Independent Contribution of Extracellular Proton Binding Sites to ASIC1a Activation*

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Background: Protons activate ASIC1a by an unknown mechanism.

Results: E79K and D345K mutants showed shifted biphasic proton activation curves. The introduction of Lys residues at both of these sites restrained to a large extent proton gating.

Conclusion: Two independent proton binding sites in the extracellular region of each subunit contribute to ASIC1a activation.

Significance: These findings are relevant to understand the process of activation of ASIC1a.

Acid-sensing ion channels (ASICs) are a group of trimeric cation permeable channels gated by extracellular protons that are mainly expressed in the nervous system. Despite the structural information available for ASIC1, there is limited understanding of the molecular mechanism that allows these channels to sense and respond to drops in extracellular pH. In this report, we employed the substituted cysteine accessibility method and site-directed mutagenesis to examine the mechanism of activation of ASIC1a by extracellular protons. We found that the modification of E238C and D345C channels by MTSET reduced proton apparent affinity for activation. Furthermore, the introduction of positively charged residues at position 345 rendered shifted biphasic proton activation curves. Likewise, channels bearing mutations at positions 79 and 416 in the palm domain of the channel showed reduced proton apparent affinity and biphasic proton activation curves. Of significance, the effect of the mutations at positions 79 and 345 on channel activation was additive. E79K-D345K required a change to a pH lower than 2 for maximal activation.

In summary, this study provides direct evidence for the presence of two distinct proton coordination sites in the extracellular region of each subunit of ASIC1a. These findings are relevant to understanding the process of activation of ASIC1a.

Acid-sensing ion channels (ASICs) are a group of trimeric cation permeable channels primarily expressed in neurons of the central and peripheral nervous system that participate in mechanosensation, nociception, synaptic plasticity, and fear conditioning (1–6). Four genes (Accn1, Accn2, Accn3, and Accn4) that encode for the ASIC subunits and their splice variants have been identified in mammals (7, 8). Functional studies revealed that ASIC subunits assemble in neurons to form homo- and heterochannels with distinct functional properties (9–12). Recently, crystallographic studies disclosed the atomic structure of a chicken ASIC1 (cASIC1) trimer in a desensitized conformation (13). The solved structure showed that each ASIC subunit has two pore-forming α-helices, cytosolic tails, and a large extracellular region with defined domains that have been termed the thumb, finger, knuckle, β-ball, and palm (Fig. 1) (13). In addition to the structural information available, there is limited understanding of the gating mechanism of these channels.

Efforts to map the sites for proton binding in ASIC1a have yielded inconclusive results. Work by Paukert et al. (14) identified several positions in human ASIC1a where neutral mutations produce modest changes in proton affinity. Jasti et al. identified a negatively charged cavity deemed the “acidic pocket” containing three pairs of acidic residues in the extracellular region of cASIC1, which they proposed were part of the proton-sensing machinery (13). However, channels bearing individual or multiple substitutions at selected positions within the acidic pocket retained proton sensitivity, suggesting that additional residues contribute to proton sensing (13–16). Recently, the Kellenberger laboratory (15) used a computational approach to examine the contribution of acidic and basic residues to proton gating based on their predicted pKa values in the solved cASIC1 structure. Their work suggested that although no single neutral substitution had a significant effect in proton affinity, multiple binding sites are necessary for activation and desensitization to occur. Lastly, we recently identified a proton coordination site in the lower palm domain of ASIC1a formed by the residues Glu79 and Glu416 (17). The results from our studies suggested that the protonation of Glu79 and Glu416 facilitates pore opening in response to extracellular acidification.

The complex architecture of ASICs as well as the labile nature of their agonist has limited our understanding of the gating mechanism of these channels. The present investigation reveals that two sites, one previously identified in the palm domain formed by the residues Glu79 and Glu416, and another
sensitive to structural changes at positions 345, contribute independently to the activation of ASIC1a by extracellular protons. The findings reported here provide new insight into the molecular mechanism underlying the function of ASIC1a.

EXPERIMENTAL PROCEDURES

Molecular Biology and Oocyte Expression—Mutations were generated in a mouse ASIC1a (mASIC1a) construct with QuikChange XL (Agilent Technologies, Santa Cruz, CA) according to the manufacturer’s instructions. All mutations were verified by direct sequencing. cRNAs were transcribed using mMESSAGE mMachine SP6 (Applied Biosystems, Carlsbad, CA). Oocytes stages 5–6 were harvested from adult female Xenopus laevis (NASCO, Plant City, FL) in accordance with a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Oocytes were injected with 0.2–6.0 ng of cRNA encoding ASIC1a mutants and maintained at 18 °C in modified Barth’s solution containing 88 mM NaCl, 10 mM KCl, 0.2–6.0 ng of cRNA encoding ASIC1a mutants and maintained at 18 °C in modified Barth’s solution containing 88 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 10 μg/ml sodium penicillin, 10 μg/ml streptomycin sulfate, and 100 μg/ml gentamycin sulfate, pH 7.4.

Electrophysiology—Two-electrode voltage clamp experiments were conducted at room temperature 1–2 days after injection. Injected oocytes were impaled with glass electrodes filled with 3 m KCl and then continuously perfused by gravity at a rate of 8–10 ml/min as described previously (18). Currents were measured at a holding potential of −60 mV with a TEV-200A amplifier (Dagan Corp., Minneapolis, MN). Data were captured with a Digidata 1440A acquisition system using pClamp 10 (Molecular Devices, Sunnyvale, CA). The recording solution contained the following: 110 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 10 mM hydrogen ion buffer. Solutions of pH 7–8 were buffered with HEPES, solutions of pH 4–6.5 with MES, and solutions of pH 2–4 with glycine.

Modification of Cys Residues—Methanethiosulphonate (MTS) reagents were dissolved in the recording solution to give a final concentration of 1 mM. Because MTS reagents decompose very quickly at basic pH, solutions containing MTS reagents buffered at pH 8.0 were prepared and used immediately. The half-time of hydrolysis for MTSET is 130 min at pH 7.0 (19). Consequently, solutions buffered at pH 7.0 were used within 60 min of preparation. The reducing reagent tris(2-carboxyethyl)phosphine (TCEP) was dissolved in the recording solution to give a final concentration of 1 mM, and the pH was adjusted accordingly. TCEP solutions were used within the same day of preparation.

Data Analysis—Data are expressed as the mean ± S.E. (n), where n indicates the number of independent experiments analyzed. A p value of <0.05 was considered statistically significant. The pH of half-maximal activation (pH<sub>50</sub>) was calculated from normalized pH-evoked peak currents plotted as a function of the extracellular pH used for activation. Unless otherwise indicated, pH-evoked peak currents were normalized to the pH-evoked peak current obtained at a pH of activation of 4. Data were fitted to a standard monotonous sigmoid dose-response curve or alternatively when appropriate to a biphasic sigmoid shape dose-response curve using GraphPad

RESULTS

ASICs are transiently activated by drops in extracellular pH and desensitize upon continuous exposure to high [H⁺] (i.e. low pH). These channels essentially reside in three conformational states: a closed resting state, an open conducting state, and a desensitized closed proton-bound state. The location of the proton sensors as well as the molecular mechanism that promotes pore opening upon proton binding has not been elucidated. We employed the substituted cysteine accessibility method to identify sites within the ectodomain of ASIC1a that undergo structural changes in response to extracellular acidification. We targeted residues in the acidic pocket, as they localize at the boundary between several domains (Fig. 1), a classical location for allosteric effector sites. mASIC1a contains an unpaired modifiable Cys at position 70 (20). The mutants used in this study were generated in a mASIC1a construct carrying a Leu at position 70. This mutation does not change the functional properties of the channel (20). As shown in Fig. 1, the acidic pocket is formed by the pairs Glu<sup>238</sup>-Asp<sup>345</sup>, Asp<sup>237</sup>-Asp<sup>349</sup>, and Glu<sup>219</sup>-Asp<sup>407</sup>. Residues in the pairs Glu<sup>238</sup>-Asp<sup>345</sup> and Asp<sup>237</sup>-Asp<sup>349</sup> form intrasubunit contacts between the finger and thumb domains. The residues Glu<sup>219</sup> and Asp<sup>407</sup> are located in the palm domain. In the desensitized state, these residues reside in close proximity to the pairs Glu<sup>238</sup>-Asp<sup>345</sup> and Asp<sup>237</sup>-Asp<sup>349</sup> of the neighboring subunit. The residues in the acidic pocket serve as binding sites for psalmotoxin, a selective ASIC1a inhibitor from the tarantula Psalmopoeus cambridgei venom (21, 22). We previously showed that mASIC1a residues in a responsive closed state at pH values above 7.4, but it desensitizes when the extracellular pH decreases below 7.2 (19).

Thus, we probed the reactivity toward MTSET of channels bearing individual Cys mutations at selected sites in the acidic cluster at pH 7.0, i.e. channels in the desensitized state, or at pH 8.0, i.e. channels in the closed state (Fig. 2). Mutant channels were activated by a drop in extracellular pH from 8.0 to 5.0 before and after MTSET treatment (see representative recording in Fig. 2a). Non-treated oocytes served as controls for these experiments. Fig. 2b shows the ratio of the pH-elicited peak current after MTSET treatment to the pH-elicited peak current before treatment for channels bearing individual mutations in the acidic pocket. The response to extracellular acidification of D345C channels was significantly reduced after MTSET treatment at pH 7.0 or 8.0 (Fig. 2, a and b). The effect of the covalent modification at position 345 manifested during activation (Fig. 2a, lower traces). These results suggest that the area surrounding Asp<sup>345</sup> experiences a conformational rearrangement during activation. No functional effects were observed after MTSET treatment with the other mutants tested (E219C, D237C, E238C, D349C, and D407C) (Fig. 2b).

To gain insight into the structural requirements for inhibition of D345C channels by thiol reactive reagents, we examined
the effect of other MTS reagents which vary in size as well as charge (Fig. 2c). For these studies, the response to extracellular acidification was assessed before and after MTS reagent treatment at pH 7.0. Fig. 2d shows the chemical formula of the transferable moiety of the thiol reactive reagents used in this study. As shown in Fig. 2c, MTSMT, MTSET, MTSES, and MTSEA treatment reduced the magnitude of the proton-gated currents in oocytes expressing D345C channels to a similar extent. These results indicate that the effect of the modification at position 345 is relatively independent of the size or the charge of the moiety covalently attached at this position.

The Modification of the Cys at Position 345 Reduces Apparent Proton Affinity for Activation—To gain insight into the mechanism by which covalent attachments at position 345 reduce proton-gated currents, we assessed the apparent proton affinity for activation of unmodified, MTSET-treated, and MTSEA-treated D345C channels. The estimated pH for half-maximal activation (pH50) was 6.10 ± 0.06 (CI 5.98–6.21) for ASIC1a (C70L) control channels, 5.98 ± 0.04 (CI 5.91–6.06) for unmodified D345C channels, and 4.53 ± 0.07 (CI 4.38–4.68) for D345C channels treated with MTSET (Fig. 3 and Table 1). Significantly, the dose-response relationship for proton activation of MTSEA-treated D345C channels was bimodal with pH50 values of 3.85 ± 0.02 (CI 3.82–3.89) and 5.62 ± 0.06 (CI 5.50–5.74) (Fig. 3). These results revealed that the covalent attachment of moieties at position 345 alters the function of a proton binding site that contributes to channel activation.

Thumb-finger Dynamics Influence Proton Gating—Asp345 resides in close proximity to Glu238 in the finger domain of cASIC1 in the desensitized state (Fig. 1). We noticed that the magnitude of the response to extracellular acidification of E238C channels increased after repeated stimulations (Figs. 2b and 4). ASIC1a contains seven disulfide bonds, three of which are located in the thumb and β-ball domains proximate to Glu238 (Fig. 1a). We hypothesized that the Cys introduced at position 238 forms a disulfide bond with an endogenous Cys, thus generating a structural change that impacts channel function. To explore this possibility, E238C channels expressed in oocytes were exposed to the reducing reagent TCEP before activation. As shown in Fig. 4a, the pretreatment of oocytes expressing E238C channels with TCEP prevented the increase in current observed after repeated stimulations. Likewise, we found that the magnitude of the peak current elicited by extracellular acidification did not increase after consecutive stimulations in oocytes expressing E238S channels (Fig. 4a and 4b).

These results indicate that the Cys introduced at position 238 forms a disulfide bond with an endogenous Cys. To determine whether a covalent attachment at position 238 affects channel activity, oocytes expressing E238C channels were treated with...
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FIGURE 2. Reactivity of residues in the acidic pocket toward MTS derivatives. a, representative recordings of experiments performed with oocytes expressing D345C channels. Whole-cell currents were elicited by a change in extracellular pH from 8.0 to 5.0 before and after MTSET treatment. Oocytes expressing mutant channels were exposed to MTSET (1 mM) at pH 7.0, i.e. channels in the desensitized state, or at pH 8.0, i.e. channels in the resting state (lower traces). Non-treated oocytes expressing D345C channels served as controls (upper traces). b, reactivity of channel bearing Cys substitutions at selected positions in the acidic pocket toward MTSET. Whole-cell currents were evoked by a change in extracellular pH from 8.0 to 5.0, as shown in a. The relative response represents the ratio of the pH-elicted peak current following MTSET treatment (or control) to the pH-elicted peak current before treatment (n = 8–17). Statistically significant differences are indicated as +, p < 0.001 (Kruskal-Wallis test following by Dunn’s multiple comparisons test). The relative response represents the ratio of the pH-elicted peak current following MTSET treatment (or control) to the pH-elicted peak current before treatment (n = 11–38). Statistically significant differences with the control are indicated as **, p < 0.01 and +, p < 0.001 (Kruskal-Wallis test following by Dunn’s multiple comparisons test). c, chemical formula of the transferable moiety of MTSET, MTSET, MTSES, and MTSEA.

FIGURE 3. Covalent attachments at position 345 reduce apparent proton affinity for activation. The covalent modification of Cys at position 345 reduces apparent proton affinity (left panel). Proton activation curves for controls (C70L) (open circles), unmodified D345C channels (red circles), MTSEA-treated D345C channels (green circles), and MTSET-treated D345C channels (filled circles). Proton-activated currents were elicited by a drop in extracellular pH from 8.0 to solutions of lower pH (n = 8–45). Peak currents for MTSEA-treated and MTSET-treated D345C channels were normalized to the peak current obtained at a pH of 3.0. Time constants of desensitization of controls, unmodified D345C, MTSEA-treated D345C channels, and MTSET-treated D345C channels (right panel). Whole-cell currents were fitted to a single exponential function as described under “Experimental Procedures” (n = 9–55).

TCEP and then exposed to MTSET (Fig. 4, c and d). The results from these experiments indicate that the covalent attachment of the transferable moiety of MTSET at position 238 inhibit to some extent proton activation.

Our studies indicate that E238C channels contain an unnatural disulfide bond. To identify the residue that forms the disulfide bond with the Cys at position 238, we substituted individually the endogenous Cys residues at positions 93, 194, 323, 335, and 343 to Ser on channels bearing E238C mutations. The introduction of individual Ser substitutions in the thumb domain at positions 323, 335, and 343 completely disrupted proton-gating. We hypothesize that the disulfide bonds in the thumb domain are required for proper protein folding and/or function. Thus, we examined the effect of TCEP on oocytes expressing single (E238C), double (C93S/E238C and C194S/ E238C) and triple (C93S/C194S/E238C) mutants (see Fig. 4e). Non-treated oocytes served as controls for these experiments. The relative response represents the ratio of the pH-evoked peak currents from two consecutive activations. Proton-gated currents increased after repetitive stimulations in oocytes expressing E238C and C93S/E238C channel, but not in oocytes expressing C194S/E238C and C93S/C194S/E238C channels. These results indicate that in E238C channels, the Cys introduced at position 238 forms a disulfide bonds with Cys194.

To gain insight into the mechanism by which covalent attachments at position 238 reduce proton-gated currents, we determined the apparent proton affinity for activation of unmodified and MTSET-treated E238C channels. E238C channels pretreated with TCEP were exposed to MTSET and then to successive activations at various pHs (Fig. 4f). The pK\textsubscript{a0} of activation for TCEP-treated E238C channels was 5.99 ± 0.06 (CI 5.86–6.12) and for TCEP-treated MTSET-treated E238C channels was 5.32 ± 0.11 (CI 5.11–5.53) (Fig. 4f and Table 1). Taken together, our data show that the covalent attachment of moieties at positions 238 and 345 reduces apparent proton affinity for activation. We propose that structural changes introduced at these positions affect molecular movements triggered by extracellular protons and associated with the activation of the channel.

Positively Charged Substitutions at Position 345 Decouple a Proton Binding Site—We generated a series of substitutions at position 345 that included positively charged, polar non-charged, and non-polar residues. We found that the proton dose-response relationship for activation of channels carrying Lys or Arg substitutions at position 345 displayed a biphasic sigmoid shape (Fig. 5a). Channels bearing residues with polar...
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Biochemical characterization of ASIC1a mutants

Proton-activated currents were elicited by a drop in extracellular pH from 8.0 to solutions of lower pH. Peak currents for control, E219K, D237K, E238C+TCEP, E238K, D345C, D345N, D349K, D407K, and 6NQ channels were normalized to the signal obtained at a pH of 4.0. Peak currents for E79K, E79Q, E219K, D237K, E238C+MTSET, D345M, D345K, D345R, D345C+MTSEA, D345C+MTSET, D345K-5NQ, and E416K channels were normalized to the signal obtained at a pH of activation of 3.0. Peak currents for E79K/D345K channels were normalized to the signal obtained at a pH of activation of 2.0. For monophasic proton dose-response activation curves, pH50–1 is the log of the concentration of protons that give half-maximal stimulatory effects for the two different phases. Peak pH 3/peak pH 4.5 represents the ratio of the peak current evoked at pH 3 to the peak current evoked at pH 4.5. Note that for those mutants with biphasic dose-response activation curves the peak pH 3/peak pH 4.5 ratio is close to 2, whereas for those mutants with monophasic dose-response activation curves, this value is close to 1. The time constants of desensitization ($\tau_d$) were estimated by fitting the current decay following activation to pH 5.0 to a single exponential function as described under “Experimental Procedures.”

To determine whether Lys substitutions at other positions in the acidic pocket enable a similar effect on proton activation, we generated proton dose-response activation curves for E219K, D237K, E238K, D349K, and D407K channels (Fig. 5b). The proton dose-response relationships for activation for these mutants were monophasic, and the estimated apparent proton affinities for activation were similar or slightly higher than those for the controls (C70L) (Fig. 5a). We hypothesize, based on the biphasic nature of the dose-response curves of D345K and D345R channels, that positively charged substitutions at position 345 disengage one of the proton coordination sites in the acidic pocket, which reduces the current at pH 3 and increases the current at pH 4.5.

### Table 1

| Mutant          | pH30–1 | pH45–2 | Peak pH 3/peak pH 4.5 | $\tau_d$ |
|-----------------|--------|--------|----------------------|---------|
| C70L-HA         | 6.10 ± 0.06 (24–45) | 0.76 ± 0.04 (12) | 1.24 ± 0.12 (19) |
| E79K            | 5.65 ± 0.09 (11–50) | 3.85 ± 0.02 | 2.26 ± 0.17 (11) | 0.75 ± 0.03 (21) |
| E79Q            | 5.80 ± 0.06 (9–24) | 3.59 ± 0.08 | 0.41 ± 0.03 (28) |
| E219K           | 6.15 ± 0.07 (12) | 1.91 ± 0.10 (13) |
| D237K           | 6.67 ± 0.07 (12) | 1.12 ± 0.11 (11) |
| D345K           | 6.20 ± 0.07 (11–12) | 0.89 ± 0.05 (10) |
| D345C           | 5.99 ± 0.06 (11–15) | 0.65 ± 0.06 (9) | 1.13 ± 0.06 (11) |
| D345N           | 5.32 ± 0.11 (10–23) | 0.68 ± 0.06 (10) |
| D345M           | 5.98 ± 0.04 (12–13) | 1.12 ± 0.06 (13) |
| D345R           | 6.22 ± 0.07 (11–23) | 0.86 ± 0.05 (11) | 0.87 ± 0.06 (10) |
| D345N+MTSET     | 5.62 ± 0.06 (13–45) | 1.59 ± 0.02 (12) | 0.84 ± 0.03 (14) |
| D345R+MTSET     | 5.69 ± 0.05 (9–52) | 3.74 ± 0.03 | 2.09 ± 0.32 (12) | 0.35 ± 0.02 (43) |
| D345R+MTSET     | 5.53 ± 0.10 (9–52) | 3.84 ± 0.02 | 1.93 ± 0.37 (12) | 0.49 ± 0.03 (16) |
| D345C+MTSET     | 4.53 ± 0.07 (10–21) | 0.49 ± 0.05 (10) |
| D345C+MTSEA     | 5.62 ± 0.06 (8–27) | 3.85 ± 0.02 | 0.41 ± 0.04 (12) |
| D349K           | 6.38 ± 0.06 (16–50) | 0.64 ± 0.05 (16) |
| D407K           | 6.27 ± 0.07 (11–24) | 1.13 ± 0.09 (11) | 0.70 ± 0.04 (11) |
| E416K           | 5.95 ± 0.28 (11–30) | 3.84 ± 0.03 | 0.87 ± 0.04 (10) | 0.93 ± 0.05 (10) |
| 6NQ             | 6.04 ± 0.05 (10–21) | 3.68 ± 0.05 | 2.06 ± 0.20 (11) | 0.35 ± 0.02 (16) |
| E79K/D345K      | 5.27 ± 0.08 (12–48) | 3.68 ± 0.05 |
| 5NQ             | 2.77 ± 0.07 (13–25) | 0.93 ± 0.02 (16) |

non-charged or hydrophobic side chains at this position such as D345N or D345M mutants showed monophasic dose-response relationships (Fig. 5a). For D345M, D345K, and D345R channels, the peak currents evoked by extracellular acidification were normalized to the peak current obtained at a pH of activation of 3.0. We noted that the peak currents evoked at pH 3 were lower than at pH 4 for control and D345N channels. Consequently, the peak currents evoked by extracellular acidification were normalized to the peak current obtained at pH 4 for these channels. This finding limited the pH values that we were able to test for control and D345N channels. Then, to determine whether control and D345N channels have biphasic proton dose-response activation curves, we estimated the ratio of the peak current evoked at pH 4.5 to the peak current evoked at a pH of 3 (see Table 1). For mutants with biphasic dose-response relationships for activation, the data showed that the first component plateaus near pH 5, whereas the second component plateaus near pH 3. Consequently, the ratio of the peak current evoked at pH 3 to the peak current evoked at pH 4.5 (peak pH 3/peak pH 4.5) provides a good estimation of the number of components in the dose-response relationship (monophasic or biphasic). The peak pH 3/peak pH 4.5 ratio was close to 2 for mutants with biphasic dose-response activation curves, and it was lower than 1 for control and D345N channels (Table 1). These results indicate that the dose-response activation curves of control and D345N have only one component. Taken together, the biphasic nature of the dose-response relationships for activation of D345R and D345K channels suggest the existence of distinct proton coordination sites in the extracellular region of ASIC1a.

To determine whether Lys substitutions at other positions in the acidic pocket have a similar effect on proton activation, we
The estimated pH values for the coordination sites of the D345K channels were 3.74 ± 0.03 (CI 3.68–3.80) and 5.69 ± 0.05 (CI 5.60–5.79). These results indicate that the Lys at position 345 does not functionally interact with the acidic residues in the pocket.
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Two Proton Coordination Sites Indepedently Contribute to ASIC1α Activation—We previously reported that Glu79 and Glu416 in the lower palm domain constitute a proton binding site and that the protonation of these residues cooperatively facilitate pore opening in response to extracellular acidification (17). The biphasic nature of the proton activation curves of the D345K and D345R mutants revealed the existence of multiple proton coordination sites in the extracellular region of ASIC1α. In our previous work, the apparent proton affinity for activation for Glu79 and Glu416 mutants was calculated from proton dose-response relationships generated for activation pH values from 7.0 to 4.0. We generated new proton dose-response relationships for activation for E79K, E79Q, and E416K channels, but in this case, we normalized the peak currents to the peak current elicited at pH of 3. Unexpectedly, the proton activation relationships for these three mutants were also biphasic when normalized to a final activation pH of 3 (Fig. 7a and Table 1). These results suggest the presence of at least two proton coordination sites in the extracellular region of the channel, one in the lower palm domain (involving Glu79 and Glu416) and another sensitive to modifications at the finger-thumb interface. To determine whether these sites contribute independently to channel activation, we generated a construct with mutations at both sites (E79K/D345K). Channels combining Lys substitutions at positions 79 and 345 have a pH50 for activation of 2.77 ± 0.07, requiring higher [H+] for activation than channels with individual mutations at these sites (Fig. 7c). The dose-response relationships for E79K/D345K channels were normalized to the response elicited by a change in extracellular pH from 8.0 to 2.0. These measurements might have an implicit error, as the peak current evoked by extracellular acidification as indicated above in (a) (n = 9–52). Peak currents for E79K and D345K channels were normalized to the signal obtained at a pH of 3.0, whereas currents for E79K/D345K mutants were normalized to signal attained at a pH of 2.0.

DISCUSSION

The molecular mechanism by which protons control ASIC1α function remains unknown, despite the structural information currently available. Although ASIC1α has a large number of protonable charged residues in the extracellular region that potentially could serve as proton binding sites, our current work indicates that a limited number of them are functionally relevant. The studies presented here show that two distinct proton coordination sites in the extracellular region of ASIC1α, one of which resides in the palm domain (involving residues Glu79 and Glu416) and another which is altered by changes at the finger-thumb interface, jointly facilitate pore opening in response to extracellular acidification (Fig. 8). The proton binding site in the lower palm domain was characterized in a previous work (17). Using substituted cysteine accessibility method
we previously showed that Cys residues introduced at positions 79 and 416 are accessible for modification at pH 8.0, i.e. channels in the closed state, but not at pH 7.0; i.e. channels in the desensitized state. These findings suggest that the lower palm domain undergoes a structural reorganization in response to extracellular acidification. In addition, we found that non-protonable substitutions at positions 79 and 416 resulted in reduced proton apparent affinity for activation. Unexpectedly, we noticed that the apparent proton affinity for activation of channels bearing Lys mutations at positions 79 and 345 (E79K/D345K) was significantly lower than the affinity of the single mutants (E79K and D345K). Furthermore, for the double mutant E79K/D345K, the maximal response to extracellular acidification is achieved at a pH of activation lower than 2.

In summary, our studies unveil the presence of two proton coordination sites in the extracellular region of ASIC1a, one in the lower palm domain and another affected by changes in the finger-thumb region. Neither of these sites by themselves is essential for proton activation. The substitution of acidic residues by polar non-charged amino acids at every position in the acidic pocket of rat ASIC1a reduced apparent proton affinity. The acidic pocket located at the interface of finger, thumb, and palm domains was proposed to be part of the cASIC1 proton-sensing machinery (13). Li et al. (16) reported that the introduction of Ala at positions 237, 238, and 350 in the acidic pocket of rat ASIC1a reduced apparent proton affinity for activation. Unexpectedly, our findings revealed that the introduction of polar non-charged amino acids at every position in the acidic pocket (E219Q/D237N/E238Q/D345N/D349N/D407N) did not alter apparent proton affinity. The substitution of acidic residues by polar non-charged residues is more conservative than the substitution by Ala employed in the work mentioned above, which may account for the different results. Based on our data, it is reasonable to conclude that the residues in the acidic pocket do not serve as proton coordination sites and are not essential for proton activation.

In summary, our studies unveil the presence of two proton coordination sites in the extracellular region of ASIC1a, one in the lower palm domain and another affected by changes in the finger-thumb region. Neither of these sites by themselves appears to be indispensable for proton gating. However, the response to extracellular acidification was significantly restrained in channels carrying substitutions at both sites, sug-
gesting that the two proton sensors contribute independently to channel activation. There is growing interest in ASICs as potential therapeutic targets since they contribute to axonal degeneration in experimental autoimmune encephalomyelitis (23), neuronal damage in brain ischemia (24), pain sensation (4, 6), and cardiac pain in myocardial ischemia (25, 26). The findings reported here are particularly relevant to understand the process of activation as well as to develop inhibitors that target the gating machinery of these channels.

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