IDENTIFICATION OF PROTEIN DOMAINS THAT CONTROL PROTON AND CALCIUM SENSITIVITY OF ASIC1A

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The acid-sensing ion channels (ASICs) open in response to extracellular acidic pH and individual subunits display differential sensitivity to protons and calcium. ASIC1a acts as a high affinity proton sensor, whereas ASIC2a requires substantially greater proton concentrations to activate. Using chimeras composed of ASIC1a and ASIC2a we determined that two regions of the extracellular domain (between 87-197 and 323-431) specify the high affinity proton response of ASIC1a. These two regions appear to undergo inter-subunit interactions within the multimeric channel to specify proton sensitivity. Single amino acid mutations revealed that amino acids around D357 play a prominent role in determining the pH dose response of ASIC1a. Within the same region, mutation F352L abolished PcTx1 modulation of ASIC1a. Surprisingly, we determined that another area of the extracellular domain was required for calcium-dependent regulation of ASIC1a activation and this region functioned independently of high affinity proton sensing. These results indicate that specific regions play overlapping roles in pH-dependent gating and PcTx1-dependent modulation of ASIC1a activity, while a distinct region determines the calcium dependence of ASIC1a activation.

The acid-sensing ion channels (ASICs) are proton-gated ion channels expressed in neurons throughout the central and peripheral nervous system (1-3). ASICs are activated by extracellular acidosis and protons act as ligands triggering channel opening (4). Disruption of the ASIC1 gene dramatically reduces proton-gated currents in central neurons and alters a variety of behaviors including fear, learning, and memory (5,6). ASIC1 also contributes to neuronal damage and death during the prolonged acidosis accompanying cerebral ischemia (7). Specifically, mice lacking the ASIC1 gene display 60% smaller lesion size compared to normal mice in models of stroke (8). Application of PcTx1, a venom peptide that prevents ASIC1a activation, is similarly neuroprotective, even when administered hours after injury (8,9). Thus, ASIC1a represents a novel pharmacological target for the prevention of neuronal death following stroke.

Mammals have four ASIC genes (ASIC1-4) that encode at least six different ASIC subunits (1-3,10). Like all members of the DEG/ENaC family, individual ASIC subunits have two transmembrane regions separated by a large cysteine-rich extracellular region. Three ASIC subunits associate to form homomeric or heteromeric channels with distinct biophysical characteristics (11-14). Specifically, ASIC1a homomeric channels activate at pHs much closer to neutral pH compared to ASIC2a homomeric channels. The high affinity proton sensitivity of ASIC1a plays a prominent role in acidosis-induced neuronal death, and modulators that alter the pH dose response of ASIC1a affect neuronal sensitivity to prolonged acidosis (8,9,15). For example, the neuroprotective venom peptide PcTx1 increases the proton sensitivity of the ASIC1a channel, allowing the channel to desensitize at neutral pH and become unresponsive.
to subsequent acidic shifts in pH (16, 17). The large extracellular region of ASIC1a is thought to be the site of proton/modulator interaction and governs the characteristics of channel gating (10, 11, 18). However, the exact molecular mechanisms defining ASIC1a activation and the protein domains that are responsible for the apparent proton sensitivity of ASIC1a remain unclear. Here, we used chimeras containing specific regions from both ASIC1a and ASIC2a to identify the specific protein regions that confer high affinity proton sensing, PcTx1 sensitivity, and calcium modulation to ASIC1a.

EXPERIMENTAL PROCEDURES

Construction of Chimeras, Site-directed mutagenesis, and Expression in Xenopus oocytes-Human ASIC1a and ASIC2a cDNAs in the pMT3 mammalian expression vector have been previously described (19, 20). These sequences correspond to Genbank accession number NM_001095 for human ASIC1a and NM_001094 for human ASIC2a. Chimeras were generated using overlap extension PCR (21). The specific locations of chosen chimeric breakpoints in human ASIC1a or ASIC2a are shown in Fig 1A and are as follows: “KR” is immediately preceding transmembrane region one (TM1) at K42 (1a) or R42 (2a); “FP” is at the conserved extracellular FPAVT sequence at F87 (1a) or F86 (2a); “KA” is at the conserved KANF sequence at K141(1a) or K140(2a); “FN” is at the conserved FNSG sequence at F197 (1a) or F196 (2a); “CN” is at the conserved CNCR sequence at C323 (1a) or C320 (2a); “TR” is at the third conserved TRY sequence at T370(1a) or T367(2a); “LL” is just before transmembrane region two (TM2) at L431 (1a) and L428 (2a). When reported in the text and figures, the numbering refers to the locations of corresponding residues in ASIC1a. Chimeras were cloned into pMT3 using unique restriction sites engineered into the PCR primers. Site-directed mutagenesis was employed on human ASIC1a using the QuikChange Kit® from Stratagene (Cedar Creek, TX). All inserts were fully sequenced prior to electrophysiological analysis (Plant-Microbe Genomics Facility, The Ohio State University, Columbus, Ohio). Qiagen Midi or Maxi prep kits (Valencia, CA) were used to prepare plasmid DNA for oocyte injection.

Unfertilized oocytes were harvested from wild-type oocyte positive Xenopus laevis females from Xenopus I (Dexter, MI) using standard procedures (22). ASIC expression plasmids at a 50-100 ng/µL concentration were injected into oocyte nuclei (located beneath the animal pole) using a PV820 Pneumatic Picopump (World Precision Instruments, Sarasota, FL). For co-expression studies, plasmids were injected at a 1:1 ratio (50 ng/µl of each construct). Oocytes were allowed to incubate at 18°C for 18-72 hours before experiments were performed.

Electrophysiology: Macroscopic currents were recorded using the two electrode voltage-clamp technique at a holding potential of -60 mV. Electrodes (0.5-2 MΩ) were filled with 3 M KCl. Current was recorded using a Warner oocyte OC-725 clamp (Warner Instruments, Hamden, CT) and either a Powerlab 4SP digitizer with CHART software (ADInstruments, Colorado Springs, CO) or an Axon Digidata 1200 digitizer and pCLAMP-8 software (Molecular Devices, Sunnyvale, CA). The standard bath solution contained 116 mM NaCl, 2 mM KCl, 5 mM HEPES, 5 mM MES, 2 mM CaCl2, 1 mM MgCl2 and pH adjusted with 1 N HCl or 1 N NaOH. The pH  of the standard bath solution was 7.4 unless otherwise indicated. Oocytes were placed in a bath chamber (500 µL) and acid applications were made using pump-driven perfusion directed towards the oocyte with a bath solution exchange rate of 2-3 ml per second (5 mls total). The cells were maintained in pH 7.4 for at least two minutes between acid applications to allow the cells to recover from desensitization. To minimize current run-down, the peak current amplitude of evoked current from acidic test pH applications was normalized to the average of the peak current amplitudes evoked from saturating acidic pH (pH 3.0 or pH 5.0) applied before and after the test pH. PcTx1- containing venom from the tarantula Psalmopoeus cambridgei was purchased from SpiderPharm (Yarnell, AZ) and diluted to 1:3,333-5,000 for experiments.

The pH0.5 was calculated by fitting the data from individual pH dose-response experiments within each oocyte using the equation: $I / I_{pHmax} = 1 / \left\{ 1 + \left( \text{EC}_{50} / [\text{H}^+] \right)^n \right\} = 1 / \left\{ 1 + 10^{-n \cdot (pH - pH_{0.5})} \right\}$, where n is the Hill coefficient. EC50 and pH0.5 are the proton concentration and pH yielding half of the saturating peak current amplitude ($I_{pHmax}$). The
pH$_{\text{max}}$ was determined as a saturating test pH for all channels within the experimental group. This was either pH 3.0 or pH 5.0. The Hill coefficient was $4.4 \pm 0.46$ ($n=18$) for ASIC1a and $1.3 \pm 0.05$ ASIC2a ($n=13$). Generally, the Hill coefficient correlated with the pH$_{0.5}$ of mutant channels with those showing smaller Hill coefficients also displaying a lower pH$_{0.5}$. However, the variability in the calculated Hill coefficient of ASIC1a limited significance substantially. To quantify desensitization, IGOR (WaveMetrics, Portland, OR) was used to fit the decay phase of acid-activated currents to a single exponential equation: $I = k_0 + k_1 \cdot e^{-t/\tau}$. The tau of desensitization ($\tau_d$) was calculated in seconds from the fitted curve. Two-tailed paired or unpaired $t$-tests were done to analyze statistical significance as appropriate. A “$p$” value less than 0.05 was considered significant.

**RESULTS**

**Two regions of the extracellular domain specify the pH sensitivity of ASIC1a**

ASIC1a is activated by pH 6.7 and shows a pH of half maximal activation (pH$_{0.5}$) of approximately 6.5. This pH$_{0.5}$ corresponds to an EC$_{50}$ for protons of 316 nM. ASIC2a requires a pH below 6.0 for activation, displays a pH$_{0.5}$ of approximately 3.9, and has an apparent EC$_{50}$ for protons of 126 $\mu$M. Thus, ASIC1a requires an almost 400-fold lower proton concentration for half-maximal activation compared to ASIC2a. To identify the protein regions responsible for this difference in apparent proton affinity, chimeras were constructed with different segments of ASIC1a and ASIC2a sequence (Fig. 1A).

All chimeras produced transient proton-gated currents when expressed in *Xenopus* oocytes. A pH dose response was assessed for each construct and the pH$_{0.5}$ (the pH of half maximal activation) was calculated. Substitution of several regions of ASIC1a with ASIC2a sequence affected the pH$_{0.5}$. In particular, exchanging the region N-terminal to the “FP” site, altered the pH$_{0.5}$ slightly (see chimeras 2FP1, 1KR2, and 1FP2) (Fig. 1B). However, two other protein regions showed a more prominent role in defining the apparent proton sensitivity of ASIC1a. First, substitution of the region between amino acids 87-197 (1FP2FN1) of ASIC1a with ASIC2a sequence resulted in a dramatic reduction in the pH$_{0.5}$ (Fig. 1B). Second, substitution in the region between amino acid 323-431 (1CN2LL1) of ASIC1a substantially diminished the proton dose response. Alternatively, exchanging the N-terminal region before amino acid 87 (2KR1), the C-terminal region after amino acid 431 (1LL2, 2LL1), or the middle region between amino acids 197 and 323 of ASIC1a with ASIC2a sequence (1FN2CN1) did little to alter the proton sensitivity.

To further define the important residues within these regions, chimeras containing only part of the regions 87-197 and 323-431 were generated. Exchanging only region 141-197 of ASIC1a with ASIC2a sequence (chimera 1K22FN1) recapitulated the proton dose response of chimera 1FP2FN1 in which the entire 87-197 region was exchanged (Fig. 1B). However, exchanging the region between 87 and 141 (1FP2KA1) showed a pH$_{0.5}$ indistinguishable from ASIC1a. Furthermore, exchanging just the N-terminal area of amino acids 323-370 (1CN2TR1) resulted in a chimera with a pH dose response indistinguishable from the chimera in which the entire region was exchanged (Fig. 1B). Exchanging amino acids 370-431 (1TR2LL1) also reduced the apparent proton sensitivity slightly. Thus, amino acids within regions 141-197 and 323-370 appear to play a dominant role in specifying the pH dose response of ASIC1a, with residues 323-370 being the most important.

**Specific amino acids between 323 and 370 determine the apparent proton affinity of ASIC1a**

Exchanging the region between amino acids 323-370 of ASIC1a with ASIC2a sequence decreased the pH$_{0.5}$ from 6.5 to 3.8, a more than 400-fold decrease in apparent proton sensitivity. There are 11 amino acids within this region that differ between ASIC1a and ASIC2a. To determine which amino acids are important for proton sensitivity, individual amino acids in ASIC1a were converted to the corresponding amino acids in ASIC2a (Fig 2A). Y342H, D347E, D351G, F352L, V354A and the double mutant E364R/M365T all displayed smaller relative currents in response to pH 6.5 and their pH$_{0.5}$ was substantially smaller compared to ASIC1a (Fig. 2B). However, the lowest pH$_{0.5}$ elicited from these single mutants was 6.19 and none of these single mutations decreased pH sensitivity to the level of
1CN2TR1 (pH0.5 = 3.8) suggesting that changes in multiple amino acids are responsible for the effect of exchanging all amino acids between 323-370 with ASIC2a sequence. To determine whether combining mutations could decrease the pH0.5 further, subunits with multiple mutations were constructed and analyzed (Fig. 2B). Only when all 11 substitutions were made was the pH0.5 of 1CN2TR1 recapitulated. These results likely indicate that all these residues contribute to high affinity proton sensing of ASIC1a.

D357 and surrounding residues play a critical role in proton-dependent gating

Amino acids with negatively charged side chains have been hypothesized to play a role in determining proton-binding (23,24). We reasoned that because both ASIC1a and ASIC2a are activated by protons (albeit at different concentrations), then conserved acidic residues might be necessary for proton-dependent gating. Therefore, we tested the effect of mutation of acidic residues within region 323-370 that are conserved between ASIC1a and ASIC2a (Fig. 3A). Surprisingly, ASIC1a mutants with alterations in acidic residues E340, E344, and E355 displayed a normal proton dose-response. However, D357A displayed a decreased response to pH 6.5 solutions (Fig. 3B). Quantification of the pH0.5 revealed that D357A showed a relatively large decrease in pH0.5 (5.92 ± 0.06 for D357A (n = 7, p = 8.5 x 10^-14 compared to ASIC1a) (Fig. 3C). Similar results were obtained with D357N suggesting the charge of the aspartic acid side chain is important.

The D357A mutation showed the largest change in pH dose-response of any single mutation examined in these studies. We reasoned that the region surrounding D357 might play a prominent role in defining the pH sensitivity of ASIC1a. Since our previous results indicated that E355A and Q358S did not alter pH sensitivity (Fig. 3C and 2B), we tested mutations that more significantly affected the amino acids. Surprisingly, mutants E355K and Q358E showed increased current in response to pH 6.7 application (Fig. 3D). Further quantification revealed that the proton dose-response of these mutant channels was shifted such that the pH0.5 was greater than ASIC1a (Fig. 3E). The pH0.5 for E355K was 6.64 ± 0.02 (n = 8, p = 0.0032 compared to ASIC1a) and the pH0.5 of Q358E was 6.66 ± 0.03 (n = 7, p = 0.00032 compared to ASIC1a). We also assessed the impact of K356A mutation and determined that pH sensitivity was slightly reduced although the data narrowly reached statistical significance. (pH0.5 of K356A = 6.48 ± 0.02, n = 8, p = 0.049 compared to ASIC1a) (Fig. 3E). These results indicate that the area around D357 plays an important role in the pH dose-response of ASIC1a.

Two regions are sufficient for high affinity proton sensing.

ASIC1a sequences between amino acids 87-197 and 323-431 are required for high affinity proton sensing. To determine whether either region is sufficient, we analyzed ASIC2a chimeras with ASIC1a sequence within these regions (Fig. 4A). The presence of ASIC1a sequence in the region between 87-197 alone did not increase the apparent proton sensitivity of ASIC2a. When the region of ASIC2a between 323-431 was exchanged with ASIC1a sequence, the pH sensitivity was increased (pH0.5 of 2CN1LL2 = 4.43 ± 0.10, n = 6, p = 0.0003 compared to ASIC2a). Replacing regions 141-197 and 323-370 (2KA1FN2CN1TR2) increased the pH0.5, but only to 5.48 ± 0.05, n = 3. However, when BOTH regions 87-197 and 323-431 of ASIC2a were exchanged with ASIC1a sequence (2FP1FN2CN1LL2), the chimera displayed a dramatic increase in proton sensitivity (pH0.5 of 2FP1FN2CN1ILL2 = 6.17 ± 0.07, n = 7, p = 1 x 10^-6 compared to ASIC2a) (Fig. 4A). Although this chimera was still less sensitive than ASIC1a (p = 0.0005), these results suggest that ASIC1a sequence in the regions between amino acid 87 and 197 as well as the region between 323 and 431 are sufficient to confer high affinity proton sensing and a pH0.5 higher than 6.0.

Our data indicate that two regions of ASIC1a must be present for a pH0.5 greater than 6.0. These results suggest these regions may interact to determine the pH sensitivity of ASIC1a. Functional ASIC channels are formed by association of three ASIC subunits. Thus, the interaction between these regions could be within the same subunit (intra-subunit) or one region of a subunit could interact with the other region from another subunit (inter-subunit). To determine whether inter-subunit interactions between these two regions affect pH sensitivity of ASICs, we co-
expressed chimeras 2FP1FN2 and 2CN1LL2 and measured proton-gated current (Fig 4B,C). Oocytes expressing both 2FP1FN2 and 2CN1LL2 displayed appreciable proton-activated current with pH 6.0 (Fig. 4B). However, very little proton-activated current was observed in response to pH 6.0 in oocytes expressing either chimera alone. The pH dose-response of oocytes expressing both chimera was also shifted toward more neutral pHs (Fig. 4C). Although the pH sensitivity of proton-gated currents when 2FP1FN2 and 2CN1LL2 were co-expressed was not as sensitive as 2FP1FN2CN1LL2, this increase in proton sensitivity suggests that regions 87-197 and 323-431 undergo inter-subunit interactions, at least in part, to specify the proton dose-response.

**F352L eliminates PcTx1 sensitivity of ASIC1a**

In addition to increased proton sensitivity, ASIC1a is also sensitive to the venom peptide PcTx1 whereas ASIC2a is not (17). PcTx1 limits ASIC1a activity by increasing the apparent proton sensitivity of ASIC1a and promoting steady-state desensitization at pH 7.4 (16,18,25). If steady-state desensitization is not induced, then PcTx1 potentiates ASIC1a activation by shifting the pH0.5 of activation to more neutral pHs (16,25). Under such conditions, ASIC1a currents evoked by non-saturating pHs are substantially enhanced in the presence of PcTx1 (Fig. 5A). We asked whether the same domains that determine high affinity proton sensing also confer sensitivity to PcTx1. ASIC1a chimeras containing ASIC2a in the region between amino acid 323-431 (1CN2LL1) were not affected by PcTx1-containing venom (Fig. 5B). Conversely, ASIC2a chimeras containing ASIC1a sequence in this same region (2CN1LL2) were enhanced by PcTx1-containing venom (Fig. 5B). These results suggest that ASIC1a sequence in the region between amino acids 323-431 is both necessary and sufficient for PcTx1 enhancement of the pH dose-response and are in agreement with recently published observations (26). To determine the specific amino acids in this region involved in the PcTx1 sensitivity of ASIC1a, we analyzed channels with mutations converting amino acids in this region of ASIC1a to amino acids present in ASIC2a. For most mutants, the effect of PcTx1 was retained (data not shown). However, ASIC1a channels with mutation F352L were unaffected by PcTx1-containing venom (the pH 6.3 response was 36.9 ± 5.0% maximal current without PcTx1 and 31.2 ± 1.8% in the presence of PcTx1, n = 5, p = 0.22) (Fig. 5C). A thorough pH dose-response analysis in the presence of PcTx1 revealed that F352L was unaffected by PcTx1 (Fig. 5D). The pH0.5 of F352L was 6.23 ± 0.01 (n = 4) in the absence of PcTx1 and was 6.21 ± 0.04 (n = 4, p = 0.72) in the presence of PcTx1. Under the same conditions, the pH dose-response of ASIC1a was substantially shifted toward more neutral pHs (pH0.5 of ASIC1a in the absence of PcTx1 was 6.59 ± 0.04, n = 5, and in the presence of PcTx1 was 6.81 ± 0.06, n = 3, p = 0.02). These results indicate that the F352L mutation eliminated PcTx1-induced potentiation of the pH dose-response of ASIC1a.

PcTx1 modulation of steady-state desensitization was also lost in F352L. Steady-state desensitization is induced by incubating cells in a slightly acidic pH (conditioning pH) for two minutes prior to pH 5.0 application (Fig 5E, F). When PcTx1 was present, steady-state desensitization of human ASIC1a was enhanced and subsequent activation of the channels produced a substantially smaller current (Fig. 5E, F). Yet, steady-state desensitization of F352L was not significantly different in the presence of PcTx1 (Fig 5G, H). Thus, the F352L mutation prevents PcTx1 modulation of the pH-dependence of both activation and steady-state desensitization.

**Calcium dependence is determined by amino acids 193-319.**

The pH dose response of ASIC1a is also markedly affected by extracellular calcium, with increasing concentrations of calcium increasing the concentration of protons required for channel activation (4,27-29). In fact, it has been proposed that calcium tonically inhibits ASIC1a activation and protons activate ASICs by facilitating calcium unbinding (23,24,30). This effect can be observed by the decrease in the relative peak current amplitude evoked by pH 6.5 in the presence of either 1.8 or 10 mM calcium (Fig. 6A). To determine whether calcium affects ASIC1a and ASIC2a similarly, we tested the calcium dependence of ASIC2a activation. Surprisingly, we find that calcium enhanced proton sensitivity of ASIC2a as indicated by the relative increase in pH 3.5-evoked currents (Fig. 6A). Quantification of the % change in normalized currents reveals a
negative change for ASIC1a and a positive change for ASIC2a with increasing calcium (Fig. 6B). We used our ASIC1a/2a chimeras to identify the regions which are responsible for the difference in calcium dependence between ASIC1a and ASIC2a. We found that exchanging amino acids 197-323 of ASIC1a with ASIC2a sequence (1FN2CN1) eliminated the ASIC1a-like response to calcium (Fig. 6B). Similarly, replacing the residues of ASIC2a between amino acids 197-323 with ASIC1a sequence (2FN1CN2) was sufficient to allow calcium inhibition of proton sensitivity (as indicated as a negative change in % normalized current in Fig. 6B), even though the channel had a pH0.5 of 4.1. 

Exchanging the regions we previously identified as playing a prominent role in the dose response to protons (FP-FN and CN-LL) did not determine the response to calcium. Thus, the region between amino acids 197-323 is responsible for the differential calcium sensitivity of ASIC1a and ASIC2a, whereas the region responsible for the pH0.5 differences lies in regions 87-197 and 323-431.

**DISCUSSION**

The apparent proton sensitivity of ASIC1a determines channel activation and the extent of depolarization and calcium influx induced by that activation. Most modulators of ASIC1a function, including zinc, redox reagents, lactate, and PcTx1, act by altering the apparent proton sensitivity of the channel (16,18,25,31-34). These compounds also impact ASIC1a-induced neuronal death (8,9,15,31). We find that two protein regions, located between amino acids 87-197 and 323-431 of the extracellular domain are both necessary and sufficient for high affinity proton sensing of ASIC1a.

The published crystal structure of desensitized chicken ASIC1a indicates that the channel is a trimer with individual subunits making multiple intra- and inter-subunit contacts (11). Each subunit has a “wrist domain” which connects the extracellular domain to transmembrane regions as well as a “β-ball”, “finger”, “palm”, and “thumb” domain. The crystal structure does not reveal a permeability pore and the exact role of each individual domain is not known (11). There is still much debate about the mechanism of ASIC activation by protons. Specifically, where the proton-bindings site(s) are located (if specific sites exist), how protons trigger channel activation, how modulators alter the apparent proton sensitivity of the channel, and the exact role of calcium in proton-dependent ASIC gating remain unanswered.

Currently, there are two different theories on the location of the proton binding sites of ASIC1a. Experiments using chimeras composed of proton-insensitive and proton-sensitive ASICs suggest that specific residues within the wrist domain of ASICs are required for proton-dependent activation (35-37). Channels that are unresponsive to protons are produced when these residues are mutated and because some of these residues can be titrated by protons, it has been suggested that they represent the proton-binding site(s). The involvement of these residues in proton-mediated activation has been confirmed by other studies looking at the role of conserved acidic residues (38). Whether these residues are required for the allosteric changes induced by proton binding or if they act as protonation sites has yet to be experimentally determined. Our experiments did not identify these regions as playing a large role in specifying high affinity proton sensing of ASIC1a.

Analysis of the crystal structure of ASIC1a has highlighted a second region, termed the “acid pocket”, as the proton-binding site (11,39). This highly acidic region contains several pairs of carboxyl-carboxylate interactions between the side chains of several amino acids. Specifically, contact between D238-D350 (D351 in human ASIC1a), E239-D346 (D347 in human ASIC1a), E220-D408, and E80-E417 define the four pairs (11). It has been suggested that protonation of these residues confers pH sensing. Although mutations to some of these residues reduce apparent proton affinity (11), mutation to others does not affect apparent proton sensitivity and individual mutations in this region do not eliminate proton-dependent gating (as mutations in the wrist domain do) (38). This could be due to redundancy between the residue pairs. Our data highlight this region and these residues as playing a role in high affinity proton sensing of ASIC1a versus ASIC2a. Specifically, our studies found that two of these residues, D347E and D351G in ASIC1a, were involved in specifying the difference in proton sensitivity between ASIC1a and ASIC2a. Thus, our data support a role for the
acid-pocket in determining apparent proton sensitivity. However, we also find that residues along the thumb and palm domains are also responsible for the low affinity proton sensing of ASIC2a. Further, we find that residues 141-197 of ASIC1a, located throughout the finger, palm and β-Ball regions, are also important for proton sensing. Except for R191, residues in this region have not been previously highlighted as playing a direct role in the acidic pocket. Thus, it appears that the entire region is important for defining the sensitivity of proton sensing.

Our data suggest that inter-subunit interactions between 87-197 and 323-431 are important for specifying the pH dose response. In the crystal structure of desensitized ASIC1a, these regions make several inter-subunit contacts, one near the base of the thumb domain and one near the tip of the thumb domain (11). The importance of D357 in acid sensing supports the idea that these inter-subunit contacts are important. Mutation of this residue decreases proton-sensitivity more profoundly than any single amino acid mutation we tested. This residue is located near K355 (K356 in human ASIC1a) and N357 (Q358 in human ASIC1a), two residues which form strong inter-subunit contacts in the crystal structure (11). Interestingly, these residues make contact around E178 and R176 of the adjacent subunit, residues which fall within the second region (87-197) we find necessary and sufficient for high affinity proton sensing. Further support that these regions are important comes from previous data indicating that D351, Q358 and N359 are required in Lamprey ASIC1a to form active ASIC1a channels, suggesting these residues are critical for proton-dependent gating (37). We find that mutation Q358E and E355K increase the apparent proton sensitivity of ASIC1a proton sensitivity. Moreover, calcium has several effects on ASIC1a activity. First, calcium at high concentration can block the channel, an effect that requires residues E425 and D432 (23). In addition, the proton-dependence of ASIC activation is inversely dependent on calcium concentration (24,28). This has led to speculation that the proton-binding site and this second calcium binding site are the same and that protons activate the channel by displacing calcium (24,27,30,41). Here, we report that the proton-gated ASIC2a does not show calcium dependence of activation, like that observed in ASIC1a and ASIC3. Although the high concentration of protons required for activation could mask any possible calcium dependence for ASIC2a gating, our studies of ASIC1a and ASIC2a chimeras indicate that the calcium dependence of activation and apparent proton sensitivity do not segregate together. For example, a chimera with a pH0.5 of 4.0 shows calcium sensitivity (2FN1CN2) and one with a
pH$_{0.5}$ of 6.5 does not (1FN2CN1). In fact, we found that the calcium dependence of ASIC1a activation involved a completely distinct region (between 193 and 323) compared to those involved in the differential pH sensitivity of ASIC1a and ASIC2a. This indicates that the sites important for calcium-dependent activation are distinct from the identified sites that specify apparent proton sensitivity and modulator action.

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**FIGURE LEGENDS**

**Fig. 1.** Two regions of the extracellular domain determine the pH dose-response of ASIC1a and 2a. **A.** Schematic of an ASIC subunit with the chimeric break points indicated (arrows). Large grey boxes represent transmembrane regions and white ovals signify conserved cysteine residues. The number of the first amino acid within the break-point motif of human ASIC1a is indicated (see methods). **B.** The calculated pH0.5 of ASIC1a/2a chimeras. On the left are schematics of the chimeras. Areas in dark grey are ASIC2a sequence and areas in white are ASIC1a sequence. Dotted lines represent the location of important break points. On the right is the graph of the pH 0.5 for each chimera. Each value represents an n of 3-10 cells. Statistical significance was assessed using an unpaired t-test. “**” represents statistical significance with a p < 0.05 compared to ASIC1a AND ASIC2a. Error bars represent the standard error of the mean (SEM).

**Fig. 2.** Conversion of amino acids within region 323-370 of ASIC1a to the corresponding residues in ASIC2a eliminates high affinity proton sensing. **A.** Schematic of an ASIC subunit with conserved cysteines indicated as white ovals. Below is the sequence of ASIC1a and ASIC2a within the region indicated by dashed lines. Amino acids 323-335 are identical between ASIC1a and ASIC2a and are not shown. Residues in bold are conserved cysteines. Residues that are different between ASIC1a and ASIC2a are underlined. **B.** The calculated pH0.5 of indicated mutants (n = 3-10). Note that the sequence of the mutant with substitution of all 11 residues is identical to chimera 1CN2TR1. Statistical significance was assessed using an unpaired t-test. “**” indicates a p <0.05 compared to human ASIC1a. Error bars represent the SEM.

**Fig. 3.** D357 and surrounding amino acids are important for proton sensitivity of ASIC1a. **A.** Schematic of an ASIC subunit with the indicated sequence of ASIC1a and ASIC2a indicated below. Amino acids in bold were mutated. **B.** Representative traces of ASIC1a and D357A in response to pH 5.0, 6.0, 6.5, and 6.7 application. **C.** The calculated pH0.5 of ASIC1a channels with mutations in acidic residues (n = 6-20). Statistical significance was assessed using an unpaired t-test. “**” represents a p <0.05 compared to ASIC1a. **D.** Representative traces of the pH 5.0 and pH 6.7 response of ASIC1a, E355K and Q358E. **E.** The pH dose-response of channels with mutations around D357. I/I0Max is the peak amplitude of current evoked by the test pH (indicated on the x-axis) divided by the peak current amplitude of the pH 5.0 response (n = 5-18).

**Fig. 4.** Two regions of ASIC1a encompassing amino acids 87-197 and 323-431 are sufficient for high affinity proton sensing and undergo intersubunit interaction. **A.** pH0.5 of ASIC2a chimeras with ASIC1a sequence. The schematic of chimeras tested is shown on left (n = 5-8). “**” indicates a p <0.05 compared to human ASIC1a. **B.** Representative traces of proton-gated currents in oocytes expressing 2FP1FN2, 2CN1LL2, or both 2FP1FN2 and 2CN1LL2. Scale bars represent 1 μA (Y-axis) and 100 seconds (X-axis). **C.** pH dose-response of proton-gated current in oocytes injected with both 2FP1FN2
and 2CN1LL2 (dark line) compared to those injected with only 2FP1FN2, 2CN1LL2, or 2FP1FN2CN1LL2 ($n = 3-10$). Error bars represent the standard error of the mean (SEM).

**Fig. 5.** *PcTx1 modulation is eliminated by the F352L mutation.* A - C. Representative traces of the *PcTx1* effect on the response to sub-maximal proton concentrations of human ASIC1a (A), chimeras 1CN2LL1 and 2CN1LL2 (B), and point mutation F352L (C). For human ASIC1a and F352L, the basal pH was maintained at pH 7.9 to eliminate *PcTx1* action on steady-state desensitization (see below). *PcTx1*-containing venom (1:3,000-1:5,000) was present in the extracellular bath solution for two minutes prior to the application of saturating acidic pH solutions. The activating pH’s chosen produced maximal (left) and sub-maximal (right) responses from the individual channels. Similar results were attained when pH 5 was used to activate 2CN1LL2 and pH 5.5 was used to activate 1CN2LL1 (not shown). D. pH dose-response of human ASIC1a and F352L in the presence and absence of *PcTx1* (1:5,000). Basal pH was maintained at pH 7.9 before application of acidic pHs. Peak current amplitude was normalized to that evoked from pH 5 in each cell ($n = 5-9$). E-F. Representative traces of *PcTx1* modulation of steady-state desensitization of human ASIC1a (E) and F352L (F). Basal pH was maintained at 7.9, dropped to the indicated conditioning pH (either 7.0 for ASIC1a or 6.9 for F352L) for two minutes, and pH 5.0 was used to activate proton-gated currents (dark bar above trace). *PcTx1* was applied for two minutes at pH 7.9 as well as during the conditioning and activating pH. Quantification of the effect of *PcTx1* on steady-state desensitization of human ASIC1a and F352L is shown in F and H, respectively. The peak current amplitude of pH 5.0-evoked currents following exposure to the conditioning test pH (either 7.0 or 6.9) was normalized to peak current amplitude following exposure to pH 7.9 solutions for two minutes ($n = 5-6$). *PcTx1* was applied as above. Statistical significance was assessed using a paired t-test. “***” indicates a $p < 0.0005$. Error bars represent the SEM.

**Fig. 6.** The calcium dependence of ASIC1a activation does not segregate with regions controlling the pH dose response. A. Representative traces of the effect of calcium on ASIC1a, ASIC2a and the indicated chimeras. Oocytes were incubated in either nominal calcium (0 mM calcium added), 1.8 mM calcium or 10 mM calcium. Proton-gated currents were activated with a pH to induce a maximal response (on left) and a sub-maximal-response (on right). Grey dotted line indicates the maximal response in the specific calcium concentration. Note that pH 6.5 evoked current is reduced compared to the maximal response as calcium concentration increases for ASIC1a and such a response is not observed with ASIC2a. B. Quantification of the calcium response. Schematic of chimeras tested is shown on left and the change in normalized current evoked from the sub-maximal pH application in either 1.8 or 10 mM calcium compared to nominal calcium is shown at right. The activating pHs used were those indicated in parentheses. Because different pH’s were used to activate current for different channels, the trend (whether positive or negative changes with increased calcium) and not the absolute values were considered for comparison.

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**FOOTNOTES**

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Fig. 2

A

H1a  PYCTPEQYKECADPALDFLVEKDOEYCVCEMPCNLTRY
H2a  PECTPEQHKECAEPALGLLAEKDNSYCCLRTPCNLTRY

B

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* 0.5

Fig. 2
Fig. 3

A

B

C

D

E

H1a

H2a

ASIC1a

D357A

ASIC1a

E344A

E340A

E355A

D357A

D357N

ASIC1a

E355K

Q358E

ASIC1a

E355K

K356A

Q358E

PyCTPEQYKECADPALDFLVEKDQEYVCEMPCLNLTRY

PFCTPEQHKECAPALGLAEXDNSNYCLCRTCPCLNLTRY

ASIC1a

E344A

E340A

E355A

D357A

D357N

ASIC1a

E355K

Q358E

0.20

0.40

0.60

0.80

1.00

1.20

0

5.0 5.5 6.0 6.5 7.0

pH

I/I

MAX

ASIC1a

E355K

K356A

Q358E

5.5 5.75 6.0 6.25 6.5

pH0.5

1 µA

25 s

1 µA

25 s

1 µA

25 s
Fig 4.
Fig. 6

A

ASIC1a
0 mM Ca\(^{2+}\) 1.8 mM Ca\(^{2+}\) 10 mM Ca\(^{2+}\)

ASIC2a
0 mM Ca\(^{2+}\) 1.8 mM Ca\(^{2+}\) 10 mM Ca\(^{2+}\)

B

ASIC1a (6.5)
1FP2FN1CN2LL1 (4.1)
2FN1CN2 (3.5)
1FN2CN1 (6.5)
2FP1FN2CN1LL2 (6.3)
ASIC2a (3.5)

% change in normalized current

42 87 197 323 431