium throughput screening and SAR campaigns.

Introduction

Acid-Sensing Ion Channels (ASICs) are voltage-insensitive Na^+ channels that are classified in a subfamily of the degenerin/epithelial Na^+ channel (ENaC) superfamily[1]. Four *ASIC* genes *(ASIC1, ASIC2, ASIC3*, and *ASIC4*) have been identified in mammals, which encode a total of six ASIC subunit isoforms, including ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4[2]. Each subunit contains two transmembrane domains and a pore-forming region (Figure 1A), and functional channels are composed of three subunits (Figure 1B). The subunits are capable of forming either homo- or heterotrimers, which exhibit different sensitivities to activation by extracellular protons[2]. ASIC subunits possess large extracellular regions that contain the proton binding site, which upon occupation can allosterically activate the channel and open the cation-conducting pore.

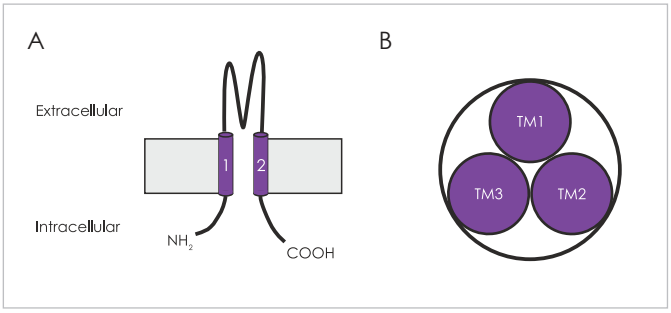


Figure 1: The putative membrane topology of ASIC subunits
(A) ASIC subunits are composed of two transmembrane domains and a pore region.
(B) Functional ASIC channels are composed of three subunits and can form homo- or heterotrimers.

ASIC1a is expressed in various regions of the brain, including the hippocampus, cerebral cortex, cerebellum, and amygdala[3] Elevated neural activity can lower the pH of the extracellular environment into a range capable of activating ASIC1a channels, which leads to an influx of Na^+ and depolarisation of the cell membrane. Additionally, homomeric ASIC1a channels are permeable to Ca^{2+} so excessive activation can contribute to neurotoxicity[4,5]. Consequently, ASIC1a has been implicated as a therapeutic target in a range of neurological indications associated with pathophysiological extracellular acidification, such as ischemia, multiple sclerosis and seizures. For example, genetic ablation of ASIC1a was capable of reducing infarct size by ~60% in an *in vivo* model of ischaemic stroke[5]. Additionally, intracerebroventricular administration of the potent ASIC1a inhibitor Hi1a markedly reduced infarct size up to 8 hours after stroke induction, which correlated with improved neurological and motor function and preservation of neuronal architecture[6].

There is also strong evidence supporting the role of ASIC1a ion channels in nociception. ASIC1 subunits are expressed in dorsal root ganglia neurons and in peripheral nerve terminals, where they respond to inflammatory-mediated changes in pH. The functional expression of ASIC1a in peripheral neurones is demonstrated by the intense and unremitting pain that can be elicited by the selective ASIC1 activator, MitTx, in patients envenomated by the Texas coral snake[7]. In contrast, the ASIC1a inhibitors, PcTx1 and Mambalgin, which are derived from spider and snake venoms respectively, reduce thermal and mechanical hyperalgesia in *in vivo* pain models[8,9]. Clinical validation was obtained by PainCeptor, whose ASIC1 inhibitor, PPC 5692, reduced heat threshold and mechanical sensitivity in a UV-B inflammatory pain biomarker & target engagement study in Ph I patients.

Given the interest in ASIC1a channels as a CNS injury and pain therapeutic target there is considerable demand for reliable assays to support drug discovery efforts. We report the development and validation of a robust and cost-effective ASIC1a assay on the QPatch48, which is a gigaseal quality automated patch clamp platform. Our ASIC1a assay can be employed to support medium throughput screening and SAR campaigns.

Results and Discussion

Concentration-dependent activation of ASIC1a by extracellular protons

The rapid activation and desensitisation properties associated with ASIC1a ion channels provide some technical challenges for the development an automated patch clamp assay. Our initial experiments were performed to evaluate the pH-dependent activation of ASIC1a ion channels. Single cells were clamped at -60 mV and ASIC1a currents were elicited with 3 second applications of extracellular solution containing five different concentrations of protons. The cells were washed three times with pH 7.4 extracellular solution (pH 7.4) and remained in pH 7.4 solution for at least 2 minutes between applications of the different concentrations of protons to allow recovery from desensitisation. Figure 2A shows representative current traces obtained in five different concentrations of extracellular protons, while Figure 2B shows the mean ± S.D. normalised data plotted against Log[proton]. The data were fit with a concentration-response curve, which yielded a half-maximal (pH_{50}) value of 219 nM H^+ (pH 6.66). This corresponds to pH_{50} values ranging from pH 6.5 to pH 6.6 reported in the literature[10].

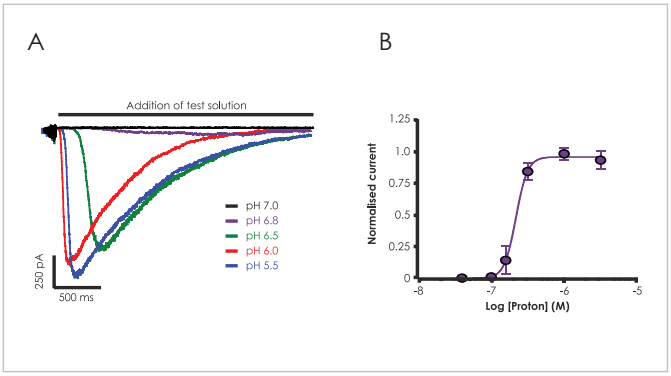


Figure 2: Proton-dependent activation of ASIC1a on the QPatch48

(A) Representative ASIC1a current traces elicited by five different concentrations of extracellular protons.

(B) Concentration-response relationship for proton-dependent activation of ASIC1a revealed an EC_{50} of pH 6.66.

The remaining experiments were conducted using pH 6.5 extracellular solution (pH 6.5) to moderately activate ASIC1a current, which is close to the calculated pH_{50} value. The rationale for using this concentration was to:

1. Avoid tachyphylaxis associated with lower pH.
2. To allow the development of an assay that could potentially distinguish between agonists, partial agonists and antagonists.

Evaluation of the stability of ASIC1a current responses with multiple stimulations

The development of an accurate screening assay requires a stable current response over multiple rounds of activation, because significant tachyphylaxis can lead to an overestimation of inhibition. Therefore, the stability of ASIC1a current on the QPatch was investigated using seven consecutive additions of pH 6.5 solution. To limit tachyphylaxis, three washes in pH 7.4 solution were applied following each 3 second application of pH 6.5. Additionally, the cells were maintained in pH 7.4 solution between applications of pH 6.5.

The current amplitude was determined following each addition of pH 6.5 and normalised to the largest current amplitude recorded in each cell. The mean (± S.D.) normalised data are presented in Figure 3B, which reveals a slight reduction in ASIC1a current amplitude between the first and second pH 6.5 applications, followed by consistently stable currents over the remainder of the experiment. A second set of experiments were conducted, where 30 μM benzamil was included in the fourth application of pH 6.5 solution to assess the reversibility of drug effects. Figure 3B demonstrates that 30 μM benzamil fully inhibited currents evoked by a pH 6.5 solution application. The maximal effect was fully reversible upon wash-off with pH 7.4 solution, and ASIC1a responses remained stable during subsequent applications of pH 6.5.

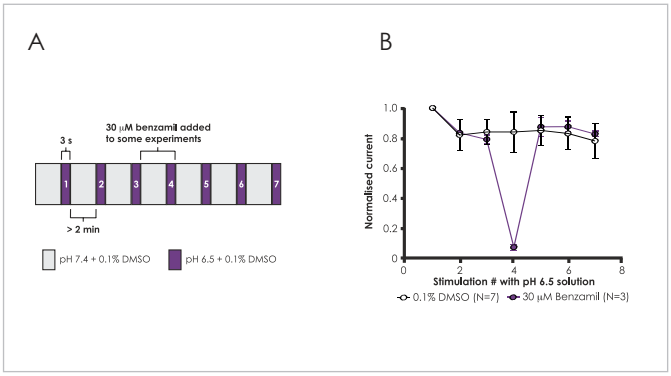


Figure 3: Stable ASIC1a recordings over multiple applications of pH 6.5

(A) Schematic of the assay paradigm used to evaluate current stability over seven applications of pH 6.5.

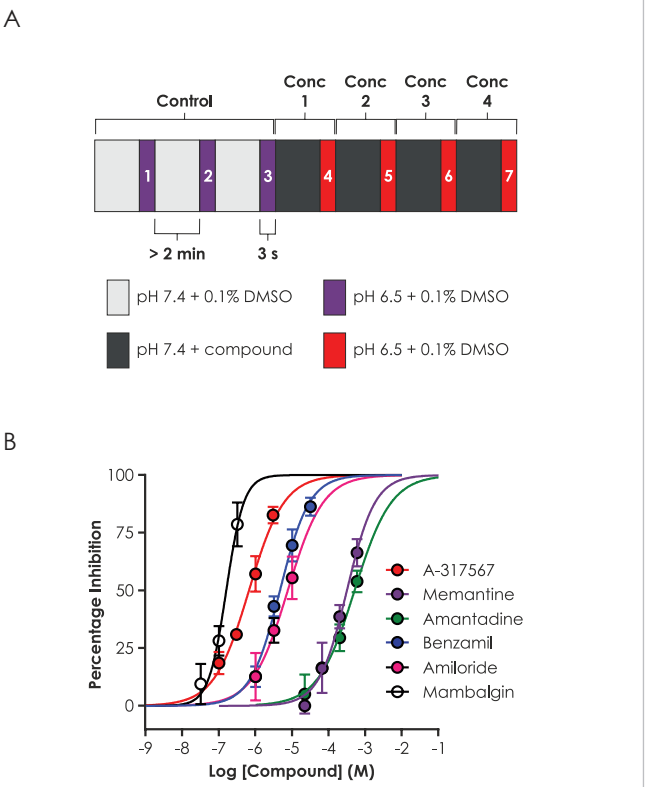
(B) Mean (± S.D.) normalised data for currents elicited over seven applications of pH 6.5. One series of experiments was conducted with all experimental solutions containing 0.1% DSMO. A second set of experiments was conducted where 30 μM benzamil was included in the fourth pH 6.5 application.

The stability of the ASIC1a ion channel current amplitude elicited with multiple additions of pH 6.5 on the QPatch48 indicated that it would be possible to develop a medium throughput automated patch clamp assay that will not overestimate inhibition because of receptor tachyphylaxis.

Pharmacological validation of ASIC1a ion channel assay

Cumulative concentration-response assays were developed and pharmacologically validated with a toolbox of ASIC1a inhibitors. Reference compounds included the peptide toxin, mambalgin, and the small molecule inhibitors, amiloride, benzamil, memantine, amantadine and A-317567[11,12,13]. Figure 4A shows a schematic outlining a four-point concentration-response assay format. The assay paradigm is the same as shown in Figure 3A, except that four increasing concentrations of compound are included in the fourth, fifth, sixth and seventh applications of pH 6.5.; additionally, compound was included in the preceding 2 minute wash period in pH 7.4 solution. This pre-incubation period allowed the test samples to reach their binding site(s); it is anticipated that test samples that activate ASIC1a channels will also be identified during this period as membrane current data is recorded after every liquid addition.

The mean inhibition (± S.D.) data for each test sample were plotted against Log[compound] and fit with a concentration-response function (Figure 4B). The half-maximal inhibitory (IC_{50}) values derived from the fits are listed in Table 1, which are consistent with values obtained from the literature. The data reveals that Metrion's ASIC1a cumulative concentration-response assays are capable of accurately identifying antagonists across a wide range of potencies and modalities.



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Compound	IC_{50} (μM)	Literature IC_{50} value (μM)
Benzamil	4.66	3.50 (Leng)
Amantadine	510.40	25% at 500 μM (Diochot)
Memantine	315.30	31% at 500 μM (Diochot)
A-317567	0.66	2.00 (Dube)
Amiloride	7.62	13.50 (Leng)
Mambalgin	0.16	0.055 (Nagaeva)

Figure 4: Pharmacological validation of ASIC1a on QPatch48

(A) Schematic showing a four-point concentration-response assay paradigm.

(B) Concentration-response relations for the toolbox compounds.

(C) IC_{50} values derived from the concentration-response fits in (A) and representative IC_{50} values obtained from the literature.

Evaluation of the efficiency of the ASIC1a assay

The efficiency of the ASIC1a assay was determined by evaluating the success metrics of a number of key assay parameters, including the percentage of wells that delivered gigaohm quality seals and the fraction of gigaohm whole-cells which were maintained during different stages of the experiment. The mean (± S.D.) data is presented in Figure 5, which also includes the percentage of wells that passed various quality control (QC) criteria. The QC'd success rate was approximately 65%, which indicated that this assay was efficient enough to be used for routine screening purposes.

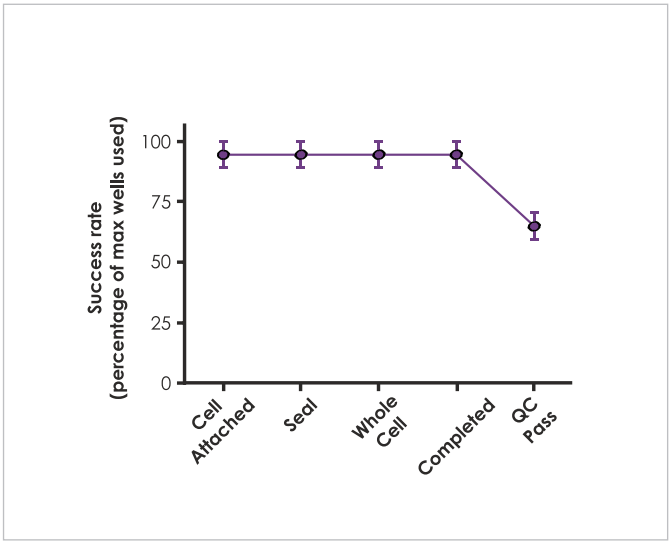

Figure 5: Success metrics for a number of key assay parameters
The mean (± S.D.) percentage success rate for a number of key assay parameters. QC pass filters included minimum seal and whole-cell resistance, minimum current expression (e.g. > -200 pA), and stable and low series resistance values.

Conclusions

Metrion undertook a series of experiments to develop and validate a screening assay for the ligand-gated ion channel, ASIC1a, on the QPatch48, a gigaseal quality automated patch clamp platform. Extracellular protons elicited rapidly-activating ASIC1a currents in a concentration-dependent manner with pH_{50} values consistent with literature values. Pharmacological validation with a toolbox of reference compounds revealed IC_{50} values that were consistent with values reported in the literature.

The work undertaken in this study, led to the creation of an accurate and cost-effective ASIC1a ion channel assay that is capable of supporting medium throughput screening and SAR campaigns.

Methods

Chinese Hamster Ovary (CHO) cells expressing human ASIC1a were obtained from B'SYS. Cells were cultured and harvested using Metrion's optimised QPatch protocols. Standard QPatch cell suspension, sealing and whole-cell protocols were utilized, with minor adjustments, to obtain a high proportion of gigaohm seals and optimised amplitudes of whole-cell ASIC1a currents.

All QPatch48 data was collected using multi-hole chips at room temperature.

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