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 Ca^{2+}- 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 Ca^{2+}) 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. 1*A*) (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 Ca^{2+}- and voltage-activated (BK, SLO1, or Maxi-K) K^+ channels (21, 22). BK channels, composed of four α-subunits, are inhibited by iberiotoxin (IbTx), charybdotoxin (CTx), and several other scorpion toxins containing the R·F/-·G·K·C motif (Fig. 1*A*). 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. 1*B*); the results were similar to previous reports (25, 26). However, when we coexpressed the two channels, BK current amplitude fell (Fig. 1 *B* and *C*) even though the amount of BK protein on the cell surface did not (Fig. 1*D*). 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. 1 *B* and *C* and 4*B* and refs. 28 and 29), it has been reported

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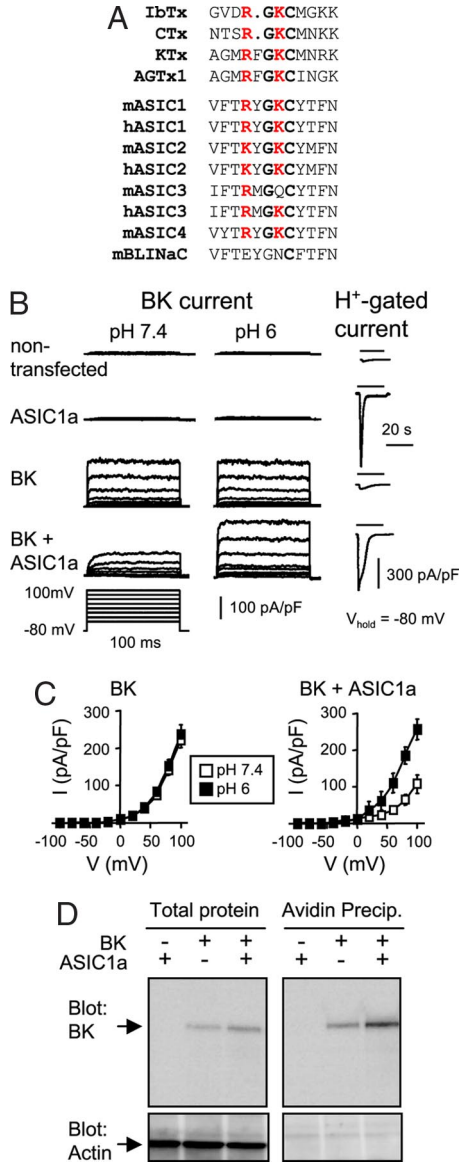

Fig. 1. ASIC1a reduces BK current, and protons relieve inhibition. (*A*) Similarity between conserved sequence in ASIC extracellular domains and sequence in scorpion toxins that block K^+ channels. Bold letters indicate conserved residues in ASIC channels and the toxins. Red indicates positively charged 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 ± 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. 1 *B* 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. 2 *A* 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. 1*A*) 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. 2*B*). 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. 2*C* 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. 2*C*). However, on switching back to pH 7.4, reinhibition of BK was markedly delayed (Fig. 2 *D* 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. 1*B* 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. 3 *A*–*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. 3*D*). 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. 1*A*), 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. 4*A*). 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. 4*B*). 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. 4*A*). Conversely, when ASIC2a contained an ASIC1a sequence (RYGKC), pH 6 solution reversed BK inhi-

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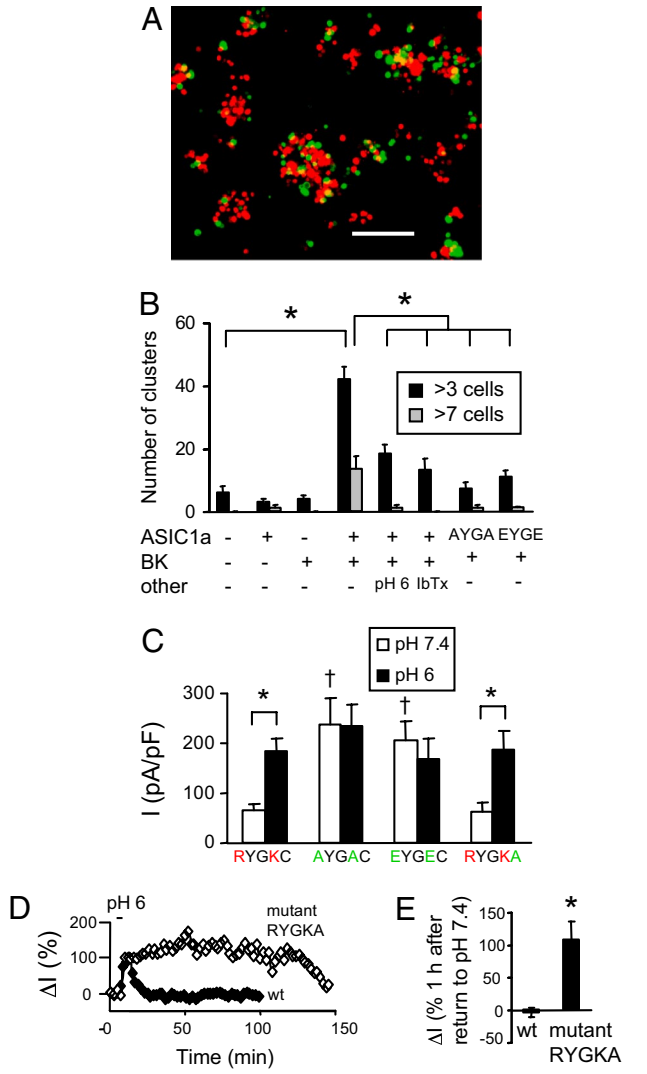

Fig. 2. The extracellular domain of ASIC1a physically interacts with and inhibits BK. (*A*) ASIC1a and BK interaction caused cell aggregation. HEK293 cells expressing mASIC1a and EGFP are green, and those expressing BK and DsRed are red. Cell clusters are shown. (Scale bar: 50 μm.) (*B*) Clustering experiment was performed with wild-type (RYGK) and mutated (AYGA and EYGE) mASIC1a as shown. Data are mean ± SEM for clusters containing more than three and more than seven cells ($n = 3$). *, $P < 0.05$ (Bonferroni test, applies to both sets of data). (*C*) BK current amplitude at +80 mV in the presence of wild-type (RYGKC) and mutated mASIC1a at pH 7.4 and 6 ($n = 6–8$). †, comparison to ASIC1a at pH 7.4 ($P < 0.05$). *, comparison between pH 7.4 and 6. Red indicates positively charged conserved amino acid residues; green indicates mutations. (*D*) Mutation of Cys-194 in mASIC1a (RYGKA) affected inhibition of BK. Bar indicates pH 6 application; otherwise pH was 7.4. Data are BK current amplitude at +80 mV. (*E*) Changes in BK current amplitude 1 h after pH 6 application. *, $P < 0.05$; $n = 6$.

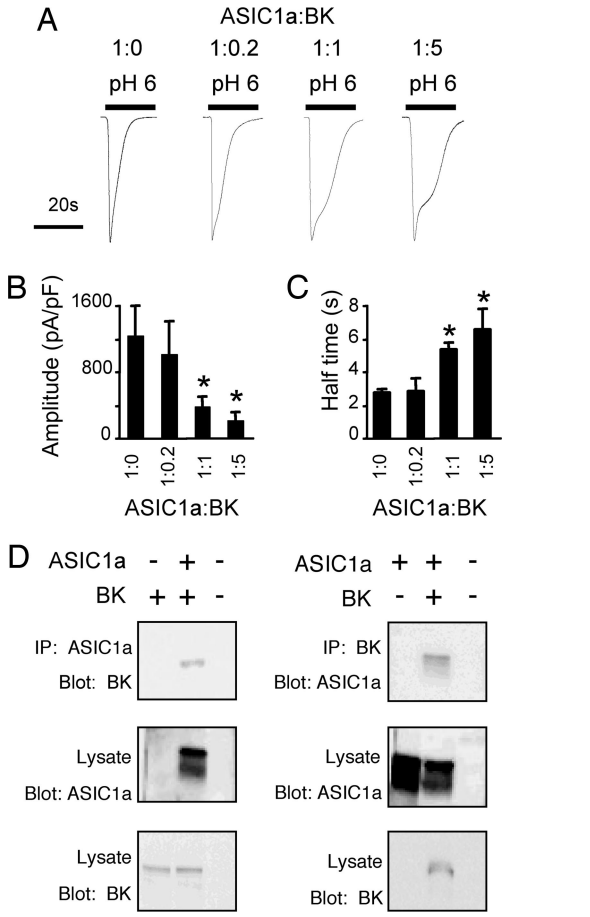

Fig. 3. The ASIC1a–BK interaction also alters ASIC1a currents. (*A*) pH 6-evoked ASIC1a inward current in transfected CHO cells. Current amplitude was normalized for illustrative purposes. Amount of mASIC1a cDNA was constant, and increasing amounts of BK cDNA were transfected at ratios shown; EGFP cDNA was used to maintain constant total cDNA for all transfections. (*B* and *C*) Peak amplitude and half-time of the ASIC1a current transient. *, $P < 0.05$; $n = 6$. (*D*) ASIC1a coimmunoprecipitates with BK. HEK293T cells were transfected with ASIC1a, BK, or both at 1:1 ratios. Data are immunoprecipitated protein or lysates blotted with indicated antibodies. ASIC1a migrated at 65 kDa, and BK migrated at 125 kDa.

bition. In contrast to the ASICs, the DEG/ENaC channel BLINaC (EYGNC), which lacks the cationic residues, failed to inhibit BK (Fig. 4*A*). These data emphasize the importance of these positively charged residues for inhibition and suggest that they might be responsible for differences between ASICs in their inhibition of BK.

To learn whether the inhibitory effect of ASICs was selective for BK channels we tested two other K^+ channels. We found that ASIC1a also inhibited Kv1.3 channels (Fig. 5 *A* and *C*). Even though pH 6 solution reduced current when Kv1.3 channels were expressed alone (35), in the presence of ASIC1a protons reversed ASIC1a-dependent inhibition and increased current. In contrast, ASIC1a failed to inhibit Kir3.1 currents (Fig. 5 *B* and *D*). These results suggest that ASICs might interact with several families of K^+ channels.

Discussion

Our results reveal a surprising regulation of ion channels. Two very different types of channel, ASIC1a Na^+ channels and BK K^+ channels, closely associated in the cell membrane and regulated each other's activity. ASIC1a inhibited BK current, and BK altered ASIC1a current kinetics.

Association of these two channels also imparted a novel response to BK channels; in the presence of ASIC1a, extracellular protons increased BK activity, even though BK is not inherently sensitive to extracellular acidosis. Our data exclude the possibility that current through ASIC channels somehow attenuated BK inhibition, because protons partially relieved BK inhibition by ASIC2b, which shows no H^+-gated current. Instead, we speculate that acidification weakened the interaction between ASIC and BK channels and/or acidification induced a conformational change in ASIC that prevented its inhibition of BK. Perhaps more consistent with the former, when we made a

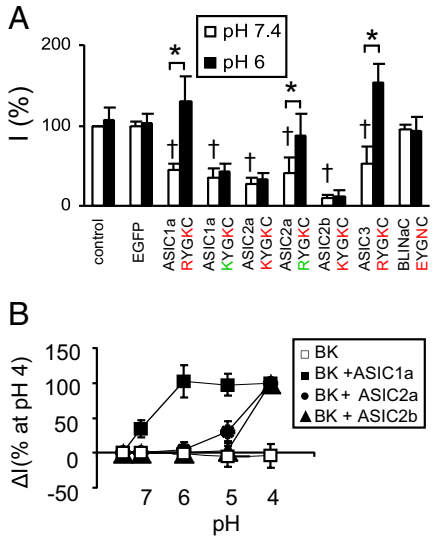

Fig. 4. Several ASICs inhibit BK channels. (*A*) Amplitudes of BK current at +80 mV measured at the end of a 100-ms voltage pulse in cells expressing BK alone, BK and EGFP as a control, and BK in the absence and presence of indicated wild-type and mutated constructs. Currents were normalized to current with BK alone at pH 7.4; $n = 6–13$. Red indicates positively charged conserved residues; green indicates mutations. †, comparison to BK alone at pH 7.4 ($P < 0.05$). *, comparison between pH 7.4 and 6 ($P < 0.05$). (*B*) Effect of pH on BK current when BK was expressed alone or with ASIC1a, ASIC2a, or ASIC2b. Data are normalized to current at pH 4; $n = 6$.

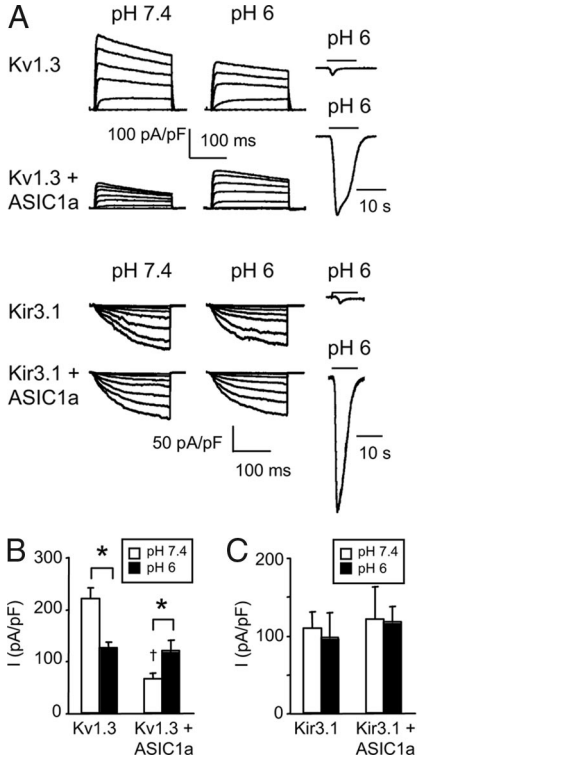

Fig. 5. ASIC1a can inhibit other K^+ channels. (*A Left*) Kv1.3 currents in the absence and presence of ASIC1a at pH 7.4 and 6. (*A Right*) Proton-activated current; cells were at pH 7.4, and bar indicates pH 6 application. Membrane voltage was held at −80 mV with steps from −40 to +60 mV in 20-mV increments. (*B Left*) Inward-rectifying currents in HEK293 cells expressing Kir3.1. Membrane voltage was 0 mV with steps from −20 to −140 mV in 20-mV increments. HEK293 cells contain some endogenous inward-rectifier current (46). (*B Right*) Proton-activated current. (*C*) Kv1.3 current density at +60 mV. $n = 6$ for Kv1.3, and $n = 22$ for Kv1.3 plus ASIC1a. †, difference from Kv1.3 alone at pH 7.4 ($P < 0.05$). *, difference between pH 7.4 and pH 6 ($P < 0.05$). (*D*) Kir3.1 current density at −120 mV. $n = 6$ for Kir3.1, and $n = 8$ for Kir3.1 plus ASIC1a.

conservative change in a single ASIC2a amino acid (Lys to Arg) to make its BK-inhibiting sequence identical to that of ASIC1a, pH 6 solution relieved inhibition by the mutant ASIC2a, as it did with ASIC1a. The reverse was also true.

The ASIC extracellular domain was involved in the interaction between ASIC and BK channels. Data from the clustering assay, the functional data showing inhibition, and the results with site-specific mutants in the functional and clustering assays all implicate the conserved cationic residues in the RYGKC sequence as critical for ASIC to interact with and inhibit BK. Because of their resemblance to residues in α-K-toxins, we speculate that those residues might be directly involved in the K^+ channel inhibition.

How do the physiological data relate to the recently published crystal structure of chicken ASIC1a (9)? At this time that relationship is uncertain. However, we can comment on the position of the RYGKC sequence. In the crystal structure the RYGKC sequence (residues 191–195 in the chicken ASIC1a sequence) sits in the region of the "β-ball." Lys-194 protrudes out away from the structure, and Cys-195 forms one of seven disulfide bonds. The RYGKC sequence is partially shielded by the "finger" and "thumb" domains and would not be exposed to interact with BK. In addition, the RYGKC sequence lies far from the membrane, making it difficult to imagine how it could interact with the outer vestibule of the BK channel, which lies close to the membrane. However, the ASIC1a structure was obtained at pH 5.6, and at this pH ASIC1a did not interact with or inhibit BK. Jasti *et al.* (9) speculated that pH changes may move the thumb domain. We wonder whether, at pH 7.4, changes in structure might allow a direct interaction with BK. In addition, data from the cell clustering assay suggest that ASIC channels on one cell might interact with BK channels on a different cell. As with interactions within a single membrane, contacts between channels located on different cells would require significant pH-dependent structural changes. Further studies of the ASIC1a conformation at higher pH may shed light on potential mechanisms. Alternatively, perhaps the RYGKC sequence does not interact directly with BK but instead is critical for another part of ASIC1a to affect BK.

Extracellular pH can fall with neural activity and in a variety of pathological states (36, 37). Our data indicate that the resulting increase in protons might enhance the activity of two very different types of channels that would have opposing effects on membrane voltage. The wide and varied expression patterns of ASIC channels (1–4) and BK and related K^+ channels (22, 23, 38–43) suggest that they might interact in a variety of cell types and brain regions. In addition, finding that more than one ASIC can inhibit BK, that ASIC1a can inhibit more than one type of K^+ channel, and that BK influences ASIC1a current suggests many intriguing opportunities to alter cell signaling, neuronal sensory function, and membrane excitability.

Materials and Methods

For details see *SI Materials and Methods*.

cDNA Constructs and Cell Culture. cDNA constructs were either previously reported or were generated by using standard procedures. HEK293 cells stably expressing BK were previously reported (25).

Biochemistry. We used standard procedures for immunoprecipitation, immunoblotting, and biotinylation assays.

Electrophysiology. We used standard procedures for whole-cell patch-clamp and analysis. Because ASIC1a can conduct Ca^{2+} and because internal Ca^{2+} can activate BK channels, we used 10 mM BAPTA in the pipette solution and

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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. 4*B*).

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

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