Identification of a Novel Protein Complex Containing ASIC1a and GABAA Receptors and Their Interregulation

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Abstract

Acid-sensing ion channels (ASICs) belong to the family of the epithelial sodium channel/degenerin (ENaC/DEG) and are activated by extracellular protons. They are widely distributed within both the central and peripheral nervous systems. ASICs were modified by the activation of γ-aminobutyric acid receptors (GABA_A), a ligand-gated chloride channels, in hippocampal neurons. In contrast, the activity of GABA_A receptors were also modulated by extracellular pH. However so far, the mechanisms underlying this intermodulation remain obscure. We hypothesized that these two receptors-GABA_A receptors and ASICs channels might form a novel protein complex and functionally interact with each other. In the study reported here, we found that ASICs were modified by the activation of GABA_A receptors either in HEK293 cells following transient co-transfection of GABA_A and ASIC1a or in primary cultured dorsal root ganglia (DRG) neurons. Conversely, activation of ASIC1a also modifies the GABA_A receptor-channel kinetics. Immunoassays showed that both GABA_A and ASIC1a proteins were co-immunoprecipitated mutually either in HEK293 cells co-transfected with GABA_A and ASIC1a or in primary cultured DRG neurons. Our results indicate that putative GABA_A and ASIC1a channels functionally interact with each other, possibly via an inter-molecular association by forming a novel protein complex.

Introduction

Acid-sensing ion channels (ASICs) belong to the family of the epithelial sodium channel/degenerin (ENaC/DEG) and are activated by extracellular protons [1]. They are widely distributed within both the central and peripheral nervous systems [2]. The activation of ASICs by protons induces sodium and/or calcium influx, giving rise to depolarization and evoking action potentials in neurons [3]. Acid-sensing ion channels (ASICs) are associated with various physiological and pathophysiological functions including regulation of synaptic plasticity [4], perception of pain [5], ischemic death of neurons [6] and the termination of seizures [7]. ASICs were modified by the activation of γ-aminobutyric acid receptors (GABAA), a ligand-gated chloride channels, in hippocampal neurons [8]. In contrast, the activity of GABAA receptors were also modified by extracellular pH [9–11]. However, the mechanisms underlying this intermodulation remain obscure. Megan et al. identified the αβ subunit TM2 residue mediating proton modulation of GABAA receptors [12,13]. Huang et al. reported that external protons regulated GABAA receptor function by direct or allosteric interaction with the GABA binding site [14]. But whether there was a direct binding site for proton within the GABAA receptor was so far unknown. We hypothesized that GABAA receptors and ASICs channels might form a novel protein complex and functionally interact with each other. In the study reported here, we found that ASICs were modified by the activation of GABAA receptors either in HEK293 cells following transient co-transfection of GABAA and ASIC1a or in primary cultured dorsal root ganglia (DRG) neurons. Conversely, activation of ASIC1a also modifies the GABAA receptor-channel kinetics. Immunoassays showed that both GABAA and ASIC1a proteins were co-immunoprecipitated mutually either in HEK293 cells co-transfected with GABAA and ASIC1a or in primary cultured DRG neurons. Our results indicate that putative GABAA and ASIC1a channels functionally interact with each other, possibly via an inter-molecular association by forming a novel protein complex. ASIC1a is specifically located in DRG neurons and function as a pain sensor, thus the interaction of GABAA and ASIC1a may contribute to pain sensation.

Results

Activation of GABAA receptors inhibits ASIC1a currents in HEK293 cells

We used a whole-cell voltage-clamp configuration to record ASIC currents in HEK293 cells co-transfected with GABAA receptor subunits (γ1 and β2) and ASIC1a in response to repeated application of a pH 6 solution. The peak amplitude of whole-cell ASIC currents (evoked with pH 6 solution) in HEK293 cells was stable, averaging 2.51±0.37 nA (n = 38). Under our recording conditions the responses to GABA (at 100 μM) were small relative
to ASICs currents (230±19 pA, n = 27) due to the small driving force on chloride at −60 mV (Figure 1A). Application of GABA reversibly inhibited ASIC currents (Figure 1A), which was largely abolished by application of a GABA_A receptors antagonist (either bicuculline or picrotoxin) (Figure 1B). To further confirm this phenomenon, we investigated whether GABA affected ASIC1a currents in HEK293 cells transfected with ASIC1a cDNA only. The result showed that GABA had no any effect on ASIC currents (Figure 1C). To clarify whether this inhibition is pH-dependent, we tested the effect of GABA on ASIC currents evoked by lowered pH (≤3.5). In general, the current evoked with pH 3.5 solution comprised of fast transient component and followed sustained component. Our results show that activation of GABA_A receptors also attenuated the peak current amplitude but enhanced the sustained current evoked with pH 3.5 solution, such effect was eliminated when GABA_A-R was blocked or HEK293 cells was transfected with ASIC1a cDNA only (Figure 2). These results suggested that activation of GABA_A receptors strongly regulates ASIC1a currents.

Activation of ASIC1a modifies the current kinetics of GABA_A current

Activation of ASIC1a reversibly altered the overall shape of GABA_A currents in HEK293 cells co-transfected with GABA_A receptor subunits (α1 and β2) and ASIC1a. Activation of ASIC1a had multiple effects on the GABA_A currents, not only was the peak amplitude of the ASIC current enhanced, but also the kinetics of the GABA_A currents were altered. Although activation of ASIC1a did not change the rise time (10%–90%) for the GABA_A currents, the time for desensitization or deactivation of GABA_A currents were markedly decreased when the pH of the extracellular solution was decreased from 7.4 to 6. After washout, the time for desensitization and deactivation was totally recovered (Figure 3A, n = 12). To exclude the direct role of proton on GABA_A currents, we transfected HEK293 cells with GABA_A receptor subunits only and did not obtain any current response to pH 6 solution although the peak amplitude of GABA_A currents was also altered (Figure 3B, n = 12). These data indicate that the functions of GABA_A receptors are modified by ASIC1a.

Co-immunoprecipitation of ASIC1a and GABA_A proteins in transfected HEK293 cells and primary cultured neurons

To investigate the underlying mechanisms of interregulation of ASIC1a and GABA_A proteins, we transiently co-transfected ASIC1a and GABA_A-R in HEK293 cells. Due to endogenous expression of ASIC1a in HEK293 cells, we transfected ASIC1a with HA tag. In Co-IP experiments, anti-HA magnetic beads are used for the immunoprecipitation of specific HA-tagged proteins expressed in HEK293 cells. Our results showed that GABA_A specifically co-precipitated with ASIC1a only in cells co-transfected with ASIC1a and GABA_A, which was confirmed by reversed Co-IP using antibodies to GABA_A-R β2 (Figure 4 A). ASIC1a endogenously expressed in HEK-293 cells [15]. Indeed, in our studies, we found that endogenous ASIC1a also co-precipitated with GABA_A-R in HEK293 cells transfected with GABA_A-R α1β2 subunits. It is well known that DRG neurons expressed both ASIC1a and GABA_A-R. To examine the possible co-expression of endogenous ASIC1a and GABA_A-R, primary DRG rat neurons were incubated with specific anti-GABA_A-R and anti-ASIC1a antibodies. The merged image indicates that ASIC1a and GABA_A-R are co-segregated with each other (Figure 4 B1). To further investigate a possible association between ASIC1a and GABA_A-R proteins, GABA_A-R was immunoprecipitated from rat DRG lysates with a polyclonal anti-GABA_A-R β2/3 antibody. The immunoprecipitated samples were probed with ASIC1a antibody. Conversely, the total DRG lysates was precipitated with ASIC1a antibody and then probed with GABA_A-R β2/3 antibody (Figure 4 B2).
Taken together, our results showed that ASIC1a and GABAA proteins co-immunoprecipitated each other.

**Interregulation of GABAA receptors and ASIC1a in DRG neurons**

Both GABAA receptors and ASIC1a channels colocalized in rat DRG neurons. To examine the interaction of endogenous ASIC1a and GABAA receptors in primary cultured rat DRG neurons, we used a whole-cell voltage-clamp configuration to record ASIC currents or GABAA currents in DRG neurons in response to application of a pH 6 solution or 100 μM GABA. GABA (100 μM) reversibly attenuated acid-evoked currents (Figure 5A, n = 12). Conversely, GABA induced current was enhanced in the presence of pH 6.0 solution (Figure 5B, n = 7).

**Figure 2. Activation of GABAA receptors attenuated the peak current amplitude and enhanced the sustained current of ASIC1a.**

A, Example traces of a fast-inactivating transient current and a sustained current of ASIC1a activated by pH 3.5. GABA (100 μM) attenuated a fast-inactivating transient current and enhanced the sustained current of ASIC1a in HEK293 cells co-transfected with GABAA receptor subunits (α1 and β2) and ASIC1a, which can totally abolished by co-application of picrotoxin (100 μM) with GABA. ASIC1a current traces were superimposed to the right (inset) (B). C, GABA had no effect on ASIC1a currents in HEK293 cells transfected with ASIC1a cDNA only. ASIC1a current traces were superimposed to the right (inset).

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Figure 3. Activation of ASIC1a modifies the current kinetics of GABA_A current. A, The representative current traces recorded from HEK293 cells co-transfected with GABA_A receptor subunits (γ_1 and β_2) and ASIC1a. ASIC1a activated by pH 6 reversibly altered the overall shape of GABA_A currents. GABA_A current traces were superimposed to the right. Red arrow indicates the current activated by pH 6 solution. B, pH 6 solution changed the peak current amplitude but not the shape of GABA_A currents in HEK293 cells transfected with cDNA of GABA_A receptor subunits only. C (cotransfected with both plasmids) and D (transfected with cDNA of GABA_A receptor subunits), bar graph showing the summarized data of rise time.
Discussion

In the study reported here, activation of GABA_A receptors strongly attenuates the peak current amplitude of ASIC currents in transfected HEK 293 cells. Conversely, Activation of ASIC1a modifies the current kinetics of GABA_A current. These modifications included enhancement of the peak amplitude of GABA_A current and slowing of channel kinetics. Similar effects were observed in primary cultured DRG neurons. Furthermore, ASIC1a is co-segregated with GABA_A proteins in either transfected HEK 293 cells or rat DRG neurons, a finding verified by the immunoblotting assays. Our overall conclusion is that ASIC1a and GABA_A interregulated each other through a conformation-dependent protein-protein interaction.

Interregulation of ASIC1a and GABA_A receptors through conformation-dependent protein-protein interaction

Modification of ASIC1a by GABA_A receptors occurred rapidly, and when the activation of GABA_A chloride channels were blocked by pharmacological blockade or genetic loss, the modifications were eliminated or largely reduced. The ASIC

![Figure 4. Co-immunoprecipitation of ASIC1a and GABA_A proteins in transfected HEK293 cells and DRG neurons. A. Co-immunoprecipitation of ASIC1a and GABA_A proteins in transfected HEK293 cells. GABA_A specifically co-precipitates with ASIC1a in cells co-transfected with ASIC1a and GABA_A (both). n = 3. B1. DRG neurons were double-labeled with anti-ASIC1a and -GABAAR antibodies. ASIC1a (red in left panel) as well as GABAAR (green in middle panel) localized to the apical membrane of DRG neurons. The merge (right) of ASIC1a and GABAAR images indicates that ASIC1a co-segregates with GABAAR in the apical membrane in neurons (yellow). Nuclei were identified by DAPI staining (blue). Scale bars equal 50 μm. B2. GABAAR precipitates with ASIC 1a in primary cultured DRG neurons. DRG neurons lysates were immunoprecipitated (IP) with anti-GABAAR β2/3 subunits polyclonal antibody in 8% SDS-PAGE gel, and the blot was then probed with anti- ASIC 1a polyclonal antibody (IB). In turn, ASIC 1a co-immunoprecipitates with GABAAR. Cell lysates were immunoprecipitated with ASIC 1a antibody in 6% SDS-PAGE gel, and the blot was probed with GABAAR β2/3 antibody. These experiments were repeated three times with identical results.](https://www.plosone.org/about/image/10.1371/journal.pone.0099735.g004)
currents also recovered rapidly (Figure 1). This study suggests that ASIC1a current is modified directly by activation of GABA A receptors. In 2011, Cheng et al. found that ASICs were reversibly inhibited by activation of GABA receptors in murine hippocampal neurons and such inhibition of ASICs required opening of the chloride channels. Therefore these authors speculate that a conformation-dependent interaction might occur between GABA receptors and ASICs [16]. Our present studies further demonstrated that these two receptors form a novel protein complex by performing the Co-IP experiments. These two receptors physically couple together and interact with each other that may depend on their conformation change. However, so far we can not exclude the possibility that an intermediate protein or regulator might participate in this receptor-receptor interaction.

Furthermore, in our present studies, we also demonstrated that pH 6 extracellular solution largely decreased the time for desensitization or deactivation of GABAA currents in HEK 293 cells co-transfected with ASIC1a and GABAA, but not in HEK293 cells transfected with GABAA cDNA only, suggesting that these two receptors couple with each other and regulate each other. External protons regulate GABAA receptor function by direct or allosteric interaction with the GABA binding site [14]. So far, we can not exclude the possibility that GABAA receptor may have a proton binding site. In summary, upon binding of GABA, the GABAA receptors undergo a conformational change and then modify current kinetics of ASIC1a by allosteric interaction with the proton binding site. Conversely, when ASIC1a is activated, the ASIC1a channels undergo a conformational change, then such change is converted to GABAA receptors. Our results indicate that putative GABAA receptors and ASIC1a channels physically couple and functionally interact with each other, possibly via an intermolecular association.

Physiological and pathophysiological implications of the interaction between ASIC 1a and GABAA receptors of all ASICs, ASIC1a appears to play a prominent role in determining current amplitude and also affects the kinetics of H+-gated current [4,17–19]. In CNS neurons, ASIC1a has been shown to be involved in synaptic plasticity, learning and memory [4,20], and in acidosis-mediated, glutamate-independent neuronal injury [6,21]. ASIC1a is expressed throughout the brain, with prominent expression in areas that receive rich synaptic input [17,20,22,23]. Moreover, ASIC1a have a higher expression in GABAergic interneurons than that in the principal neurons [7,24]. Given that the GABAA receptors are the predominant inhibitory ionotropic receptors in the CNS, the interaction between ASIC1a and GABAA receptors may occur at numerous locations and could be involved in a number of brain functions. Furthermore, ASIC1a is specifically located in DRG neurons and functions as a pain sensor, thus the interaction of GABAA and ASIC1a may contribute to pain sensation.

Materials and Methods

Transfection of HEK293

HEK 293 cells were cultured in DMEM (HyClone) supplemented with 10% fetal bovine serum (HyClone), 1% penicillin/streptomycin at 37°C in a 5% CO2 incubator. Cells were transfected with pcDNA3.0 constructors encoding ASIC1a and/ or GABAA, using Lipofectamine TM 2000 (Invitrogen) according to the manufacturer’s instructions. All recordings were made 24 to 48 h after transfection in the GFP-positive cells.

DRG Cell Isolation and Culture

All the animal experiments were approved by the Medical Ethics Committee of Shandong University (number ECAES-DUSM 2012029). Adult Wistar rats were euthanized by cervical
dislocation and the entire spinal columns were removed. Bilateral DRGs were collected and washed twice with 1 L-15 medium (Gibco, Gaithersburg, MD). They were then incubated in 10 ml L-15 medium containing 10 mg collagenase type 1 (Sigma, St. Louis, MO) and 0.25 ml 0.25% Trypsin (HyClone, Thermo scientific, USA) at 37 °C for 50 min. DRGs were removed from the enzyme solution, centrifuged for 5 min at 1,000 revolutions/min, washed twice with L-15 medium, and transferred to 2 ml L-15 medium containing 10% FBS. The ganglia were triturated with a suction pipe for 3-min and then centrifuged for 50 seconds at 1,000 revolutions/min. Supernatants were placed into 35 mm diameter Petri dishes. The cells were then cultured at 37 °C in a 5% CO2 incubator (Thermo Forma, Hamilton, NJ, USA). In our study, we chose freshly isolated neurons from rat DRGs in the range of 15–30 μm diameter to test the effect of GABA on acid-evoked currents or pH on GABA-induced current.

Electrophysiological Recordings

Whole-cell voltage-clamp and current-clamp recordings were performed at room temperature (22±25 °C) using a computer amplifier (MultiClamp 700B; Axon, New York, NY, USA) and a Digidata (1440A; Axon). Patch pipettes were filled with intracellular solution containing (in mM): KCl 140, MgCl2 2.5, HEPES 10, EGTA 11 and Na2ATP 5 with pH adjusted to 7.4 using NaOH. Cells were bathed in extracellular saline containing (in mM): NaCl 150, KCl 5, CaCl2 2.5, MgCl2 2, HEPES 10, D-glucose 10 with pH adjusted to 7.4 using NaOH. The resistance of the recording pipettes was in the range of 5–8 MΩ. The series resistance was compensated for 70–80% after establishing a whole-cell configuration. The membrane potential was held at −60 mV throughout the recordings unless otherwise specified. Current-clamp recordings were obtained by switching to current-clamp mode after a stable whole-cell configuration was formed in the voltage-clamp mode. In this experiment, only cells with a stable resting membrane configuration 36 h post-transfection. HEK293 cells were lysed in 1 ml of lysis buffer (1% Triton X-100, 50 mM Tris buffer pH 7.5), and the samples were loaded and run in 8% SDS-PAGE gel. The precipitates were transferred to a PVDF membrane and immunoblotted by anti-GABAAR antibody (Millipore). The blots were developed by the enhanced chemiluminescence kit. Rat DRG or HEK293 cells were lysed in lysis buffer (1% Triton X-100, 50 mM Tris buffer pH 7.5) and a mixture of protease inhibitors. Anti-ASIC1a antibody or anti-GABAAR β2/3 antibody was immunoprecipitated with protein A/G-agarose beads (Santa Cruz Biotechnology). The protein complexes were immunoprecipitated with antibody cross-linked protein A/G-agarose beads. After incubating the lysates with cross-linked antibody, the beads were pelleted and washed three times in lysis buffer (1% Triton X-100, 50 mM Tris buffer pH 7.5), and the samples were loaded and run in 8% SDS-PAGE gel. The precipitates were transferred to a PVDF membrane and immunoblotted by anti-GABAAR β2/3 antibody (Millipore) or anti-ASIC1 antibody (Sigma). The blots were developed by the enhanced chemiluminescence kit.

Drug Application

All drugs were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Except picrotoxin (dissolved in DMSO), all drugs were initially made up as stock solutions in distilled water and subsequently diluted in the external solution of the cells at a maximum of 1:1000 to achieve their final working concentrations.

Data Analysis

Data were expressed as mean ± SEM and compared statistically using paired t tests by Sigma Plot 10.0. A p<0.05 was required for the results to be considered statistically significant.

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Author Contributions

Conceived and designed the experiments: JL. Performed the experiments: DZ NN ZL HS CW DC. Analyzed the data: DZ NN ZL JL. Contributed reagents/materials/analysis tools: CW DC. Wrote the paper: JL.

References

1. Krishtal OA, Pidoplichko VI (1981) A receptor for protons in the membrane of sensory neurons may participate in nociception. Neuroscience 6: 2599–2601.
2. Krishtal O (2003) The ASICs: signaling molecules? Modulators? Trends Neurosci 26: 477–485.
3. Mainer J, Baron A, Lazdunski M, Voilley N (2002) Proinflammatory mediators, stimulators of sensory neuron excitability via the expression of acid-sensing ion channels. J Neurosci 22: 10662–10670.
4. Wemmie JA, Chen J, Adesokan CC, Hrncova-Hagerman AM, Price MP, et al. (2002) The acid-activated ion channel ASIC contributes to synaptic plasticity, learning, and memory. Neuron 34: 463–477.
5. Chen CC, Zimmer A, Sun WH, Hall J, Brownstein MJ, et al. (2002) A role for ASIC1 in the modulation of high-intensity pain stimuli. Proc Natl Acad Sci U S A 99: 8992–8997.
6. Xiong ZG, Zhu XM, Chiu XP, Minami M, Hey J, et al. (2004) Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels. Cell 118: 607–618.
7. Ziemann AE, Schneider MK, Albert GW, Severson MA, Howard MA, 3rd, et al. (2008) Seizure termination by acidosis depends on ASIC1a. Nat Neurosci 11: 816–822.
15. Gunthorpe MJ, Smith GD, Davis JB, Randall AD (2001) Characterisation of a human acid-sensing ion channel (hASIC1a) endogenously expressed in HEK293 cells. Pflugers Arch 442: 668–674.
16. Chen X, Whissell P, Orser BA, MacDonald JF (2011) Functional modifications of acid-sensing ion channels by ligand-gated chloride channels. PLoS One 6: e21970.
17. Waldmann R, Champigny G, Bassilana F, Heurteaux C, Lazdunski M (1997) A proton-gated cation channel involved in acid-sensing. Nature 386: 173–177.
18. Askwith CC, Wemmie JA, Price MP, Rokhlina T, Welsh MJ (2004) Acid-sensing ion channel 2 (ASIC2) modulates ASIC1 H+–activated currents in hippocampal neurons. J Biol Chem 279: 18296–18305.
19. Escoubas P, De Weille JR, Lecoq A, Diochot S, Waldmann R, et al. (2000) Isolation of a tarantula toxin specific for a class of proton-gated Na+ channels. J Biol Chem 275: 25116–25121.
20. Wemmie JA, Askwith CC, Lamani E, Cassell MD, Freeman JH, Jr., et al. (2003) Acid-sensing ion channel 1 is localized in brain regions with high synaptic density and contributes to fear conditioning. J Neurosci 23: 5496–5502.
21. Yermolaieva O, Leonard AS, Schneider MK, Abboud FM, Welsh MJ (2004) Extracellular acidosis increases neuronal cell calcium by activating acid-sensing ion channel 1a. Proc Natl Acad Sci U S A 101: 6752–6757.
22. Garcia-Anoveros J, Derfler B, Neville-Golden J, Hyman BT, Corey DP (1997) BNaC1 and BNaC2 constitute a new family of human neuronal sodium channels related to degenerins and epithelial sodium channels. Proc Natl Acad Sci U S A 94: 1459–1464.
23. Alvarez de la Rosa D, Krueger SR, Kolar A, Shao D, Fitzsimonds RM, et al. (2003) Distribution, subcellular localization and ontogeny of ASIC1 in the mammalian central nervous system. J Physiol 546: 77–87.
24. Weng JY, Lin YC, Lien CC (2010) Cell type-specific expression of acid-sensing ion channels in hippocampal interneurons. J Neurosci 30: 6548–6558.