The intracellular N-terminus domain of the acid-sensing ion channel 1a participates in channel opening and membrane expression

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List of abbreviations

amino acid (AA)
Acid-sensing ion channels (ASICs)

C kinase-1 (PICK1)

Enhanced chemiluminescence (ECL)

Green fluorescent protein (GFP)

N-2-Hydroxyethylpiperazine-N-2-Ethane Sulfonic Acid (HEPES)

three-dimensional(3D)

one-way analysis of variance (ANOVAs)

phosphate-buffered saline (PBS)

PBS-Tween (PBST)

polyvinylidene difluoride (PVDF)

PSD-95, Disc-large, and ZO-1 (PDZ)

Radio-Immunoprecipitation Assay (RIPA)

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
ABSTRACT

Acid-sensing ion channels (ASICs) are widely expressed in the nervous system. The intracellular C-terminus of ASIC1a has many sites involved in regulating its expression and the opening mechanism, but the role of the intracellular N-terminus domain is poorly understood. Here, we explored the correlation of ASIC1a intracellular N-terminus with membrane expression and gate opening. We modified the N-terminus structure of ASICs by deletion/truncation/mutation strategies and transfected the recombinant plasmids into CHO cells. Protein expression was analyzed with immunofluorescence, western blots, and patch-clamp experiments. Deleting the entire N-terminus decreased the membrane expression of channel proteins, and ion channel opening was lost. Deleting sections of the N-terminus also decreased membrane expression suggested that all areas were significant, with no single or group of amino acid residues playing a decisive role in regulating ASIC1a membrane expression. In terms of gate opening, five amino acid (AA) residues from AA 16 to AA 20 participated in gate opening, and isoleucine at AA 18 was the most important. The whole N-terminus of ASICs participates in the membrane expression of ASIC1a, and five amino acid residues (AA 16 to 20) are involved in the gate opening mechanism.

Keywords: Acid-sensing ion channels; Ion channel gating; N-terminus; membrane expression; function
Significance Statement

The whole N-terminus of ASICs participates in the membrane expression of ASIC1a, and five amino acid residues (AA 16 to 20) are involved in the gate opening mechanism.
Introduction

Acid-sensing ion channels (ASICs) are cationic channels that are widely expressed in the nervous system. ASICs are essential for many physiological processes such as synaptic transmission and dendritic spine development and are involved in many pathophysiological conditions that involve tissue acidosis, such as ischemic stroke, epileptic seizures, and multiple sclerosis (Zeng et al., 2014). Since ASICs were first discovered (Waldmann et al., 1997), their structure-function correlation has been the focus of investigators’ attention. ASICs are a homo-oligomer or hetero-oligomer composed of 3 protein subunits, and each subunit has a two-transmembrane structure, including intracellular N-terminus, C-terminus, two transmembrane domains, and one large extracellular domain (Chu et al., 2011). The 3D structures of ASICs extracellular and transmembrane domains have been resolved by crystallography (Gonzales et al., 2009; Jasti et al., 2007), but this method's limitations mean that the intracellular structure could not be observed. The function of ASICs depends on the number of channels on the cell surface. So, the dynamic control of surface ASICs and understanding their membrane expression and gating mechanism is critical for understanding their function (Zeng et al., 2014). Thus, other methods are needed to reveal the correlation between ASICs intracellular domain and channel function.

There are currently six known ASIC isoforms (Zeng et al., 2014). The 1A subtype is widely found within the nervous system and is a critical channel subunit with suggested roles in synaptic physiology (Wemmie et al., 2013). Studies revealed that there is a PDZ structural domain (Dev, 2007) and phosphorylation site (Gao et al., 2005; Leonard et al., 2003) that combine with protein interacting with C kinase-1 (PICK1) (Baron et al., 2002) at the C-terminus (AA 564-528) of ASIC1a. These regions participate in regulating the activity and function of the channel. The
relationships between the function and structure of ASIC1a have been examined, including acid sensitivity (Immke and McCleskey, 2003; Paukert et al., 2008; Smith et al., 2007), the structure of channel size (Coscoy et al., 1999; Leonard et al., 2003), regulation ion and binding site (Shaikh and Tajkhorshid, 2008; Sherwood et al., 2009), desensitization mechanism (Coric et al., 2003), coupling sites of ligand binding and gating (Li et al., 2009).

On the other hand, there are fewer investigations on the N-terminus domain (AA 1-41). Comparison among the different family members indicated that the region before the ASIC transmembrane domain I (intracellular N-terminus) was highly conserved in the DEG/ENaC superfamily (Grunder et al., 1997). Chimera experiments with ASIC2a and ASIC2b indicated that the N-terminus of ASIC2a is responsible for gate opening and defined that nine amino acids were responsible for ion permeability and sodium ion selectivity (Coscoy et al., 1999). Cysteine mutation and intracellular modification suggested that the N-terminus region of ASIC1a participates in channel opening and gating. Alanine at AA 22, isoleucine at AA 33, phenylalanine at AA 34, and arginine at AA 43 (in the first transmembrane domain) were reported to contribute to the internal pore (Pfister et al., 2006).

These studies all suggest that the N-terminal is essential for channel expression and pore opening. Therefore, in this study, we aimed to explore further the function of the N-terminus region of ASIC1a. We modified the N-terminus of ASIC1a by deletion/truncation/mutation and investigated its impact on CHO cells.

**Results**

*ASIC1a intracellular N-terminus participates in channel function*

Firstly, the N-terminus region was deleted and exogenously expressed in CHO cells.
Immunofluorescence, western blot, and electrophysiological methods were used to explore the roles of the N-terminus region in the membrane expression of channel protein and gate opening. We found that when the N-terminus region was deleted, the membrane expression level of the channel protein was decreased (Figure 1), and ion channel opening was lost (Figure 2). To visually observe the membrane expression of ASIC1a, we co-transfected an antibody to myc-CD8α coupled with red fluorescence (as a marker of the peripheral cell membrane) and an ASIC1a plasmid expressing GFP. This suggested that after N-terminus was deleted, the membrane expression of ASIC1a was significantly decreased (Figure 3).

**Influence of regions of the ASIC1a intracellular N-terminus on membrane expression of the channel protein**

To further explore the influence of the N-terminus region on membrane expression of ASIC1a, we truncated/deleted the N-terminus-corresponding regions by different strategies and explored the membrane expression at various sites. First, we gradually truncated 10 amino acids (as a unit) from N-terminus (the final section was 11 amino acids). We found that the membrane expression gradually decreased, and when all the amino acids were deleted, the expression level was the lowest (Figure 4A-4C). Then we cut off 10 amino acids as a unit (the final section was 11 amino acids) on their own and found that no fragment influenced the membrane expression of ASIC1a (Figure 4D-4F). Finally, we cut off combinations of different units (10 amino acids as a unit) and found that although membrane expression decreased, there was no significant difference among the strategies (Figure 4G-4I). To avoid the interference of GFP close to the cell membrane when deleting the N-terminus of ASIC1a in pEGFP-C3, we used pEGFP-N1 vector to fuse the GFP protein with the C-terminus. Even with GFP on the
other end of the protein, we obtained similar results (Figure 5). The above results suggested that the N-terminus region of ASIC1a participates in the membrane expression of the channel protein. But there was no specific or group of amino acid residues that played a decisive role in regulating ASIC1a membrane expression.

**Influence of ASIC1a intracellular N-terminus on the channel gate opening**

To explore the influence of the N-terminus region on ASIC1a channel gate opening, we used similar strategies for membrane expression. We gradually truncated a unit (10 amino acids) from ASIC1a each time, and then cut off a unit (10 amino acids). We found that once two units (20 amino acids) or more were truncated, the ASIC1a channel could not open (Figure 6A, 6B). When two units (AA 21-30, AA 31-41 amino acids) were cut off, the channel could not be open either (Figure 6C, 6D). Interestingly, we found that truncation of the first 20 amino acids and cutting off 10 amino acids (AA 11-20) might cause loss of gate opening. This suggested an important membrane body within these ten amino acids (11-20) that participates in gate opening. Thus, we established four mutants: two mutants with AA 11-15 and 16-20 cut off, two mutants with AA 11-15 and 16-20 substituted with alanine (11A15, 16A20, the 20th in the wild type protein was alanine). The results of electrophysiology indicated that AA 11-15 did not participate in the regulation of channel opening and cutting off or mutation could not cause opening function loss either. However, AA 16-20 participated in the channel’s opening, and both cutting off and mutation could cause opening function loss (Figure 6E, 6F). To clarify which amino acid played the central role, the amino acids in the membrane body were mutated into alanine (V16A, S17A, I18A, and Q19A). It turned out that the mutation of isoleucine at AA 18 was the most significant, and channel function was lost.
entirely. The opening function was recovered toward the two sides, showing V type. This suggested that the isoleucine at AA 18 is the critical regulation point for gate opening of the channel protein. The adjacent amino acids at AA 16, 17, 19, and 20 might participate in the function. However, it has been reported that when isoleucine at AA 18 was mutated into cysteine, channel opening was not influenced (Pfister et al., 2006), which seems to be contradictory to our results. Therefore, we performed mutation of the isoleucine to different amino acids (mutation into alanine (I18A), glycine (I18G), valine (I18V), serine (I18S), threonine (I18T), and cysteine (I18C)). We found that only mutation into cysteine could open the channel, the other five amino acids lost opening function, and further eliminated the membrane expression results (Figure 7).

Discussion

This study aimed to explore the correlation of the ASIC1a intracellular N-terminus domain with membrane expression and gate opening. Deleting the entire N-terminus region decreased the membrane expression of channel proteins, and ion channel opening was lost. Deletion of sections of the N-terminus region also decreased membrane expression. Still, no single or group of amino acid residues was identified as playing a decisive role in regulating ASIC1a membrane expression. Five amino acid residues from AA 16 to 20 participated in gate opening, and isoleucine at AA 18 was the most important.

Previous studies suggested that the N-terminal region of ASIC1a is likely to be essential for membrane binding and channel opening (Coric et al., 2003; Coscoy et al., 1999; Li et al., 2009; Shaikh and Tajkhorshid, 2008; Sherwood et al., 2009). Nine amino acids in the N-terminus of ASIC2a are responsible for ion permeability and
sodium ion selectivity (Coscoy et al., 1999). Cysteine mutation and intracellular modification suggest that the N-terminus of ASIC1a participates in channel opening and gating and alanine at AA 22, isoleucine at AA 33, phenylalanine at AA 34, and arginine at AA 43 (in the first transmembrane domain) contribute to the internal pore (Ho et al., 1989). Directed mutagenesis is a useful tool for investigating regions of ion channels that cannot be resolved structurally. This approach has shown that the C-terminal section of ASIC1a is essential for various regulation mechanisms. For example, mutating the PDZ binding motif increased surface expression and current density of ASIC1a. Mutating either an RRGK motif or a KEAKR motif reduced ASIC1a surface expression and acid-activated current density. Mutating or deleting the RRGK motif also reduced pH sensitivity and the rate of desensitization of ASIC1a (Jing et al., 2013).

Using a layer-by-layer progressive and whole first and local later strategy, we explored the correlation of N-terminus of ASIC1a with membrane expression and gate opening. We found that the entire N-terminus participated in the membrane expression of ASIC1a. However, no single or group of amino acids was found to be vital. The present study cannot explain the specific mechanism of N-terminal’s involvement in ASIC1A protein membrane expression. In terms of gate opening, we discovered that five amino acids from AA 17 to 20 participated in the gate opening of ASIC1a, especially isoleucine at AA 18. Of concern with this result was that Pfister et al. showed that during cysteine scanning, isoleucine at AA 18 did not participate in the channel’s opening (Pfister et al., 2006). Therefore, we mutated this residue to other amino acids and found that the mutation into other amino acids except for cysteine all stopped the channel from opening. The possible reason could be that cysteine has a sulfhydryl group that may participate in a redox reaction. The formation of a disulfide

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bond will influence the spatial conformation of the protein. However, alanine does not have this characteristic. Indeed, to test this hypothesis in the future, we intend to introduce an intercellular oxidizing agent to oxidize cysteine, and break the possible disulfide bond, and see if this influences electrophysiological tests.

The present study was designed only to determine whether the N-terminus of the protein was involved in the expression of ASIC1a, not to determine the mechanisms involved. Still, since ASIC1a undergo post-translational modifications (Yang et al., 2014; Zeng et al., 2014), it is reasonable to assume that the important changes in protein size induced by deletions at different sites could be due to post-translational modifications being affected. Especially, the Δ1-10 ASIC1a displayed a smaller protein size, and it can hypothesize that the amino acid 1-10 region is required for post-translational modifications, but the exact nature of that modification will have to be examined in future studies.

In conclusion, this study confirmed that the intracellular N-terminus of ASIC1a is essential for membrane expression and ion channel opening. We also identified isoleucine at AA 18 in the N-terminus domain of ASIC1a as vital for gate opening. This suggests a new target for designing and developing new drugs aimed at regulating ASIC1a function.

**Materials and methods**

**Cell culture**

Exogenous ASIC1a gene transfection and expression were performed in CHO cells (a gift from Tianle Xu from Shanghai Jiao Tong University School of Medicine). The CHO cells were cultured at 37°C in an incubator (Thermo, USA) under 5% CO₂. F12 culture medium (Gibco, USA) containing 1 mM L-glutamine, 10% FBS, 50 μg/mL...
streptomycin and 50 U/mL penicillin was used. Half of the medium was replaced every 2-3 days according to cell growth. When the cells were passaged, the original culture medium was discarded, the cells were washed with PBS three times, and trypsin (0.125%) was added for 2-3 min. F12 culture medium containing serum was added to terminate the digestion, and cells adhering to the culture dish were washed off and centrifuged at 1000 rpm/min for 5 min. The supernatant was discarded, and the cells were resuspended in the culture medium. The cells were seeded in a culture dish at $1 \times 10^5$/35 mm dish, or $5 \times 10^5$ cells/60 mm dish.

**Plasmid mutation**

ASIC1a plasmids (pEGFP-N1 and pEGFP-C3) were obtained from Tianle Xu from Shanghai Jiao Tong University School of Medicine as gifts. Site-directed mutation, deletion, and insertion were performed in the ASIC1a DNA sequence (based on overlap extension PCR (Ho et al., 1989)). When insertion could not be done by site-directed mutation, PCR fragments with restriction sites were introduced and then inserted by sticky digestion. DNA sequencing was performed in the protein-coding region of all the established mutants, and protein expression was validated by western blot.

**Cell transfection**

When CHO cells reached 70%-80% confluence, the plasmids' transfection was performed by lipofection, following the instructions (HilyMax liposome transfection reagent, Dojindo Laboratories, China). About 7-9 μg and 3-4 μg plasmid was added to 60 mm and 35 mm culture dishes, respectively. At 4 h after transfection, the culture medium containing liposome was discarded, and the cells were washed with PBS and
cultured with maintenance medium. At 24-48 h after transfection, immunocytochemical staining, protein extraction, and electrophysiological experiments were performed. When two different plasmids were co-transfected, the plasmids were mixed at the same concentration.

**Immunocytochemistry**

CHO cells were seeded onto clean cover glass in a 35-mm culture dish. At 24 h after plasmid transfection, the culture medium was discarded, and the cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min, and then washed with PBS three times (5 min each time). The required antibody (diluted with 0.3% Triton-X100+1% FBS+ PBS) was added, and the cells were incubated at 4°C overnight. After incubation, the cells were washed with PBS three times (5 min each time), and then the secondary antibody marked with fluorescein and diluted with PBS was added. The cells were incubated at room temperature for 2 h, washed with PBS three times (5 min each time), avoiding light. The cover glass was then sealed with glycerin and observed under a fluorescence microscope. PBS was used to replace the primary antibody as a control.

**Western blot**

At 24 h after plasmid transfection of CHO cells in a 60-mm culture dish, the medium was discarded. The cells were washed with PBS at 4°C three times (on ice), and the PBS was removed. RIPA lysis buffer (140 mM NaCl, 50 mM HEPES, 1 mM EDTA, 1% SDS, 1% Triton X-100, 10 μM/ml aprotinin, 1 μM/ml PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin, cocktail protease inhibitor) was added to the well. The cells were scraped into the lysis buffer on ice and allowed to lyse for 15 min. The cell fragments
and lysis buffer were collected into a 1.5-mL tube, centrifuged at 4°C at 13,000 rpm/min for 20 min. The supernatant was collected, and 4× SDS loading buffer was added. The sample was boiled in a metal bath (95°C) for 5 min and stored at -80°C. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 4% stacking and 10% separation polyacrylamide gels were prepared. Each lane was loaded with a sample of less than 25 μL, and the gel was run at 60 mV for about 30 min and then 90 mV until the end. The protein was transferred by the semi-dry transfer method from the separation gel to a polyvinylidene difluoride (PVDF) membrane (voltage 20 mV, about 1.5 h). The PVDF membrane was sealed in PBS-Tween (PBST) solution containing 20% skim milk at room temperature for 1 h, with primary antibody diluted as recommended by the manufacturers (ASIC1a antibody was from Millipore, other antibodies were from Santa Cruz) and incubated under rotation at 4°C overnight. The membrane was washed with PBST by shaking three times (5 min each time), the diluted secondary antibody was added and incubated at room temperature for 2 h. The membrane was then washed with PBST three times (5 min each time), and the secondary antibody was identified with enhanced chemiluminescence (ECL) in the dark and the signal recorded with X-ray film. Image-Pro Plus 6.1 software was used to analyze the image and collect data after the film was scanned.

**Biotinylation of membrane proteins**

At 24 h after plasmid transfection of CHO cells in a 60-mm culture dish, the medium was discarded, and the cells were washed with PBS (pH 8.0) three times. The PBS was removed, then biotin (Thermo dilution ratio 1:200) was added and incubated at 4°C for 30 min. The cells were further washed with PBS containing 0.1 M glycine
(pH 7.4) three times and with PBS (pH 7.4) three times. Then the procedure for western blotting was then followed.

**Electrophysiology**

The whole-cell recording mode under voltage clamp was used in this study. The constituents of electrode internal fluid (pH 7.2) were 30 mM NaCl, 120 mM KCl, 0.5 mM CaCl$_2$, 1 mM MgCl$_2$, 2 mM Mg-ATP, 5 mM EGTA, using 10 mM HEPES as buffer system (Sigma, USA). The pH was adjusted with Tris-base to the target pH. The fluid was filtered and stored at -20°C. The constituents of the common extracellular solution fluid (pH 7.4) were 124 mM NaCl, 5 mM KCl, 1.2 mM KH$_2$PO$_4$, 24 mM NaHCO$_3$, 1.3 mM MgSO$_4$, 2.4 mM CaCl$_2$, 10 mM glucose, using 10 mM HEPES as buffer system (Sigma, USA). HCl or NaOH was used to adjust the pH. The solution was stored at 4°C. Before use, the solution was returned to room temperature and saturated with 95% O$_2$ and 5% CO$_2$. The osmotic pressure of all the extracellular fluids was adjusted using sucrose to 320-330 mOsm/L. Water resistance of a glass microelectrode (Narishige Co., Ltd., Japan) was adjusted to 3.5-5.5 MΩ, using the 2-step vertically drawing method. A Y system was used to perfuse the drug, dependent on gravity, which guaranteed rapid recording of the solution environment around the cells within seconds and had less influence on the extracellular environment. Firstly, a positive voltage was given in the glass electrode. After the cells were selected, the electrode's tip was gently pressed on the cell surface by a step-wise microcontroller. The positive voltage was removed, and the cell membrane was adsorbed on the tip of the electrode. Self-compensating of series resistance was given (70%-90% of the series resistance could be compensated). The negative voltage was gradually given, followed by pipette holding potential (generally -60 mV). After a giant impedance
beyond GΩ was formed, a negative voltage was used to break the cell membrane, interworking intracellular fluid, and electrode fluid and forming whole-cell record. All the recorded signals were collected by an amplifier (Axon 700A patch-clamp amplifier, Axon Instruments, USA) and then input into a computer by a digital-analog converter (Digidata 1320A digital to analog converter, Axon Instruments, USA). PCLAMP 9.0 software was used to collect data and adjust the setting, and Clampex 9.0 was used to analyze the data.

**Statistical analysis**

All data were analyzed by SPSS 21.0 software (IBM Corp., USA). Continuous variables complied with normal distribution, and the data were expressed as mean ± SD. Comparison between groups was analyzed using Student’s t-tests, and that among groups was analyzed using one-way analysis of variance (ANOVAs). Pairwise comparison among groups was analyzed with Dunnett’s post hoc test. Bilateral P<0.05 was termed as statistical significance for the power of the test.
References


Grunder S, Firsov D, Chang SS, Jaeger NF, Gautschi I, Schild L, Lifton RP and


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**Conflict of interest**

The authors declare that they have no conflicts of interest with the contents of this article.

**Author contributions**

*Participated in research design: Wen Li, Xiaomu Wang.*

*Conducted experiments: Wen Li, Xiaomu Wang.*

*Contributed new reagents or analytic tools: Wen Li, Xiaomu Wang.*

*Performed data analysis: Xiandong Meng, Dong Wei.*

*Wrote or contributed to the writing of the manuscript: All author*
Figure legend

Figure 1. The membrane expression of ASIC1a after deletion of N-terminus. (A) Western bolt bands of membrane protein and total protein expressed by ASIC1a (wild type and deleted N-terminus) in CHO cells. (B) Quantification results. Endogenous transferrin receptor (TfR) and endogenous α-tubulin were used as internal references of membrane protein and cytoplasmic protein, respectively. n_{WT}=6, n_{ΔN}=7, ***P<0.001 vs. wild type.

Figure 2. The inward current generated after N-terminus deletion from ASIC1a induced at pH 6.0. The results indicated that pH 6.0 could not generate inward current after N-terminus deletion. The inset illustrated inward current generated by wild type or N-terminus deleted ASIC1a induced under pH 6.0. n_{WT}=6, n_{ΔN}=5, ***P<0.001 vs. wild type.

Figure 3. Expression of wild type and N-terminus deleted ASIC1a on CHO cells. (A and D) Total protein expression of ASIC1a with GFP green fluorescence; (B and E) Cell membrane with myc-CD8α red fluorescence. There was no colocalization between green fluorescence (ASIC1a) and red fluorescence (cell membrane) after N-terminus was deleted. Scale bar: 10 μm.

Figure 4. Influence of deleting different sections of the N-terminus on the expression of ASIC1a in CHO cell membranes. (A, D, and G) The different deletion strategies of the N-terminus of ASIC1a. (B, E, and H) Western blot bands of membrane protein and total protein expressed in CHO. (C, F, and I) Quantification of the western blots. NS indicates no statistical difference, n_{WT}=5, n_{Δ1-10}, n_{Δ1-20}, n_{Δ1-30}, n_{Δ1-41}=7.
Δ1 -10, Δ1 -20, Δ1 -30, Δ1 -41 = 7, nΔa+c,Δb+c,Δa+d,Δb+d,Δc+d=6, *P<0.05 vs. wild type; ***P<0.001 vs. wild type.

Figure 5. Influence of different fusion GFP locations on the expression of ASIC1a after N-terminus deletion. (A) Western blots illustrated that membrane expression was lacking in both vectors after the N-terminus was deleted from ASIC1a. The results of inward current induced at pH 6.0 indicated that no current was recorded with both vectors. nN1-ASIC1a WT, C3-ASIC1a WT = 7, nN1-ASIC1a ΔN, C3-ASIC1a ΔN = 6, *P<0.05 vs. wild type.

Figure 6. Three N-terminus deletion/mutation strategies and electrophysiological analysis. (A, C, and E) Different N-terminus deletion/mutation strategies. (B, D, and F) Statistical results of the electrophysiological analysis, which illustrated when more than 20 amino acids were cut off, and AA 16-20 were deleted or mutant, no current would be generated in the channel. nWT = 6, nΔ1-10, Δ1-20, Δ1-30, Δ1-41 = 7, nΔ1-10, Δ11-20, Δ21-30, Δ31-41 = 7, nΔ11-15, Δ16-20, 16A20 = 6, NS indicates no statistical difference, *P<0.05 vs. wild type, ***P<0.001 vs. wild type.

Figure 7. Critical roles of AA 18 in gate opening of ASIC1a. (A) Current curve induced under pH 6.0. (C) Western blots bands of membrane protein. (B and D) Quantification of current and membrane protein. nWT = 6, nV16A, S17A, I18A, Q19A = 7, nH18A, H18C, H18G, I18S, I18T, I18V = 7, NS indicates no statistical difference, ***P<0.001 vs. wild type.
Figure 1

A

<table>
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B

Relative Membrane Protein

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**Asic1a**

α-Tubulin

GAPDH

***
Figure 2

Relative $I_{pH6.0}$ Current Density

WT  AN

pH6.0  pH6.0

1.0  0.0

2nA  10s

***
Figure 4

A

D

G

B

E

H

C

F

I

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Figure 5

A

B

Relative Current Density

NI-ASIC1a WT  NI-ASIC1a AN  C3-ASIC1a WT  C3-ASIC1a AN

Surface

Total

*
Figure 7

A

WT  V16A  S17A  I18A  Q19A

pH6.0

B

Current Density

C

Mutants

I  A  C  G  S  T  V

ASIC1a(surface)

ASIC1a(total)

α-tublin

GAPDH

D

Relative I_{max} Current Density

WT  I18A  I18C  I18G  I18S  I18T  I18V