

Acid-sensing ion channels interact with and inhibit BK K⁺ channels

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Acid-sensing ion channels (ASICs) are neuronal non-voltage-gated cation channels that are activated when extracellular pH falls. They contribute to sensory function and nociception in the peripheral nervous system, and in the brain they contribute to synaptic plasticity and fear responses. Some of the physiologic consequences of disrupting ASIC genes in mice suggested that ASIC channels might modulate neuronal function by mechanisms in addition to their H⁺-evoked opening. Within ASIC channel's large extracellular domain, we identified sequence resembling that in scorpion toxins that inhibit K⁺ channels. Therefore, we tested the hypothesis that ASIC channels might inhibit K⁺ channel function by coexpressing ASIC1a and the high-conductance Ca²⁺- and voltage-activated K⁺ (BK) channel. We found that ASIC1a associated with BK channels and inhibited their current. Reducing extracellular pH disrupted the association and relieved the inhibition. BK channels, in turn, altered the kinetics of ASIC1a current. In addition to BK, ASIC1a inhibited voltage-gated Kv1.3 channels. Other ASIC channels also inhibited BK, although acidosis-dependent relief of inhibition varied. These results reveal a mechanism of ion channel interaction and reciprocal regulation. Finding that a reduced pH activated ASIC1a and relieved BK inhibition suggests that extracellular protons may enhance the activity of channels with opposing effects on membrane voltage. The wide and varied expression patterns of ASICs, BK, and related K⁺ channels suggest broad opportunities for this signaling system to alter neuronal function.

Acid-sensing ion channels (ASICs) are voltage-insensitive cation channels expressed in both central and peripheral neurons (1–4). ASICs are activated by extracellular protons, and several agents modify the response. Since their discovery in the early 1980s (5), they have been implicated in many physiologic processes, including nociception, mechanosensation, synaptic plasticity, and fear (1–4). They have also been implicated in pathological conditions including ischemic stroke (6, 7) and multiple sclerosis (8). These channels are formed from homomultimeric and heteromultimeric combinations of ASIC1a, -1b, -2a, -2b, and -3 subunits. Individual subunits contain short intracellular N and C termini, two transmembrane domains, and a large extracellular domain of ≈370 aa (including 14 conserved cysteines) that shows substantial sequence conservation across the degenerin/epithelial Na⁺ channel (DEG/ENaC) family. The recently published crystal structure of chicken ASIC1a shows that three subunits form a channel (9).

Activation by a reduced extracellular pH suggested that these Na⁺-conducting channels (ASIC1a also conducts Ca²⁺) would depolarize membrane voltage and thereby modify neuronal activity. In some cases the data are consistent with this action (10–12). However, some observations have not seemed to fit this proposition. For example, some results obtained in mice with disrupted ASIC genes do not seem consistent with predictions for channels producing depolarizing currents; ASIC gene disruptions and dominant-negative ASIC transgenics can paradoxically increase acid-mediated nociceptive behaviors and mechanoreceptor responses (10, 13, 14). Moreover, despite ASIC1a localization at postsynaptic membranes in central neurons, these

channels have not yet been shown to cause depolarization in response to synaptic activity (15, 16).

These incongruities caused us to wonder whether ASICs might modify neuronal function through another mechanism, and so we examined the amino acid sequence of ASICs. In the extracellular domain we identified a highly conserved sequence (R/K·Y/M·G·K·C) that resembled the part of scorpion α-K-toxins that blocks K⁺ channels (R/F/·G·K·C) (Fig. 1A) (17). In α-KTx toxins, the Lys side chain plugs the channel pore, the Arg interacts with residues in the outer vestibule of K⁺ channels, and the Cys forms a disulfide bond that stabilizes toxin structure (17–19). Although it seemed clear that the ASIC extracellular domain did not resemble an α-KTx toxin, the conserved sequence led us to hypothesize that ASICs might interact with and inhibit K⁺ channels. (After we performed these studies, the structure of chicken ASIC1a was reported; below we discuss the location of these residues in the crystal structure.) Along a similar line, Tavernarakis and Driscoll (20) reported that *Caenorhabditis elegans* degenerins and vertebrate ENaCs have amino acid sequences in their extracellular domains that show similarity to a Na⁺ channel toxin.

Results

ASIC1a Interacts with and Inhibits BK Channels. To test the hypothesis that ASICs inhibit K⁺ channels, we coexpressed ASIC1a with high-conductance Ca²⁺- and voltage-activated (BK, SLO1, or Maxi-K) K⁺ channels (21, 22). BK channels, composed of four α-subunits, are inhibited by iberitoxin (IbTx), charybdotoxin (CTx), and several other scorpion toxins containing the R/F/·G·K·C motif (Fig. 1A). These channels influence neuronal excitability, neurotransmitter release, hormone secretion, cochlear cell tuning, redox sensing, and smooth muscle tone (21–24).

Expressing BK in HEK293 cells produced depolarization-activated K⁺ currents, and mASIC1a generated H⁺-gated currents (Fig. 1B); the results were similar to previous reports (25, 26). However, when we coexpressed the two channels, BK current amplitude fell (Fig. 1B and C) even though the amount of BK protein on the cell surface did not (Fig. 1D). We obtained similar results in CHO cells and with hASIC1a (data not shown).

Protons induce conformational changes in ASIC extracellular domains and activate these channels (9, 27). Although extracellular acidosis had little effect on BK channels expressed alone (Figs. 1B and C and 4B and refs. 28 and 29), it has been reported

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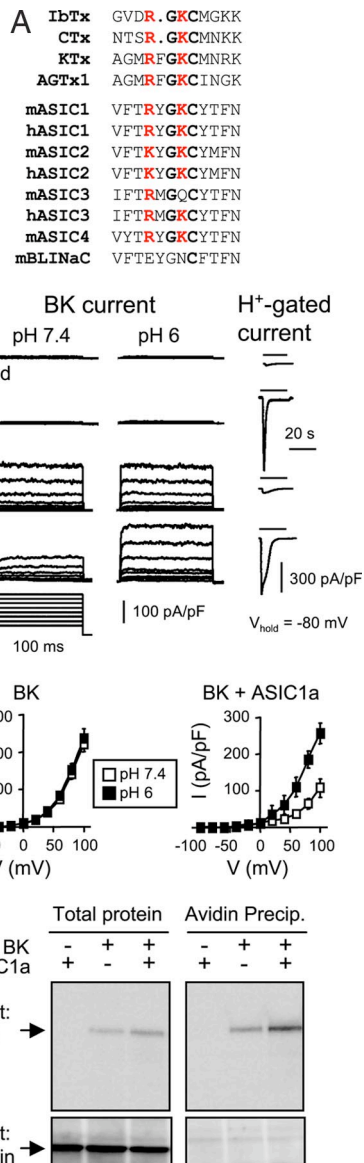


Fig. 1. ASIC1a reduces BK current, and protons relieve inhibition. (A) Similarity between conserved sequence in ASIC extracellular domains and sequence in scorpion toxins that block K^+ channels. Bold letters indicate conserved residues in ASIC channels and the toxins. Red indicates positively charged conserved Arg and Lys residues. (B) BK currents at pH 7.4 and 2 min after change to pH 6 in HEK293 cells that were not transfected ($n = 4$), were transfected with mASIC1a ($n = 6$), were stably transfected with BK ($n = 8$), or were transfected with both BK and ASIC1a ($n = 14$). Proton-activated currents are shown in *Right*. Cells were kept at pH 7.4, and the bar indicates pH 6 application. Similar results were obtained with human ASIC1a (data not shown). Consistent with a previous report, HEK293 cells contained small endogenous H^+ -gated currents (45). (C) Current–voltage relationships for BK current in the absence ($n = 8$) and presence ($n = 14$) of mASIC1a. Data are mean \pm SEM. In the presence of mASIC1a, currents at pH 7.4 were different from those in cells with BK alone, and BK currents in the presence of ASIC1a at pH 6 and pH 7.4 were different at voltages over +40 mV ($P < 0.05$, repeated-measures ANOVA). (D) BK protein levels in the absence and presence of ASIC1a. HEK293T cells were transfected as indicated. Lysate (total protein) and biotinylated proteins were blotted with anti-BK and anti-actin antibodies.

to partially relieve CTx block (30). We tested extracellular acidosis and found that reducing pH to 6 doubled BK current amplitude in cells expressing both BK and ASIC1a (Fig. 1B and C). Thus, ASIC1a inhibited BK channel activity, and extracellular protons reduced the effect.

The ASIC1a Extracellular RYGKC Sequence Is Important for Interaction with BK. If the ASIC1a extracellular domain inhibits BK, then the extracellular domains of the two channels should interact. To test this prediction, we labeled cells expressing BK with DsRed and cells expressing ASIC1a with EGFP. We then mixed the cells together to test for cluster formation. In this assay using living cells, only the extracellular domains of proteins delivered to the cell surface will be accessible for interaction. In addition, the assay can detect weak interactions because clustering may result from multiple weak contacts between cells. We found clusters of red and green cells (Fig. 2A and B). Omitting either channel strikingly attenuated clustering. In addition, protons reduced clustering, just as they had reduced ASIC1a inhibition of BK channels. These data suggest that the extracellular domain of ASIC1a binds the extracellular surface of BK.

Earlier work showed that mutating the positively charged Arg and Lys residues in the pore-blocking motif of IbTx and CTx (Fig. 1A) to either neutral (Ala) or negative (Glu) amino acids greatly reduced their affinity for voltage-gated K^+ channels (31–33). Likewise, the corresponding mutations in the ASIC1a sequence decreased clustering (Fig. 2B). Adding IbTx also reduced clustering, suggesting that IbTx and ASIC1a might compete for BK binding. These results suggest that the extracellular domain of ASIC1a may physically interact with BK.

These results predicted that mutating conserved residues in the ASIC1a sequence would also have functional effects. Indeed, changing the cationic residues to Ala or Glu prevented BK current inhibition [Fig. 2C and supporting information (SI) Fig. 6]. The Cys in the R·F·-G·K·C sequence forms a disulfide bridge that stabilizes scorpion toxin structure, thereby positioning Arg and Lys to interact with BK. Mutating that Cys dramatically reduced CTx affinity for BK (19). We found that mASIC1a–C194A still inhibited BK current and pH 6 solution relieved inhibition (Fig. 2C). However, on switching back to pH 7.4, reinhibition of BK was markedly delayed (Fig. 2D and E), suggesting that loss of the Cys may have destabilized the structure around the RYGK sequence. These data identify ASIC1a residues that are key for BK inhibition.

The Interaction with BK Affects ASIC1a Current. In addition to the effect of ASIC1a on BK, we noticed that BK affected ASIC1a currents. Fig. 1B shows that BK altered the kinetics of mASIC1a H^+ -gated currents. Transfecting increasing amounts of BK cDNA reduced ASIC1a current amplitude and prolonged the time for desensitization (Fig. 3A–C). Thus, ASIC1a and BK exerted reciprocal effects on each other's function.

BK alteration of ASIC1a current raised the possibility that the two channels closely associate. To test this, we immunoprecipitated ASIC1a and found that it coprecipitated BK (Fig. 3D). BK also precipitated ASIC1a. We speculate that proximity in the plasma membrane positions ASIC1a and BK channels where they can interact.

Other ASIC and K^+ Channels Interact. Because other ASIC channels share a similar conserved sequence (Fig. 1A), we asked whether they would also inhibit BK current. Like ASIC1a (with an RYGKC sequence), hASIC3 (RMGKC) inhibited BK currents and pH 6 solution relieved inhibition (Fig. 4A). Previous studies showed that ASIC2a is less sensitive to pH reductions than ASIC1a or -3 and that acid fails to open ASIC2b (26, 34). We found that both ASIC2a and -2b (KYGKC) inhibited BK current but pH 6 solution failed to reverse the effects. However, more severe pH reductions attenuated the BK inhibition (Fig. 4B). When we changed the ASIC1a RYGK sequence to match that of ASIC2a (KYGKC), the mutant channel behaved like ASIC2a: it inhibited BK current, but pH 6 solution failed to relieve inhibition (Fig. 4A). Conversely, when ASIC2a contained an ASIC1a sequence (RYGKC), pH 6 solution reversed BK inhi-

performed some experiments in nominally Ca^{2+} -free extracellular solution. Under these conditions protons still increased BK current in cells expressing ASIC1a and BK. Similar results were obtained in experiments using EGTA instead of BAPTA. More direct evidence that Ca^{2+} was not required came from two other experiments. (i) ASIC2a inhibition of BK was partially relieved by protons even though ASIC2a does not conduct Ca^{2+} . (ii) Protons partially relieved BK inhibition by ASIC2b, which shows no H^{+} -gated current.

Although ASIC1a can conduct H^{+} , the stimulating effect of intracellular H^{+} on BK channels has been observed only in the absence of divalent cations (29), and in our experiments the intracellular solution contained 4.8 mM Mg^{2+} . In addition, dropping pH to values <6 elicited no additional relief of BK inhibition by ASIC1a (Fig. 4B).

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Cell Clustering Assay. We used methods similar to those previously reported (44).

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