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Acid sensing ion channels regulate neuronal excitability by inhibiting BK potassium channels

Elena Petroff [a,*], Vladislav Snitsarev [a], Huiyu Gong [b], Francois M. Abboud [c]

[a] Department of Biology and Molecular Biology, Montclair State University, Montclair, NJ 07043, USA
[b] Department of Neonatology, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA
[c] Department of Internal Medicine and Cardiovascular Research Center, Department of Physiology and Biophysics, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA

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ABSTRACT

Acid sensing ion channels (ASICs), Ca^{2+} and voltage-activated potassium channels (BK) are widely present throughout the central nervous system. Previous studies have shown that when expressed together in heterologous cells, ASICs inhibit BK channels, and this inhibition is relieved by acidic extracellular pH. We hypothesized that ASIC and BK channels might interact in neurons, and that ASICs may regulate BK channel activity. We found that ASICs inhibited BK currents in cultured wild-type cortical neurons, but not in *ASIC1a/2/3* triple knockout neurons. The inhibition in the wild-type was partially relieved by a drop in extracellular pH to 6. To test the consequences of ASIC–BK interaction for neuronal excitability, we compared action potential firing in cultured cortical neurons from wild-type and *ASIC1a/2/3* null mice. We found that in the knockout, action potentials were narrow and exhibited increased after-hyperpolarization. Moreover, the excitability of these neurons was significantly increased. These findings are consistent with increased BK channel activity in the neurons from *ASIC1a/2/3* null mice. Our data suggest that ASICs can act as endogenous pH-dependent inhibitors of BK channels, and thereby can reduce neuronal excitability.

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1. Introduction

Neuronal excitability is an important factor in determining neuronal function. The activity of BK (Ca^{2+} and voltage-activated potassium channel) has profound effects on neuronal excitability. Functional or heritable gain of function of BK has been associated with change in the shape of action potentials, increased frequency of action potential firing, and spontaneous seizures in both humans and mice [1,2]. ASIC channels, generally considered to be sensory channels, have also been implicated in changing neuronal action potential firing by contributing to membrane depolarization through small sustained inward currents induced by acidification of the environment [3–5]. Moreover, a number of studies report increases in the sensory response in ASIC knockout mice [6–9], which is hard to understand if ASICs act as depolarizing channels.

We have previously shown that ASIC proteins, in addition to their channel properties, can directly interact with and regulate activity of BK channels in heterologous expression systems [10]. This regulation is pH dependent, and partial relief of BK inhibition by the more acid sensitive ASIC1a can be observed at pH 7 with the full effect at pH 6, whereas relief of inhibition by ASIC2 requires pH 5 and lower [10]. ASIC and BK channels co-exist in neurons in many parts of the brain [11–14]. We hypothesized that an interaction between these channels may happen in vivo, that this interaction may regulate BK channel activity and, through this regulation, neuronal firing properties.

2. Materials and methods

2.1. ASIC1a/2/3 null mice

The generation of *ASIC1a/2/3* −/− mice has been described [9]. Each of previously generated ASIC1a, 2, and 3 null lines [6,13,15] was backcrossed for 10 generations onto a C57BL/6J background to generate congenic lines and then crossed to one another to generate a congenic C57BL/6J line missing *ASIC1a* and *2* (*ASIC1a/2* null mice), and *3* (*ASIC1a/2/3* −/− mice).

Abbreviations: ASIC, acid sensing ion channel; BK, Ca^{2+} and voltage-activated potassium channel; EGTA, ethylene glycol tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; BAPTA, 1,2-bis(o-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IbTx, iberiotoxin.

* Corresponding author. Address: Department of Biology and Molecular Biology, Montclair State University, 1 Normal Ave., Montclair, NJ 07043, USA. Fax: +1 (973) 655 7047.

E-mail address: petroffe@mail.montlcair.edu (E. Petroff).

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2.2. Antibodies

Anti-h/r/mBK monoclonal antibody was purchased from BD Biosciences Pharminogen (San Jose, CA). Anti-h/r/m Actin goat polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Biochemistry

The whole mouse brain lysates were prepared as a homogenate in 5 ml volumes of lysis buffer containing 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and a protease inhibitor cocktail (Roche, Indianapolis, IN), then centrifuged at 15,000 rpm for 30 min at 4 °C to remove insoluble material. Following ultracentrifugation, 500 μg of detergent-soluble protein was pre-cleared and immunoprecipitated using anti-BK antibody and protein A-Sepharose. The 10 μg of total lysate was resolved by SDS–PAGE, the membrane was blocked with 5% BSA in TTBS at room temperature for 1 h, and then probed with BK antibody (1:200) for 2 h at room temperature. The blots were washed three times in Tris-buffered saline containing 0.05% Tween 20 (TTBS) and incubated with horseradish-peroxidase (HRP)-labelled protein A (1:10,000) (Pierce, Rockford, IL) or anti-mouse IgG HRP (Amersham, Piscataway, NJ). After further washing 5 times with TTBS, bound antibody was detected by enhanced chemiluminescence (ECL) (Pierce, Rockford, IL).

2.4. Neuronal culture

Mouse cortical neuron cultures were prepared from postnatal day 1 mouse pups as described previously [16] with some modification. Cortical tissue from 4 to 5 pups were pooled, dissociated by enzymatic treatment, 0.15% trypsin for 8 min at 37 °C, and plated on 12 mm cover slips (200–300 cells/mm^2) coated with poly-D-lysine/laminin (BD BioCoat, BD Biosciences, Bedford, MA) in culture medium Neurobasal A containing B27, L-glutamine, and 5.0% horse serum (Gibco Life Technologies, Gaithersburg, MD). After 4 h, the cultured cells were moved into culture medium without horse serum supplement and maintained in culture for 9–10 days before the experiments.

2.5. Electrophysiology

Whole-cell patch clamp recordings were performed and analysed as previously described [17] using Axopatch 200 amplifier and pClamp9 software (Molecular Devices, Sunnyvale, CA). The pipette resistance was 3–4 MΩ, and the holding potential was −80 mV. BK current was measured using voltage steps from −60 to +80 mV in 20 mV increments, 3–5 min after the solution change to avoid potential contribution of transient ASIC currents activated by a drop in the bath pH. The composition of the pipette solution was (in mM): K aspartate 116, KCl 10, NaCl 5, $MgCl_2$ 4, $MgSO_4$ 0.8, ATP 4, BAPTA 10, HEPES 10, pH 7.2 (KOH), and 300 mOsm (mannitol). The extracellular solution for BK experiments contained: NaCl 116, KCl 5.4, $MgSO_4$ 0.8, $CaCl_2$ 1.8, glucose 5.6, HEPES 13, MES 13, pH 7.4 or 6 (NaOH), and 300 mOsm (mannitol). The pH 6 solution was applied using a fast perfusion system (Warner Instruments Inc., Hamden, CT) with the perfusion pipette positioned 50 μm from the cell body.

The sharp microelectrode studies were performed at 37 °C in Earl's balanced salt solution (Sigma, St. Louis, MO) bubbled with 5% CO_2/95% air to maintain pH 7.4. Current (usually around −0.2 nA) was injected to maintain the membrane voltage at −80 mV. Depolarizing current injections of 0.2, 0.3, and 0.4 nA were administered, and the membrane voltage was recorded.

2.6. Statistics

Statistical analysis was performed using GB-STAT software (Dynamic Microsystems, Silver Spring, MD). For normally distributed data sets (Shapiro–Wilk test), data are shown as mean ± SEM. Paired *t*-test was used to compare two treatments on the same cell, and repeated measures ANOVA was used to compare multiple treatments on the same cell. For data sets that were not normally distributed the data are presented as standard box plot charts. The comparison between such data sets was done using Wilcoxon rank-sum with Mann–Whitney *U* test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. ASICs do not alter BK protein level in brain

ASIC and BK channels both display wide distributions throughout the brain [11–14] indicating the potential for in vivo interaction. We used comparison between wild-type and triple knockouts (*ASIC1a/2/3* −/−) because central neurons express ASIC1a, -2a, -2b, and small amounts of -3 [14,18–20], all of which inhibit BK [10]. In cultured cortical neurons, disruption of these genes eliminated H^+-evoked currents that are typical for ASICs (Fig. 1A). The BK protein levels in brain were not altered (Fig. 1B).

3.2. ASICs inhibit BK channels in cortical neurons

To test if the presence of ASIC channels affects the BK channel activity, we measured K^+ currents in cortical neurons from wild-type mice and from mice with disrupted *ASIC1a*, *2*, and *3* genes (Fig. 2A). We found that the current amplitudes at different voltage steps were significantly increased (Fig. 2A–C). To avoid any potential artefacts, no channel inhibitors were applied, so the fast inward current at the beginning of the voltage pulse and an occasional postsynaptic current were present in the recordings. Because of this, the current amplitudes were measured at the end of the 200 ms voltage step to allow for inactivation and minimal input of other K^+ channels to the predominantly BK current. To further assess the specific BK current in these neurons, we measured the response to a selective BK channel blocker iberiotoxin (IbTx). As shown in Fig. 2A and B, *ASIC1a/-2/-3* null neurons had 6-fold more IbTx-sensitive current than wild-type. These results suggest that ASICs inhibit neuronal K^+ channels and that, in the absence of ASICs in the *ASIC1a/2/3* −/−, BK channels were available for IbTx block. Reducing pH to 6 caused a small but significant increase in K^+ current amplitude in wild-type, but, in *ASIC1a/-2/-3* null neurons the currents were larger to start with and did not change with pH 6 (Fig. 2A and C).

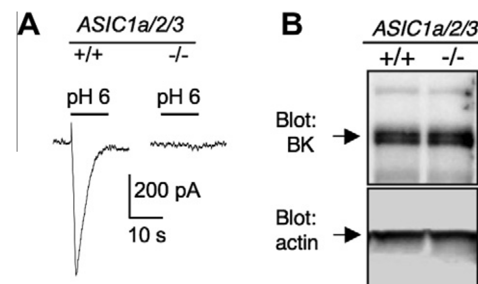

Fig. 1. Absence of ASIC1a, 2 and 3 does not affect BK channel expression. (A) Proton-gated currents in wild-type and *ASIC1a/2/3* −/− cortical neurons, V_{hold} = -−80 mV, pH 6 application is shown by the bar, *n* = 6. (B) BK protein levels in the wild-type and *ASIC1a/2/3* −/− brain detected by Western blot.

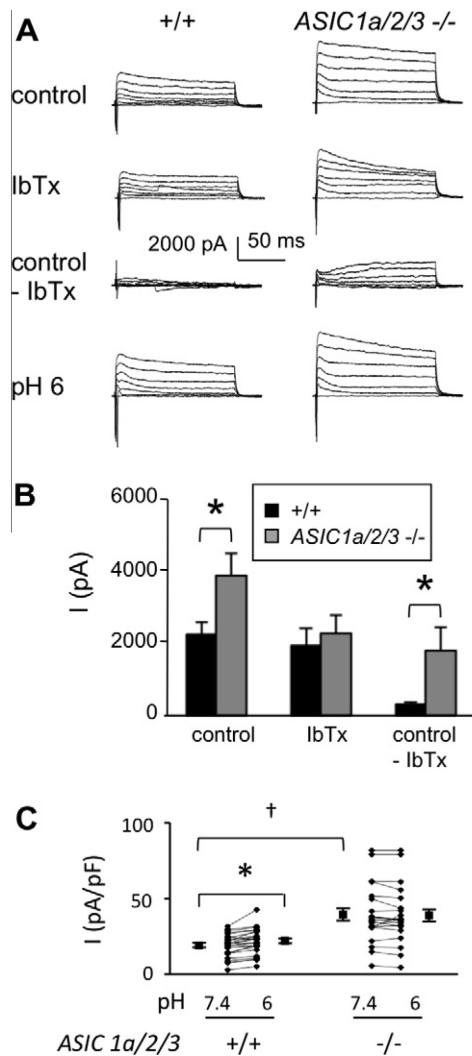

Fig. 2. ASIC channels inhibit BK currents in cortical neurons. (A) Representative K^+ currents in cortical neurons from wild-type and *ASIC1a/2/3* null mice. The cells were held at −80 mV, and currents resulting from 20 mV voltage steps between −20 to +80 mV are shown. The currents were measured at pH 7.4 (control), pH 6, and at pH 7.4 in the presence of 50 nM IbTx applied for 10 min before the measurement. The IbTx-inhibitable (BK) current component was revealed by subtracting the currents in the presence of IbTx from control. (B) Current amplitudes at +80 mV in cortical neurons under conditions described in A. Current amplitudes were measured at the end of 200 ms depolarizing voltage steps. $^*P < 0.05$ in unpaired *t*-test. (C) Individual current amplitudes at +80 mV in neurons from wild-type and *ASIC1a/2/3* null mice at pH 7.4 and 6. The average ± SEM is also shown. The respective current voltage relationships are shown in Supplementary Figure 1. $^{*,\dagger}P < 0.05$ in paired and unpaired *t*-tests, respectively. The data in B and C are from 25 wild-type and 22 *ASIC1a/2/3* −/− neurons.

3.3. Loss of ASICs increases neuronal excitability

Several studies have demonstrated that elevating BK channel activity increases neuronal excitability [1,2,21]. Excitability increases because BK channels speed up and enhance repolarization of the action potential, allowing a shorter interval before the next action potential [1,2,11,21]. To learn whether the loss of ASICs and the resulting increase in BK current would have a similar effect, we compared action potential firing and excitability of the cultured cortical neurons from wild-type and *ASIC1a/2/3* null mice. To avoid potential artefacts of cell perfusion and to preserve the intracellular signalling mechanisms involved in regulation of excitability, we used sharp microelectrode recordings of the membrane potential. In addition, to generate a more physiological environment, the studies were performed at 37 °C in Earl's balanced salt solution bubbled with 5% CO_2/95% air. The resting membrane potential was not significantly different in wild-type (−63.9 ± 11.4 mV) and *ASIC1a/2/3* null (−59.4 ± 8.1 mV) neurons. With current injections of increasing amplitude, neurons cultured from *ASIC1a/-2/-3* null mice showed increased firing compared to wild-type neurons (Fig. 3A and B). The triple knockout neurons also exhibited narrower action potentials and increased after-hyperpolarization which were significantly more sensitive to the IbTx application than in the wild-type (Fig. 3C–E). These changes in repetitive firing and action potential kinetics in the *ASIC1a/-2/-3* null neurons are characteristic of an increased BK activity [1,2,21] suggesting that, in wild-type, ASICs may reduce excitability of central neurons by inhibiting BK channels.

4. Discussion

Our data suggest that at least some populations of ASIC and BK channels interact in neurons and that ASICs inhibit BK currents in the wild-type neurons. The lowering of the extracellular pH to 6 lead to an increase in outward K^+ currents. This is consistent with the relief of inhibition of BK channels described previously [10] and, in addition, may possibly involve other potassium channels, such as voltage-gated potassium channels, that can also be inhibited by ASICs [10]. The observed increase in current at pH 6 in wild-type neurons was significant but not dramatic. This may be due to the presence of both ASIC1 and ASIC2 in the wild-type neurons with ASIC2a requiring much lower pH to relieve the inhibition of BK [10]. When ASIC1a, 2, and 3 were all missing, the inhibitory effects on BK current or pH regulation were not observed (Fig. 2A and C). Moreover, despite similar BK protein level in wild-type and ASIC triple knockout brain, the amount of BK-specific, IbTx-inhibitable current representing BK channels available for the toxin block, is significantly increased in the triple knockouts.

The action potential firing in cortical neurons from *ASIC1a/2/3* null mice displays typical characteristics of neurons with increased BK channel activity, i.e. increased after-hyperpolarization, narrow action potentials and increase in number of action potential per current injection (Fig. 3). Previous studies have also shown that mutations and treatments that lead to increased BK activity result in faster repolarization, more pronounced hyperpolarization, and increases repetitive firing presumably by speeding up the recovery of the voltage gated Na^+ channels required to fire the next action potential [1,2]. Interestingly, in the study with ASIC1a knockouts, no increase in neuronal excitability or seizure threshold was observed [22]. In our experiments, with additional deletion of ASIC2 and ASIC3, we observed the increased excitability. With ASIC 1 and 2 being most abundant in the brain, this indicates a potential important role of ASIC2, a stronger inhibitor of BK channel [10], as BK regulator in brain.

In addition, our data suggest that when brain pH drops under physiological (activity, synaptic transmission) or pathophysiological (trauma, seizure) conditions, activity of BK channel is expected to increase, and neuronal excitability may change. This is in agreement with the recent data on seizure-induced gain of function of BK channels and firing activity in neocortical pyramidal neurons [23]. Thus, interaction with ASICs may allow for the extracellular pH to control activity of BK channel, which is not normally sensitive to the extracellular acidification [22,24,25].

We speculate that similar mechanism of regulation of BK activity and excitability by ASICs may be present in other neurons. Our data may potentially help to explain some of the puzzling aspects of ASIC function in sensory responses and in the peripheral nervous system. Stimulation by protons suggested that these Na^+ conducting channels would depolarize membrane voltage and thereby

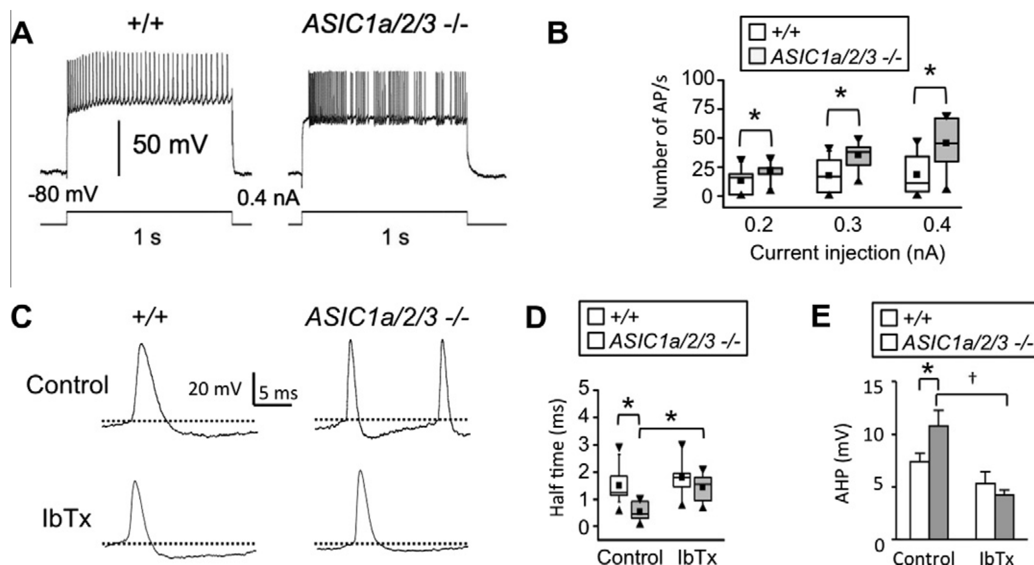

Fig. 3. ASICs regulate neuronal excitability. (A) Representative traces of action potential firing in response to 0.4 nA current injections in cortical neurons from wild-type and *ASIC1a/2/3* −/− mice. (B) Number of action potentials in wild-type and *ASIC1a/2/3* −/− neurons at different current injection steps. *P < 0.05. (C) Representative shapes of the action potentials in wild-type and *ASIC1a/2/3* −/− neurons at 0.4 nA current injections. (D) Action potential half time (measured as a duration at half amplitude of the AP) at 0.4 nA current injection. *P < 0.05. (E) After-hyperpolarization amplitude in wild-type and *ASIC1a/2/3* −/− neurons at 0.4 nA current injections. *,†P < 0.05 in unpaired and paired *t*-tests, respectively. The data in A–E are from 35 wild-type and 25 *ASIC1a/2/3* −/− neurons.

initiate neuronal activity. However, reducing ASIC function with *ASIC* gene disruptions and dominant-negative *ASIC* transgenes have in some cases shown paradoxically increased acid-mediated nociceptive behaviours and mechanosensitive responses accompanied, in some cases, by increased nerve activity [6–9]. We speculate that ASIC effects on K^+ channels and neuronal excitability might account for these apparently contradictory effects. Thus, interaction of ASIC and BK channels in neurons presents a novel function of ASICs as regulators of BK activity, a novel physiological mechanism of regulation of action potential firing, and as such, a new pharmacological target for diseases associated with altered neuronal excitability.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.114.

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