B'SYS GmbH

ASIC1a

TABLE OF CONTENTS

1 BACKGROUND

1.1 The ASIC1a channel

Cation channel with high affinity for sodium, which is gated by extracellular protons and inhibited by the diuretic amiloride. Also permeable for Ca^{2+}, Li^{+} and K^{+}. Generates a biphasic current with a fast inactivating and a slow sustained phase. Mediates L-glutamine-independent Ca^{2+} entry into neurons upon acidosis. This Ca^{2+} overloading is toxic for cortical neurons and may be in part responsible for ischemic brain injury. Heteromeric channel assembly seems to modulate channel properties. Functions as a postsynaptic proton receptor that influences intracellular Ca^{2+} concentration and calmodulin-dependent protein kinase II phosphorylation and thereby the density of dendritic spines. Modulates activity in the circuits underlying innate fear.

1.2 B'SYS' CHO ASIC 1a Cells

B'SYS has designed a new CHO ASIC1a cell line with constitutive coexpression of human Acid-sensing ion channel 1a (=Amiloride-sensitive cation channel 2). The human ASIC1a cDNA was cloned and transfected into CHO cells and then the functional properties of the ASIC1a channels validated by means of the patch-clamp technique. Results are outlined in section 3.

2 PRODUCT SHIPMENT

2.1 Product Format

CHO cells stably transfected with recombinant human ASIC 1a channel:

- 1 x 0.5 mL aliquots of frozen cells at 2.2 E+06 cells/mL
- Cells are frozen in complete medium with 10% DMSO

2.2 Mycoplasma Certificate

B'SYS periodically tests cells for presence of mycoplasma by means of highly sensitive PCR based assays. All delivered cells are free of mycoplasma.

3 VALIDATION OF CHO ASIC 1A CELLS

3.1 Electrophysiology

ASIC 1a currents were measured by means of the patch-clamp technique in the whole-cell configuration. The bath solution contained (in mM) NaCl 137, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, D-glucose 10, HEPES 10, pH (NaOH) 7.40. The pipette solution consisted of (in mM) KCl 130, $MgCl_2$ 1, MgATP 5, HEPES 10, EGTA 5, pH (KOH) 7.20. After formation of a GΩ seal between the patch electrodes and individual ASIC1a stably transfected CHO cells, the cell membrane across the pipette tip was ruptured to assure electrical access to the cell interior. During manual patch-clamping, all solutions applied to cells were continuously perfused and maintained at room temperature. As soon as a stable seal could be established desensitizing currents were measured upon low pH stimulation at a holding potential of -80 mV (Fig. 1). The cell was stimulated after a minimum rest of 30 s.
The cells were stimulated with solutions of increasing pH (6.5 – 4.5, 0.5 increments). The resulting pH_{50} was determined as 6.07 (n=6). This value is in good agreement with data from literature (Hesselager et al. 2004).

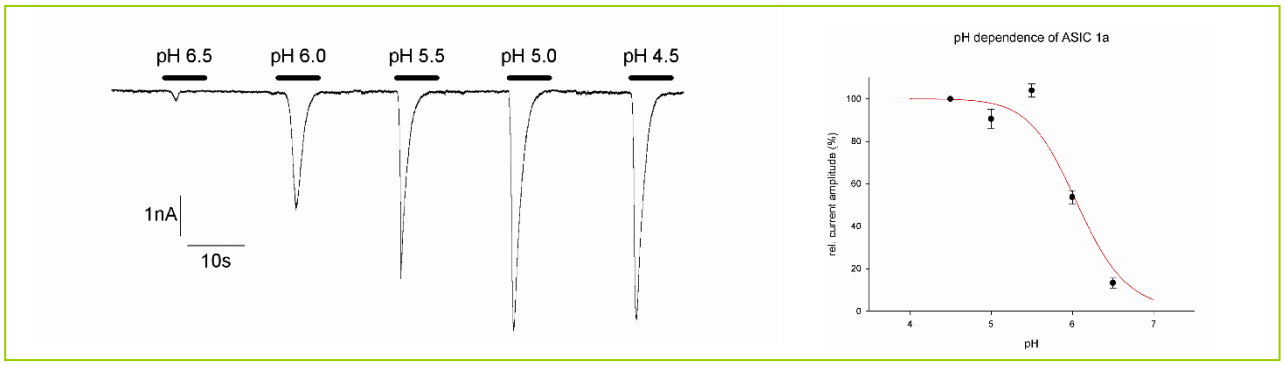

Fig. 1: manual patch-clamping: pH activation of ASIC 1a currents decreasing pH at a holding potential of -80 mV. No currents were recorded in untransfected cells (data not shown). The pH_{50} was determined to be 6.07 (n=6)

The cells were validated for automated patch-clamping using the Q-Patch system. The same solutions like for manual patch-clamping were used, but cells were clamped to -60 mV. First the effect of decreasing pH was analyzed. The cells were incubated for 10 s in solutions with decreasing pH. Between two stimulation cells were kept in a bath solution (pH7.4) for at least 30 s.
The pH_{50} was determined to be 6.65 ± 0.18 (Hill coefficient 2.37 ± 0.33, n=7)

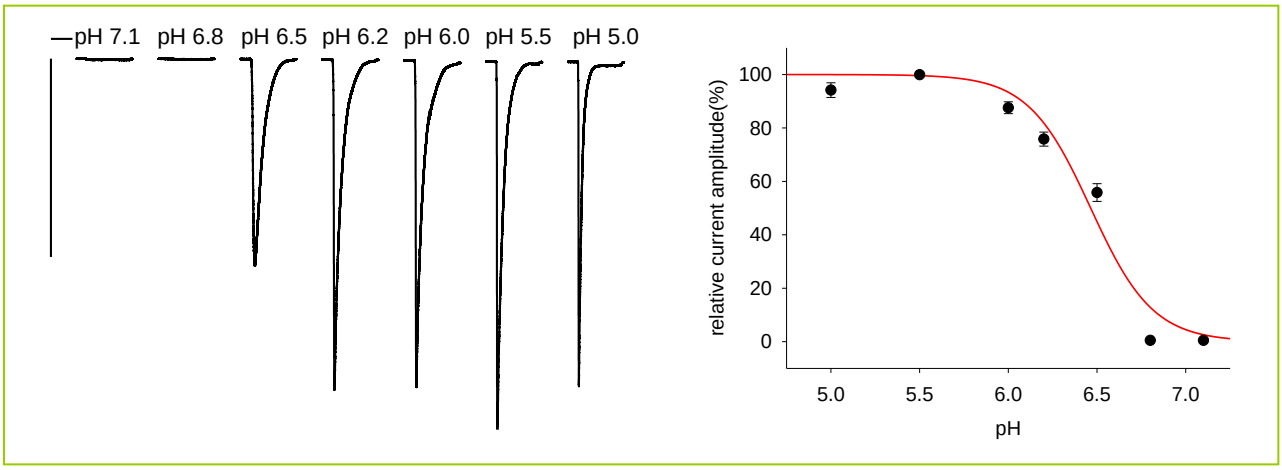

Fig2: automated patch-clamping: pH activation of ASIC 1a currents upon decreasing pH at a holding potential of -60 mV. The pH_{50} was determined to be 6.65 (n=7). Scale bars: 5 s, 5.0 nA

For the pharmacological validation, the dose response curve for Amiloride was recorded. Concentrations between 3.0 µM and 1000 µM were tested at a holding potential of -60 mV. The channels were activated with pH6.0. Under these conditions, the IC_{50} was determined as 20.13 µM (Hill coefficient: 1.29).

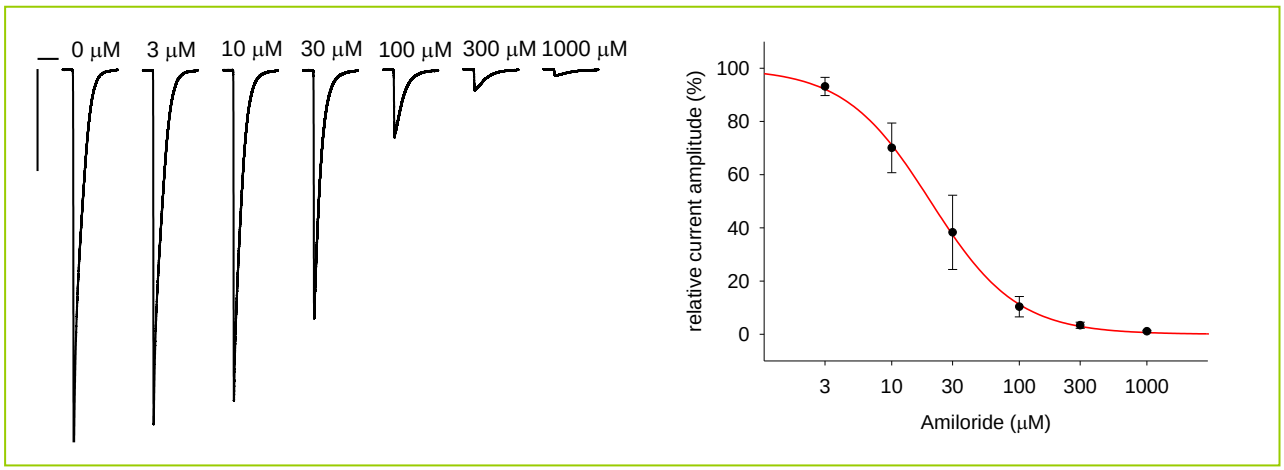

Fig3: automated patch-clamping: Amiloride blocked the ASIC 1a currents in a concentration dependent way. The IC_{50} was determined to be 20.13 µM. Currents were stimulated with pH6.0 at a holding potential of -60 mV. Scale bars: 5 s, 5.0 nA

3.2 Patch-clamp Success Rates

The patch-clamp properties of the CHO ASIC 1a cell line were elucidated at typical working passage numbers (passage 7-16).
For manual patch-clamping, a total of 17 cells were analyzed. Success for establishment of on-cell configuration was defined as follows: > 1 GΩ. The whole-cell configuration was not accepted if the membrane resistance was below 500 MΩ. A successful recording had to be free of rundown effects and variations in series resistance.

- On-cell successful: 94% (n=17)
- Whole-cell successful: 85% (n=17)
- Recording (15 min) successful: 76% (n=17)

For automated patch-clamping (Q-Patch):

- On-cell successful: 91% (n=32)
- Whole-cell successful: 88% (n=32)
- Recording (15 min) successful: 75% (n=32)

4 CELL CULTURE CONDITIONS

4.1 General

CHO ASIC 1a cells are incubated at 37°C in a humidified atmosphere with 5% CO_2 (rel. humidity > 95%). The cells are continuously maintained and passaged in sterile culture flasks containing F12 (HAM) medium supplemented with 9% fetal bovine serum, 0.9% Penicillin/Streptomycin solution and 250 µg/mL G-418 suplhate. The CHO ASIC1a cells are passaged at a confluence of about 80%. For electrophysiological measurements the cells are seeded onto e.g. 35 mm sterile culture dishes containing complete medium.

- All solutions and equipment coming in contact with the cells must be sterile.
- Use proper sterile technique and work in a laminar flow hood.
- Be sure to have frozen cell stocks at hand before starting experiments.
- Cells should be split every 2-3 days at 70% - 80% confluency at 1:3 to 1:5 ratio.

4.2 Recommended Complete Medium

- F12 (HAM) with Glutamine or GlutaMAX I or Glutamine
- 10% FBS
- 1.0% Penicillin/Streptomycin

4.3 Antibiotics

- CHO ASIC 1a clones were selected under 750 µg/mL G-418 sulphate antibiotic pressure.
- To cultivate CHO ASIC 1a cells, also a reduced antibiotic pressure (100 to 250 µg/mL) can be used.
- To separate CHO ASIC 1a cells from untransfected cells, use 750 µg/mL G-418 sulphate.

Remark: The permanent application of high antibiotic pressure has no effect on current density.

4.4 Thawing Cells

- Remove vial of cells from liquid nitrogen and thaw quickly at 37°C.
- Decontaminate outside of vial with 70% ethanol.
- Transfer cells to a T-25 culture flask containing 5.0 mL complete medium.
- Incubate cells at 37°C for 4-6 h to allow the cells to attach to the bottom of flask.
- Aspirate off the medium and replace with 5.0 mL complete medium & antibiotics.
- Antibiotics: 100 - 250 µg/mL G-418 suplhate.
- Incubate cells and check them daily until 70% - 80% confluency is reached.

4.5 Splitting Cells

- When cells are 70% - 80% confluent remove medium.
- Wash cells once with 1x PBS to remove excess medium.
- Add 1x Trypsin/EDTA and incubate 30 s at room temperature.
- Remove Trypsin/EDTA quickly and incubate cells for 2 min at 37°C.
- Detach cells, add complete medium and pipet up and down to break clumps of cells.
- Passage cells into new flask with complete medium and antibiotics at 1:3 to 1:5 ratio.
- Use remaining suspension for counting the cells.

4.6 Freezing Medium

- Mix 0.9 mL fresh complete medium and 0.1 mL DMSO for every 1 mL freezing medium.
- Sterilize freezing medium by means of appropriate micro filter (0.1 µm – 0.2 µm). Or use sterile DMSO.

4.7 Freezing Cells

- Prepare fresh freezing medium and keep it on ice.
- Cells should have 80% - 90% confluency prior to freezing.
- Remove the complete medium.
- Wash cells once with 1x PBS to remove excess medium.
- Add 1x Trypsin/EDTA and incubate 30 s at room temperature.
- Remove Trypsin/EDTA quickly and incubate cells for 2 min at 37°C.
- Detach cells, add complete medium and pipet up and down to break clumps of cells.
- Pellet cells with centrifuge and carefully aspirate off medium.
- Resuspend cells at a density of approximately 2.0 E+06 cells per mL with fresh freezing medium.
- Aliquot 0.5 mL of cell suspension into each cryovial.
- Overnight incubate cells in a styropor box at –80°C.
- The next morning transfer cryovial in liquid nitrogen tank for long-term storage.

4.8 Stability of CHO ASIC 1a cells

CHO ASIC 1a cells stably express functionally active ASIC 1a channels over 23 passages. Under recommended cell culture conditions no variation in current density was observed over 23 cell splitting cycles.

5 ASIC 1A SEQUENCE

5.1 Human ASIC 1a Accession Number NM_020039.2

Cloned cDNA sequence of human ASIC 1a subunit was error-free and identical with NM_020039.4 sequence coding for NP_064423 (Uniprot: P78348-1, Hvariant 1, Asic 1a long)

```
atggaactgaaggccgaggaggaggaggtgggtggcgtccagccggtgagcatccaggcc
 M  E  L  K  A  E  E  E  E  V  G  G  V  Q  P  V  S  I  Q  A
ttcgccagcagctccacactgcacggcctggcccacatcttctcctacgagcggctgtct
 F  A  S  S  S  T  L  H  G  L  A  H  I  F  S  Y  E  R  L  S
ctgaagcgggcactgtgggccctgtgcttcctgggctcgctggctgtgctgctgtgtgtg
 L  K  R  A  L  W  A  L  C  F  L  G  S  L  A  V  L  L  C  V
tgcacggagcgtgtgcagtactacttccactaccaccatgtcaccaagctcgacgaggtg
 C  T  E  R  V  Q  Y  Y  F  H  Y  H  H  V  T  K  L  D  E  V
gctgcctctcagcttaccttccctgctgtcacgctgtgcaacctcaacgagttccgcttt
 A  A  S  Q  L  T  F  P  A  V  T  L  C  N  L  N  E  F  R  F
agccaagtctccaagaatgacctgtatcatgctggggagctgctggccctgctcaacaac
 S  Q  V  S  K  N  D  L  Y  H  A  G  E  L  L  A  L  L  N  N
aggtatgagataccagacacacagatggcagatgaaaagcagctggagatactgcaggac
 R  Y  E  I  P  D  T  Q  M  A  D  E  K  Q  L  E  I  L  Q  D
aaagccaacttccgcagcttcaaacccaaacccttcaacatgcgtgagttctacgaccga
 K  A  N  F  R  S  F  K  P  K  P  F  N  M  R  E  F  Y  D  R
gctgggcacgacattcgagacatgctgctcctcctgccacttccgggggggaggtctgcagc
 A  G  H  D  I  R  D  M  L  L  S  C  H  F  R  G  E  V  C  S
gctgaagacttcaaggtggtcttcacacgctatggaaagtgctacacgttcaactcgggc
 A  E  D  F  K  V  V  F  T  R  Y  G  K  C  Y  T  F  N  S  G
cgagatgggcggccgcggctgaagaccatgaagggtgggacgggcaatgggctggaaatc
 R  D  G  R  P  R  L  K  T  M  K  G  G  T  G  N  G  L  E  I
atgctggacatccagcaggacgagtacctgcctgtgtgggggggagactgacgagacgtcc
 M  L  D  I  Q  Q  D  E  Y  L  P  V  W  G  E  T  D  E  T  S
ttcgaagcaggcatcaaagtgcagatccatagtcaggatgaacctcctttcatcgaccag
 F  E  A  G  I  K  V  Q  I  H  S  Q  D  E  P  P  F  I  D  Q
ctgggctttggcgtggccccaggcttccagacctttgtggcctgccaggagcagcggctc
 L  G  F  G  V  A  P  G  F  Q  T  F  V  A  C  Q  E  Q  R  L
atctacctgcccccaccctggggcacctgcaaagctgttaccatggactcggatttggat
 I  Y  L  P  P  P  W  G  T  C  K  A  V  T  M  D  S  D  L  D
ttcttcgactcctacagcatcactgcctgccgcatcgactgtgagacgcgctacctggtg
 F  F  D  S  Y  S  I  T  A  C  R  I  D  C  E  T  R  Y  L  V
gagaactgcaactgccgcatggtgcacatgccaggggatgccccatactgtactccagag
 E  N  C  N  C  R  M  V  H  M  P  G  D  A  P  Y  C  T  P  E
cagtacaaggagtgtgcagatcctgctctggacttcctggtggagaaggaccaggagtac
 Q  Y  K  E  C  A  D  P  A  L  D  F  L  V  E  K  D  Q  E  Y
tgcgtgtgtgaaatgccttgcaacctgacccgctatggcaaagagctgtccatggtcaag
 C  V  C  E  M  P  C  N  L  T  R  Y  G  K  E  L  S  M  V  K
atccccagcaaagcctcagccaagtacctggccaagaagttcaacaaatctgagcaatac
 I  P  S  K  A  S  A  K  Y  L  A  K  K  F  N  K  S  E  Q  Y
ataggggagaacatcctggtgctggacattttctttgaagtcctcaactatgagaccatt
 I  G  E  N  I  L  V  L  D  I  F  F  E  V  L  N  Y  E  T  I
gaacagaagaaggcctatgagattgcagggctcctgggtgagctgctgatgacacctgtc
```

```
 E  Q  K  K  A  Y  E  I  A  G  L  L  G  E  L  L  M  T  P  V
cccttctcatgccatgggcatggcgtggctccctatcatccaaaagcagggtgctcactt
 P  F  S  C  H  G  H  G  V  A  P  Y  H  P  K  A  G  C  S  L
ctgtcccatgagggtcctccaccccagaggcccttccccaaaccctgttgtcttggtgac
 L  S  H  E  G  P  P  P  Q  R  P  F  P  K  P  C  C  L  G  D
atcgggggccagatggggctgttcatcggggccagcatcctcacggtgctggagctcttt
 I  G  G  Q  M  G  L  F  I  G  A  S  I  L  T  V  L  E  L  F
gactacgcctacgaggtcattaagcacaagctgtgccgacgaggaaaatgccagaaggag
 D  Y  A  Y  E  V  I  K  H  K  L  C  R  R  G  K  C  Q  K  E
gccaaaaggagcagtgcggacaagggcgtggccctcagcctggacgacgtcaaaagacac
 A  K  R  S  S  A  D  K  G  V  A  L  S  L  D  D  V  K  R  H
aacccgtgcgagagccttcggggccaccctgccgggatgacatacgctgccaacatccta
 N  P  C  E  S  L  R  G  H  P  A  G  M  T  Y  A  A  N  I  L
cctcaccatccggcccgaggcacgttcgaggactttacctgc
 P  H  H  P  A  R  G  T  F  E  D  F  T  C
```

5.2 List of Asic1 splice variants available at B'SYS

NCBI nucleotide sequence	NCBI protein sequence	Uniprot	B'SYS internal name
NM_001095.4	ASIC1 isoform b, NP_001086.2	P78348-2, Hvariant 2, Asic1a	ASIC1a short
NM_020039.4	ASIC1 isoform a, NP_064423	P78348-1, Hvariant 1	ASIC1a long
NM_001256830.2	ASIC1 isoform c, NP_001243759.1	P78348-3, Hvariant 3, Asic1b	ACIC1b

6 CONTACT INFORMATION

6.1 Contact Address for Technical Support & Ordering Information

- B'SYS GmbH
  Technology Center Witterswil
  Benkenstrasse 254 B
  4108 Witterswil
  Switzerland

  Tel: +41 61 721 77 44
  Fax: +41 61 721 77 41
  Email: info@bsys.ch
  Web: www.bsys.ch