1-4-2008

Candidate amino acids involved in H+ gating of acid-sensing ion channel 1a

Martin Paukert
University of Tübingen

Xuanmao Chen
University of New Hampshire, Durham, Xuanmao.Chen@unh.edu

Georg Polleichtner
University of Würzburg

Hermann Schindelin
University of Würzburg

Stefan Grunder
University of Tübingen

Recommended Citation

Paukert M, Chen X, Polleichtner G, Schindelin H, Grunder S. Candidate amino acids involved in H+ gating of acid-sensing ion channel 1a. The Journal of biological chemistry. 2008;283(1):572-81. doi: 10.1074/jbc.M706811200. PubMed PMID: 17981796.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 1, pp. 572–581, January 4, 2008

Candidate Amino Acids Involved in H^+ Gating of Acid-sensing Ion Channel 1a*

Received for publication, August 15, 2007, and in revised form, October 31, 2007 Published, JBC Papers in Press, November 1, 2007, DOI 10.1074/jbc.M706811200

Martin Paukert[‡1], **Xuanmao Chen**[‡§], **Georg Polleichtner**[§], **Hermann Schindelin**[¶], **and Stefan Gründer**[‡§2]

From the [‡]*Department of Physiology II, University of Tübingen, Gmelinstrasse 5, 72076 Tübingen, the* [§]*Department of Physiology II, University of Würzburg, Röntgenring 9, 97070 Würzburg, and the* [¶]*Rudolf Virchow Center for Experimental Biomedicine and Institute of Structural Biology, University of Würzburg, Versbacher Strasse 9, 97078 Würzburg, Germany*

Acid-sensing ion channels are ligand-gated cation channels, gated by extracellular H^+. H^+ is the simplest ligand possible, and whereas for larger ligands that gate ion channels complex binding sites in the three-dimensional structure of the proteins have to be assumed, H^+ could in principle gate a channel by titration of a single amino acid. Experimental evidence suggests a more complex situation, however. For example, it has been shown that extracellular Ca^{2+} ions compete with H^+; probably Ca^{2+} ions bound to the extracellular loop of ASICs stabilize the closed state of the channel and have to be displaced before the channel can open. In such a scheme, amino acids contributing to Ca^{2+} binding would also be candidates contributing to H^+ gating. In this study we systematically screened more than 40 conserved, charged amino acids in the extracellular region of ASIC1a for a possible contribution to H^+ gating. We identified four amino acids where substitution strongly affects H^+ gating: Glu^{63}, His^{72}/His^{73}, and Asp^{78}. These amino acids are highly conserved among H^+-sensitive ASICs and are candidates for the "H^+ sensor" of ASICs.

Acid-sensing ion channels (ASICs)[3] are cation channels that are gated by extracellular H^+ (1, 2). A rise in the H^+ concentration opens ASICs, and the continued presence of H^+ desensitizes them. Desensitization has time constants from less than 100 ms in fish ASICs (3, 4) to several seconds in mammalian ASIC2a (5).

ASIC subunits have a simple topology: short cytoplasmic tails, two transmembrane domains (TM1 and TM2), and a large (>350 amino acids) extracellular region (6). The recently determined crystal structure of a chicken ASIC1 deletion mutant (7) reveals a trimeric arrangement, which is characterized by a high degree of asymmetry in the hexahelical transmembrane region. This structure was obtained at acidic pH and most likely represents a desensitized-like conformation and therefore does not allow direct identification of the H^+ sensor. The extracellular region is composed of five subdomains, which are connected to the membrane-spanning region via an apparently flexible wrist. As predicted (8), this region is stabilized by cysteine bridges formed by 14 conserved cysteines. The structure also confirms electrophysiological experiments suggesting that the second transmembrane domain (9–11) and a pre-TM1 domain (12, 13) contribute to the ion pore, although residues from TM1 also line the pore.

In mammals, four *asic* genes code for at least six ASIC subunits (ASIC1a, 1b, 2a, 2b, 3, and 4) (2, 14), which assemble into homo- or heterooligomeric channels. Among them, homomeric ASIC1a and ASIC3 are the most H^+-sensitive (1, 15); ASIC2b and ASIC4 cannot be activated by H^+ under physiological conditions (16, 17). ASIC1a and ASIC3 are half-maximally activated at pH 6.6. To serve as H^+ sensors under physiological conditions, these channels cannot be much more sensitive, because otherwise they would be chronically desensitized by resting concentrations of H^+ (18, 19). For both channels, it has been shown that the apparent H^+ affinity depends on the extracellular concentration of Ca^{2+} (19, 20); low Ca^{2+} concentrations increase apparent H^+ affinity, and high Ca^{2+} concentrations decrease affinity. This behavior is readily explained by a competition between Ca^{2+} and H^+ for binding to the channel. More specifically, it has been proposed that there is a common Ca^{2+}/H^+-binding site at the outer entrance to the ion pore and that H^+ would displace Ca^{2+} from this binding site, unblocking the ion pore (20). In this model the Ca^{2+} ion itself would be the gate. This model, although very attractive, seems to be too simple. First, substituting a ring of negative charges at the outer entrance to the ion pore relieves open channel block by Ca^{2+}, yet neither constitutively opens channels nor abolishes H^+ gating and Ca^{2+} modulation of H^+ gating (21). Second, detailed analysis of single channel events of fish ASIC favored changes in allosteric conformations as the gating mechanism (22). In summary, available evidence suggests that there are two binding sites for Ca^{2+} in the large extracellular region of ASICs: one at the outer entrance to the ion pore mediating open channel block and another one whose occupation stabilizes the closed state of the channel at low (resting) H^+ concentrations (21). Ca^{2+} has to be displaced from both sites for channel opening.

The binding site for Ca^{2+} at the outer entrance to the ion pore has been identified in rat ASIC1a; it is constituted by

* This work was supported by Deutsche Forschungsgemeinschaft Grants GR1771/3-3 and GR1771/3-4 (to S. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence may be addressed: Solomon H. Snyder Dept. of Neuroscience, Johns Hopkins University, 725 N. Wolfe St., WBSB 1001, Baltimore, MD 21205. Tel.: 410-955-6949; Fax: 410-955-6942; E-mail: mpauker1@jhmi.edu.

[2] To whom correspondence may be addressed: Dept. of Physiology II, Röntgenring 9, D-97070 Würzburg, Germany. Tel.: 49-931-31-6046; Fax: 49-931-31-2741; E-mail: stefan.gruender@uni-wuerzburg.de.

[3] The abbreviations used are: ASIC, acid-sensing ion channel; HA, hemagglutinin; RLU, relative light unit; TM, transmembrane domain; MES, 2-(*N*-morpholino)ethanesulfonic acid.

Glu^{425} and Asp^{432} (21). These two residues are conserved in all known ASICs, except H^+-insensitive rat ASIC4. In analogy to most other Ca^{2+}-binding proteins, it is very likely that acidic amino acids (Glu or Asp) contribute also to the second Ca^{2+}-binding site. Regarding the mechanism of the Ca^{2+} dependence of ASIC activation, it could be that H^+ activates the channel by direct titration of the carboxylates of one or both Ca^{2+}-binding sites. Furthermore, it is unclear whether such protonation/Ca^{2+} unbinding is sufficient to open the channels or whether H^+ has to bind somewhere else on the channel to trigger an additional conformational change in an allosteric manner. In the ASIC1 crystal structure, three pairs of acidic amino acids within a suspicious acidic pocket were proposed to constitute a crucial part of the H^+ sensor (7).

The purpose of the present work was to identify (*a*) amino acids contributing to the putative second Ca^{2+}-binding site and (*b*) candidate amino acids contributing to allosteric changes upon binding of H^+. To this end, we systematically screened charged residues within the extracellular region of ASIC1a. We chose ASIC1a as a model and *Xenopus* oocytes as an expression system because homomeric ASIC1a generates large current amplitudes in *Xenopus* oocytes. Our study, however, did not identify the three pairs of acidic residues identified on the basis of the structure as important for H^+ gating of ASIC1. We rather identified a few amino acids, clustered in the post-TM1 region, that are crucial for H^+ gating of ASIC1a.

EXPERIMENTAL PROCEDURES

Electrophysiology—cDNAs for rat ASIC1a and ASIC2a have been described previously (13, 23). Point mutations were introduced by recombinant PCR using standard protocols with Pwo DNA polymerase (Roche Applied Science). All of the PCR-derived fragments were entirely sequenced.

Part of the ovaries of adult *Xenopus laevis* females were surgically removed under anesthesia. Anesthetized frogs were killed after the final oocyte collection by decapitation. Animal care and experiments followed approved institutional guidelines at the Universities of Tübingen and Würzburg.

The follicular membrane was removed by digestion with collagenase type II (Sigma; 1 mg ml^{-1}) for 60–120 min. Synthesis of cRNA, maintenance of *X. laevis* oocytes, and recordings of whole cell currents were done as previously described (21). We injected amounts of cRNA as indicated in Figs. 2 and 3. For co-expression of wild-type ASIC1a and 2a, we injected equal amounts (0.01 ng) of cRNAs of the two subunits. For co-expression of wild-type ASIC2a and mutant ASIC1a, we injected 0.01 ng of ASIC2a and 0.5 ng of mutant ASIC1a cRNAs. The bath solution for two-electrode voltage clamp contained 140 mM NaCl, 10 mM HEPES; concentrations of divalent cations ($CaCl_2$ or $MgCl_2$) were as indicated in the figure legends. HEPES was replaced by MES buffer where appropriate. Because H^+ affinity of ASIC1 is modulated by extracellular Ca^{2+}, we kept the Ca^{2+} concentration always constant (1.8 mM) *between* low pH activation and changed it only *during* low pH activation. This may slightly affect the shape of the blocking curve and the IC_{50}. Holding potential was −70 mV. All of the measurements were performed at room temperature (20–25 °C).

Determination of Surface Expression—The hemagglutinin (HA) epitope (YPYDVPDYA) of influenza virus was inserted in the extracellular loop of ASIC1a, and surface expression was determined as previously described (23). The oocytes were injected with 1 ng of cRNA. Relative light units (RLUs)/s were calculated as a measure of surface expressed channels. RLUs of HA-tagged channels were at least 1000-fold higher than RLUs of untagged channels. The results are from two independent experiments with oocytes from two different frogs; at least six oocytes were analyzed for each experiment and each condition.

Data Analysis—The data were analyzed with the software IgorPro (WaveMetrics, Lake Oswego, OR). For each experiment, the oocytes from at least two different batches of frogs were used. For whole oocyte currents, pH response curves (for H^+ activation and steady-state desensitization) were fitted with a Hill function,

$$I = r + (I_{max} - r)/(1 + (pH_{50}/[H^+])^a) \quad \text{(Eq. 1)}$$

where I_{max} is the maximal current, r is the residual current, pH_{50} is the pH at which half-maximal activation is achieved, and a is the Hill coefficient. Time constants characterizing desensitization were determined with a mono-exponential function.

The results are reported as the means ± S.E. They represent the means of n individual measurements on different oocytes. Statistical analysis was done with the unpaired Student's t test. For pH response curves, current peak amplitudes were normalized to the largest peak amplitude of a recording. H^+ activation of ASIC1a undergoes tachyphylaxis (23). In our study, tachyphylaxis was variable among batches of oocytes as well as among different mutant channels. To reduce the impact of tachyphylaxis on the apparent EC_{50} of H^+ activation, we performed recordings with increasing as well as decreasing concentrations of H^+. We then took such a representative pair of recordings and normalized the respective current peak amplitudes by an empirical set of factors with the aim to minimize differences among the two recordings. We then normalized all recordings from that particular type of ion channel and batch of oocytes with the same factors.

RESULTS

Mutational Screen of Acidic Amino Acids Identifies Two Residues within the Post-TM1 Domain That Are Critical for H^+ Activation—We first focused on acidic amino acids. By site-directed mutagenesis, we replaced 31 conserved aspartates and glutamates (Fig. 1) by asparagine and glutamine, respectively, the chemically most similar nontitratable amino acids. We expressed mutant channels in *Xenopus* oocytes and tested their function by two-electrode voltage clamp. Because some mutations might render channels more sensitive to H^+ activation, we generally used a conditioning pH of 7.8. For each mutant channel we determined the pH of half-maximal activation (EC_{50}) and the maximal peak current amplitude at saturating pH. In addition, we determined the EC_{50} value with near physiological concentrations of divalent cations (1.8 mM Ca^{2+} and 1.0 mM Mg^{2+}) and with low concentrations of divalent cations (0.1 mM Ca^{2+} and no Mg^{2+}) in the acidic solution. This tested

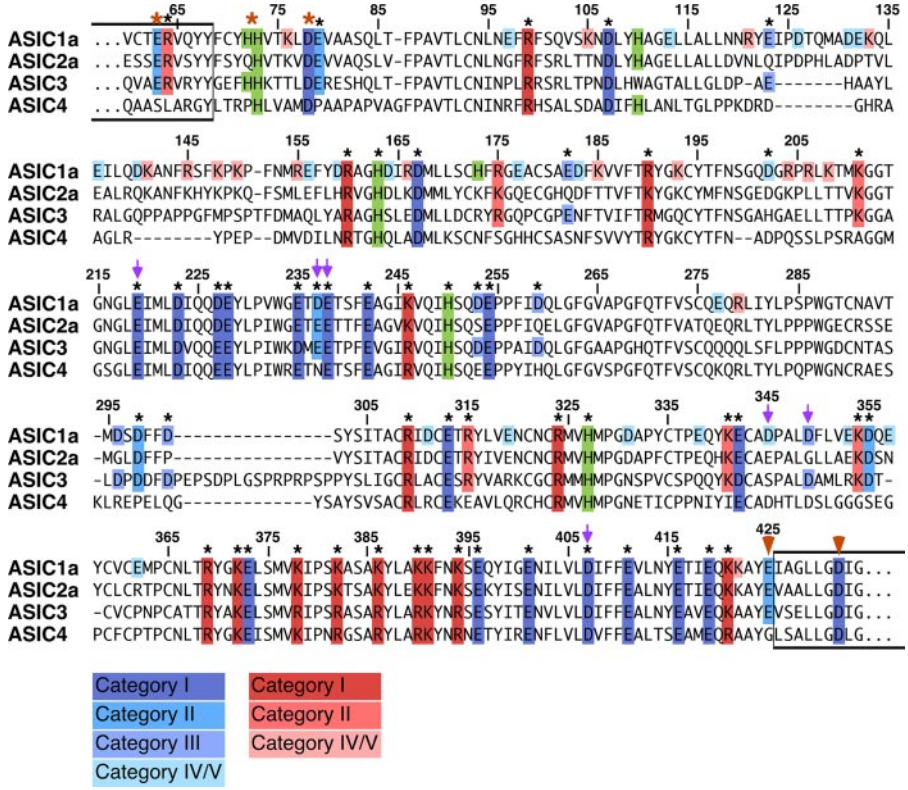

FIGURE 1. **Sequence alignment highlighting the amino acids substituted in this study.** The amino acid sequences of the extracellular regions of rat ASIC1a, 2a, 3, and 4 are aligned. The numbering refers to ASIC1a. Amino acids that have been exchanged in this study are marked by *black asterisks*; amino acids whose substitution impaired H^+ activation are highlighted by *orange asterisks*, and amino acids from the acidic pocket are shown with *mauve arrows*. The extracellular loop of ASIC1a contains 56 aspartate or glutamate residues (*blue boxes*); Glu^{425} and Asp^{432} (*orange arrowhead*) mediate open channel block by Ca^{2+} (21). 20 of the remaining residues are completely conserved among ASIC1a, ASIC2a, ASIC3, and ASIC4 (category I). Five are conserved among ASIC1a, ASIC2a, and ASIC3 (category II), and a further seven are conserved only among ASIC1a and ASIC3 (category III). Finally, 15 Asp or Glu residues are present in ASIC1a but not in ASIC3 (category IV), and seven exist exclusively in ASIC1 (category V). We engineered 31 ion channels carrying individual substitutions: we substituted all category I and II residues, five of seven category III residues, and from category IV we included Asp^{202} in our screen because ASIC3 also has a titratable amino acid, histidine, at this site. In addition, the extracellular loop of ASIC1a contains seven His residues (*green boxes*), and 38 Lys or Arg residues (*red boxes*). Two of the histidines are not conserved in ASIC3 and are therefore weak candidates for a conserved H^+ sensor; we individually substituted the five remaining histidines to alanine or asparagine. Furthermore we individually substituted by methionine all lysines and by glutamine all arginines that are conserved between ASIC1a and ASIC3 (the one exception is Arg^{175}).

whether apparent H^+ affinity of a particular mutant channel was still dependent on Ca^{2+} (19).

The results from this first set of experiments are summarized in the *left* and *right panels* of Fig. 2. All single mutant channels could be activated by H^+ and most had maximal current amplitudes that were comparable with or even larger than wild-type ASIC1a. Several substitutions, clustered in the post-TM1 region, reduced the current amplitude up to 2-fold (Fig. 2). Substituting Asp^{355} with Asn resulted in channels that were remarkable in two aspects. First, it required much larger amounts of RNA to get robust currents with this mutant. Second, H^+ sensitivity was no longer dependent on extracellular Ca^{2+} ($p = 0.6$; Fig. 2). The significance of these observations, however, is unclear because when we substituted Asp^{355} with Ala the mutant channel expressed more readily and was activated by H^+ in a Ca^{2+}-dependent manner ($p < 0.01$; Fig. 2).

The crystal structure of ASIC1 revealed an acidic pocket in the extracellular domain (7). Pairs of acidic amino acids that cluster in this acidic pocket are Asp^{237}-Asp^{349}, Glu^{238}-Asp^{345}, and Glu^{219}-Asp^{407} (7). Of these six amino acids four were included in our screen (Fig. 1), at least one from each pair. None of these four substitutions impaired H^+ gating of ASIC1a. Three substitutions (E219Q, E238Q, and D407N) did not alter apparent H^+ affinity; only D237N decreased affinity (Fig. 2). Moreover, for all four mutants apparent affinity was still shifted by Ca^{2+}, although this shift was small for E238Q. Thus, this initial screen did not support a crucial role in H^+ gating for any individual acidic pair.

It was surprising that, although the selection of residues for our initial screen had been very broad, none of the mutations seriously impaired H^+ activation. We reasoned that Glu^{425} and Asp^{432} might constitute a redundant Ca^{2+}-binding site that maintained the H^+ sensitivity of mutant channels. To test this hypothesis, we introduced all of the above mentioned individual mutations also into the ASIC1a-E425G/D432C background, resulting in triple mutant channels, as summarized in the *middle* and *right panels* of Fig. 2. To measure E425G/D432C currents of comparable size as wild-type currents, we needed to inject 5–10-fold more RNA (compare color code in Fig. 2). In many cases the introduction of individual mutations into the double mutant channel led to a further (up to 8-fold) reduction of the maximal current amplitude. All of the mutants were, however, still H^+-sensitive, with two exceptions: triple mutant channels carrying substitutions at residues Glu^{63} (category II; see legend to Fig. 2) or Asp^{78} (category I) did not produce reliable H^+ activated currents, even after injection of maximal amounts of RNA (10 ng). A more detailed analysis of these mutants is described below.

In addition to H^+ insensitivity of triple E63Q and triple D78N channels, our screen revealed that H^+ activation of triple E79Q channels was insensitive to Ca^{2+}; EC_{50} was identical with physiological and low concentrations of divalent cations (Fig. 2, *right panel*). A D78N/E79Q double mutant also showed no longer a dependence on Ca^{2+} of its apparent H^+ affinity (not shown), although both individual mutations did (Fig. 2, *right panel*). Therefore, we also determined for the triple E79Q mutant steady-state desensitization with physiological and low concentrations of divalent cations. In this case, Ca^{2+} shifted the concentration response curve by 0.2 pH units (not shown), sim-

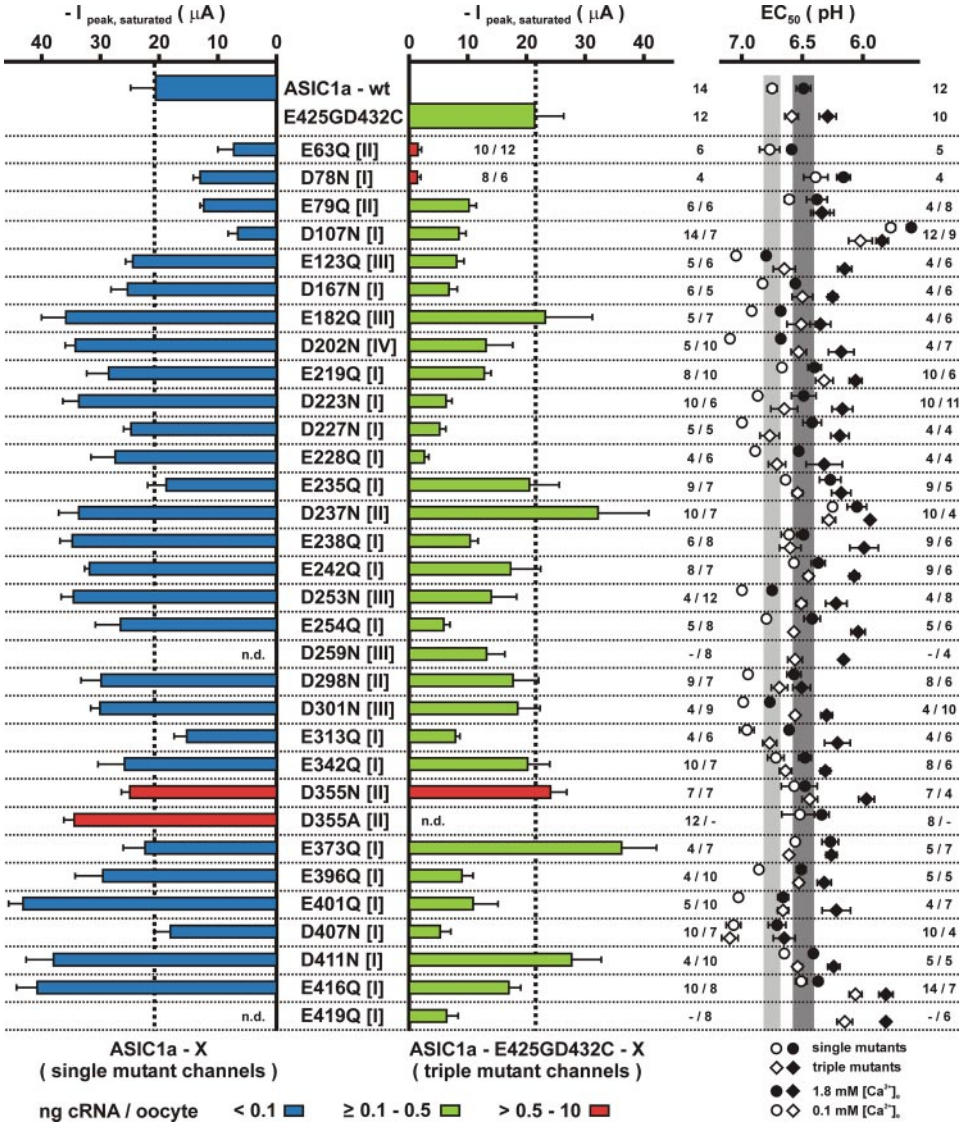

FIGURE 2. **Effect of substituting acidic candidate residues.** *Left panel*, *bars* represent means ± S.E. of peak current amplitudes induced by saturating H^+ concentrations. Test solutions contained 1.8 mM Ca^{2+} and 1.0 mM Mg^{2+}; $V_{hold} = -70$ mV. The *bars* to the *left* represent wild-type channel or mutant channels carrying the indicated single substitutions. The *bars* to the *right* represent E425G/D432C double mutant channel or triple mutant channels carrying E425G/D432C plus the indicated additional substitution. The *labels I–IV* indicate the degree of conservation of respective residue among ASIC subtypes. *n.d.*, not determined. *Right panel*, *symbols* and *bars* represent means ± S.E. of pH values for half-maximal H^+ activation. The numbers of recordings from different oocytes are indicated on the *left side* for 0.1 mM Ca^{2+} and on the *right side* for 1.8 mM Ca^{2+}/1.0 mM Mg^{2+}. The *gray bars* represent EC_{50} values for the wild type (*light gray*, 0.1 mM Ca^{2+}; *dark gray*, 1.8 mM Ca^{2+}/1.0 mM Mg^{2+}). An explanation of the color code and symbols is given at the *bottom*.

ilar to wild-type ASIC1a. Thus, evidence that Glu^{79} is involved in Ca^{2+} binding was not conclusive.

Acidic amino acids from the acidic pocket (7) were unsuspicious also as triple mutants: the triple E219Q, D237N, and E238Q substitutions decreased the apparent H^+ affinity; however, several other substitutions resulted in the same behavior (Fig. 2), and a decrease in H^+ affinity by itself does not imply a crucial role in H^+ gating. Triple D407N had a slightly increased H^+ affinity, which is unexpected if Asp^{407} forms a pair with Glu^{219} that is important for H^+ gating. Moreover, the apparent affinity was shifted by Ca^{2+} for all four triple mutants.

So far, our mutational screen showed that individual substitution of any conserved acidic amino acid, including those contributing to amino acid pairs in the acidic cluster of ASIC1, does not impair H^+ gating of ASIC1a. Substitution of Glu^{63} and Asp^{78} in the E425G/D432C background, however, led to nonfunctional channels. These two amino acids, therefore, are candidates for amino acids involved in H^+ gating.

Mutational Screen of Basic Amino Acids Identified a Pair of Histidines within the Post-TM1 Domain That Is Crucial for H^+ Activation—Besides acidic amino acids, basic amino acids are candidate targets for protonation and could therefore contribute to an allosteric mechanism of H^+ gating of ASICs. With its pK value around 6, histidine is a prime candidate to constitute a sensor for H^+ activation of ASIC1a under physiological conditions. We considered also lysine and arginine residues because their more basic pK values could be shifted toward the physiological range, depending on their local environment within the channel protein. We individually substituted five conserved histidines (Fig. 1) by alanine or asparagine. Furthermore we individually substituted methionine for all lysines, which are conserved between ASIC1a and ASIC3, and glutamine for all arginines, which are conserved between ASIC1a and ASIC3 (the one exception is Arg^{175}) yielding a total of 20 mutants. The results of the functional analysis of these mutant channels are summarized in Fig. 3. Among these mutants, only K211M was insensitive to H^+ activation.

The crystal structure revealed that Lys^{211} reaches deeply into a neighboring subunit to bind a Cl^- ion that is buried within the extracellular loop of ASIC1 (7). The Cl^- ion may have a role in assembly and gating of ASICs (7). To distinguish between these possibilities, we determined expression of this mutant at the oocyte surface. We inserted an HA epitope into the extracellular loop of ASIC1a wild type and of the K211M mutant and used a monoclonal anti-HA antibody and a luminescence assay to compare the surface expression of HA-tagged channels. This assay revealed that surface expression of the K211M mutant was significantly ($p = 0.04$) reduced compared with wild-type channels (Fig. 4). The observed reduction in surface expression by about 25%, however, cannot explain the dramatic reduction in current amplitude, suggesting that K211M has a predomi-

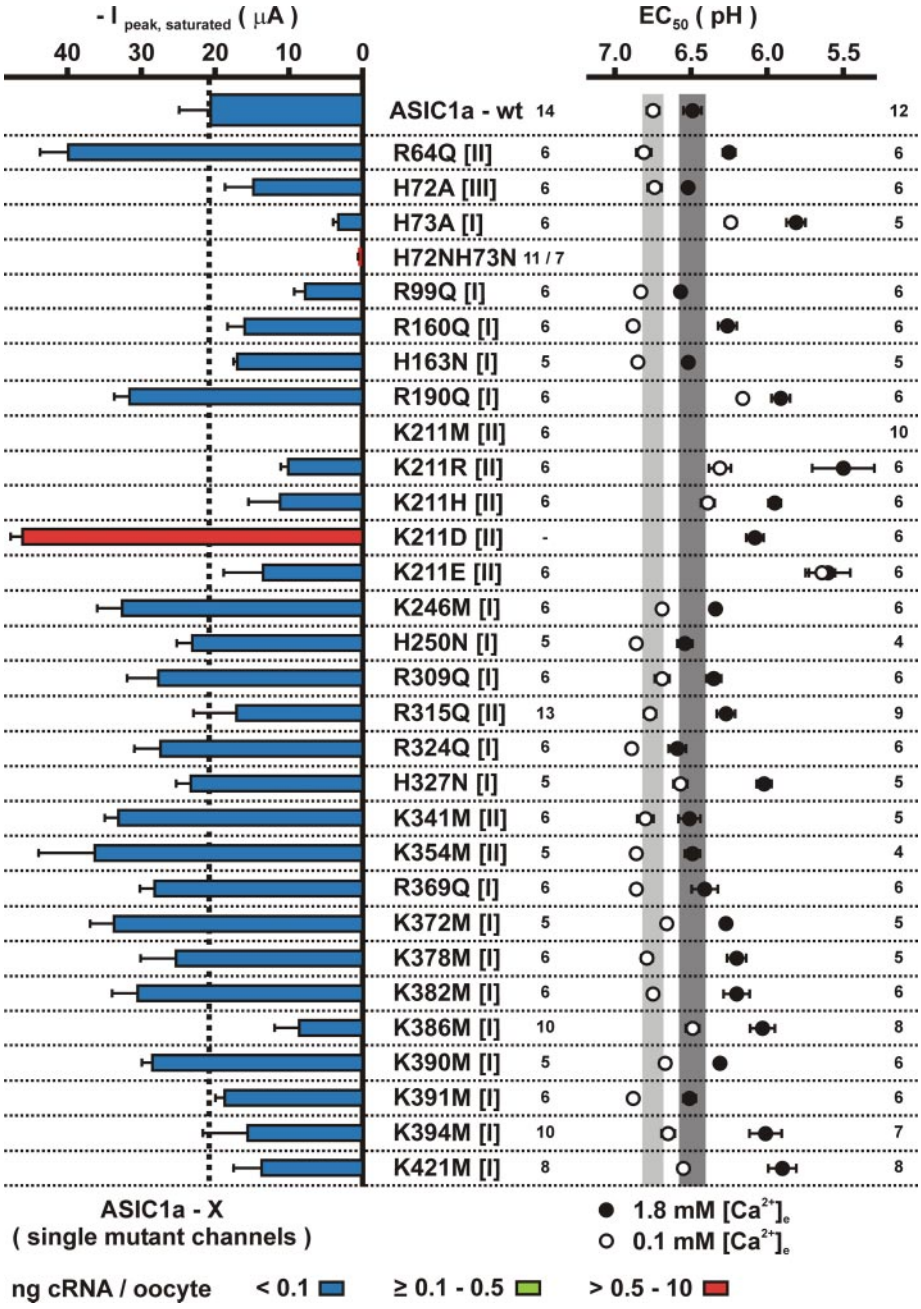

FIGURE 3. **Effect of substituting basic candidate residues.** *Left panel*, *bars* represent means ± S.E. of peak current amplitudes induced by saturating H^+ concentrations for wild-type channel or mutant channels carrying the indicated single substitutions. Test solutions contained 1.8 mM Ca^{2+} and 1.0 mM Mg^{2+}; $V_{hold} = -70$ mV. The *labels I–III* indicate the degree of conservation of respective residue among ASIC subtypes. *Right panel*, *symbols* and *bars* represent means ± S.E. of pH values for half-maximal H^+ activation. The numbers of recordings from different oocytes are indicated on the *left side* for 0.1 mM Ca^{2+} and on the *right side* for 1.8 mM Ca^{2+}/1.0 mM Mg^{2+}. The *gray bars* represent EC_{50} values for the wild type (*light gray*, 0.1 mM Ca^{2+}; *dark gray*, 1.8 mM Ca^{2+}/1.0 mM Mg^{2+}). An explanation of the color code and symbols is given at the *bottom*.

nant effect on gating rather than on assembly. To further investigate the role of Lys^{211}, we replaced this residue also by Asp, Glu, His, or Arg. In contrast to K211M, all of these substitutions resulted in H^+-sensitive channels that were, however, up to 10-fold less H^+-sensitive than wild type (Fig. 3). Thus, although these results suggest a role for Lys^{211} in gating of ASIC1a, they show that a protonation/deprotonation cycle between a neutral and a positively charged residue at position 211 cannot be crucial for H^+ sensitivity.

Substitution of His^{72} in ASIC2a renders this channel H^+-insensitive (24), suggesting that it could be involved in the pH sensor of ASICs. It is not the sole determinant of ASIC2a H^+ sensitivity, however (24). His^{73} of ASIC1a corresponds to His^{72} of ASIC2a. Substitution of His^{73} by Ala in our screen resulted in H^+-sensitive channels (Fig. 3), as previously found by others (24); maximal current amplitude of this mutant was, however, reduced 5-fold. In contrast to ASIC2a, ASIC1a has another His residue, His^{72}, immediately adjacent to His^{73}. Individual substitution of His^{72} by Ala resulted in functional channels; in contrast, combined substitution of His^{72} and His^{73} by Asn produced mutant channels that could no longer be activated by H^+ (Fig. 3). The more conservative substitution of these two His residues by the basic amino acid lysine resulted in ion channels that were still sensitive to H^+ activation, however, with dramatically reduced amplitude (not shown). These findings confirm that His^{72}/His^{73} could be an element of the H^+ sensor; in contrast to ASIC2a, in ASIC1a this crucial element would be constituted of two redundant His residues.

Detailed Analysis of the Amino Acids That Are Critical for H^+ Activation—Our screen identified the following amino acid substitutions that strongly impaired H^+ activation: E63Q and D78N in the E425G/D432C background and the H72N/H73N double mutant. All four amino acids cluster in a post-TM1 region, where they are highly conserved in ASICs. Fig. 5*A* shows a sequence comparison of the post-TM1 region of 19 ASICs from different species; for comparison, the sequence of proton-insensitive BLINaC (25), a channel closely related to ASICs, is also shown. The sequences were assigned to one of two groups: proton-sensitive (*green*) and proton-insensitive (*red*) ASICs. Proton sensitivity correlates well with the presence of a His residue at position 72/73 and a charged residue at positions 63 and 78. Exceptions are zASIC1.1 (4) that does not have a His residue here and zASIC4.1 (4) that does neither have Glu^{63} nor Asp^{78}; for zASIC4.1 we have, however, recently shown

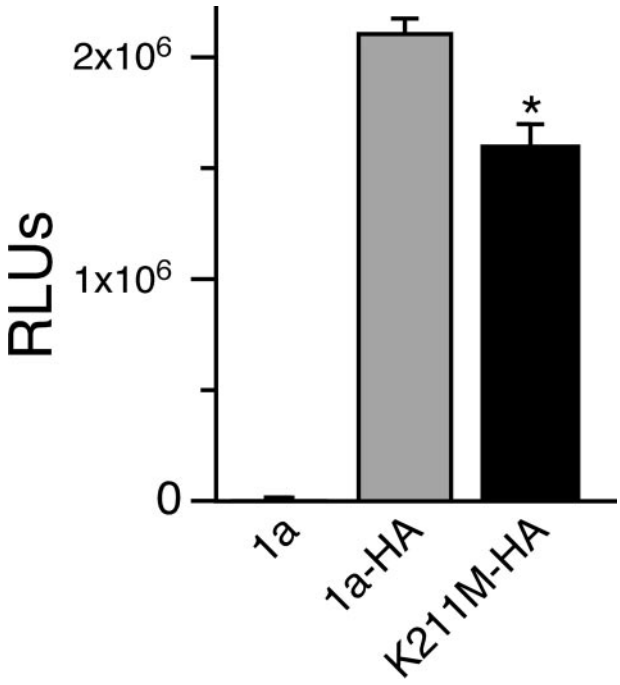

FIGURE 4. **The K211M mutant is present on the oocyte surface.** Surface expression of HA-tagged ASIC1a and ASIC1a-K211M (means ± S.E.) is shown. Untagged ASIC1a served as a control (first column). The results are expressed as RLUs/oocyte/s. n = 16; *, $p < 0.05$.

that the amino acids at these two positions are crucial for its activity (26).

In the top part of Fig. 5*B*, we show for comparison representative current traces of single substitutions at the critical sites. Wild-type ASIC1a rapidly activated and completely desensitized within less than 10 s (time constant $\tau = 1116 \pm 137$ ms, $n = 9$). Channels with individual substitutions at Glu^{63}, Asp^{78}, His^{72}, or His^{73} behaved similarly, except that desensitization of D78N was faster ($\tau = 506 \pm 25$ ms, $p < 0.05$, $n = 4$).

E425G/D432C double mutant channels are no longer blocked by Ca^{2+} but still show the typical transient ASIC current (Fig. 5*B*) (21). As shown in the *bottom panel* of Fig. 5*B*, after long H^+ stimulation washout of the acidic solution often induced a small "off" current for E425G/D432C channels. Introduction of the substitution E63Q or D78N into the E425G/D432C background resulted in channels for which the typical transient ASIC current was basically lost; the same was true for the H72N/H73N mutant (Fig. 5*B*). Sometimes oocytes expressing these channels showed a tiny transient inward current at the beginning of the acidification, like in the example in Fig. 5*B*, and there was always a small off current upon washout of the acidic solution. Thus, these mutants still showed some response to acid. One has to keep in mind, however, that 100-fold more cRNA was injected for these mutants than for wild-type ASIC1a. Therefore, we cannot exclude that the response to acid of the oocytes expressing these mutants was an unspecific effect caused by heavy overexpression of channels. In contrast to substitution of Asp^{78}, substitution in the E425G/D432C background of the directly adjacent Glu^{79} revealed currents very similar to those through E425G/D432C channels (Fig. 5*B*).

We tested whether H^+ insensitivity of these three mutants was due to a lack of surface expression. Only for triple E63Q the surface expression was significantly reduced to about 40% of wild-type levels (Fig. 5*C*); for triple D78N and H72NH73N surface expression was only slightly reduced. Considering that we injected equal amounts of mutant and wild-type cRNA for determination of surface expression but 100-fold higher amounts of mutant than wild-type cRNA for functional measurements (Figs. 2 and 3), this result shows that H^+ insensitivity of the mutants was not due to reduced surface expression.

Presence of triple E63Q, triple D78N, and H72N/H73N on the cell surface and absence of ASIC currents suggests that these residues could be specifically involved in H^+ gating. For E63Q and D78N we considered an involvement in Ca^{2+} binding. To find more direct evidence for an involvement in Ca^{2+} binding we first combined the two individual substitutions to yield mutant E63Q/D78N. Application of pH 4.0 to ASIC1aE63Q/D78N-expressing oocytes induced robust (>10 μA) transient inward currents (Fig. 6*A*), showing that this mutant channel was H^+-sensitive. Second, we assessed whether an increased Ca^{2+} concentration in the conditioning solution could rescue triple E63Q and triple D78N channels and render them H^+-sensitive. This was not consistently the case (not shown). Third, we introduced positive charges at positions 63 and 78 (mutants E63K and D78K). We reasoned that the positive charge might repel Ca^{2+}, facilitating H^+ binding also to single mutant channels. Both mutant channels were functional and H^+-sensitive (Fig. 6*B*). Apparent affinity for H^+ of mutant E63K was indeed significantly ($p < 0.05$) increased by about 0.15 pH units; full activation was reached already at pH 6.5 (Fig. 6*B*), a result expected for a channel with reduced Ca^{2+} affinity. In contrast, apparent H^+ affinity of mutant D78K was significantly ($p < 0.01$) decreased by more than 1 pH unit. In summary, our more detailed analysis of amino acids Glu^{63} and Asp^{78} shows that if Glu^{63} and Asp^{78} contributed to a Ca^{2+}-binding site, this site would not be essential.

H^+-insensitive ASIC1a Mutants Contribute to Heteromeric Channels with ASIC2a—We then assessed whether H^+-insensitive mutant ASIC1a subunits still contributed to heteromeric channels with ASIC2a and, if so, whether such heteromeric channels would have altered H^+ gating. Heteromers between ASIC1a and ASIC2a can be distinguished from homomeric ASIC2a by an about 5-fold faster desensitization kinetics and an increased H^+ sensitivity (Fig. 7) (5). Co-injection of mutant ASIC1a subunits with wild-type ASIC2a in all cases resulted in H^+-sensitive channels that desensitized significantly faster than homomeric ASIC2a (Fig. 7*A* and Table 1), showing that all mutant ASIC1a subunits successfully incorporated into a heteromeric complex. This allowed us to assess their effect on H^+ gating. We reasoned that if substituted amino acids contributed to Ca^{2+} binding, H^+ should replace Ca^{2+} ions more easily in these mutants, leading to increased apparent H^+ affinity of heteromeric channels. In contrast, if substituted amino acids contributed to allosteric changes upon proton binding, we expected a reduced proton affinity.

H^+ sensitivity of heteromeric channels containing E425G/D432C was similar to heteromeric channels containing wild-type ASIC1a (Table 1). Additional introduction of E63Q or D78N, however, reduced H^+ sensitivity by up to 1 pH unit (Fig. 7*B* and Table 1). It was difficult to obtain complete concentra-

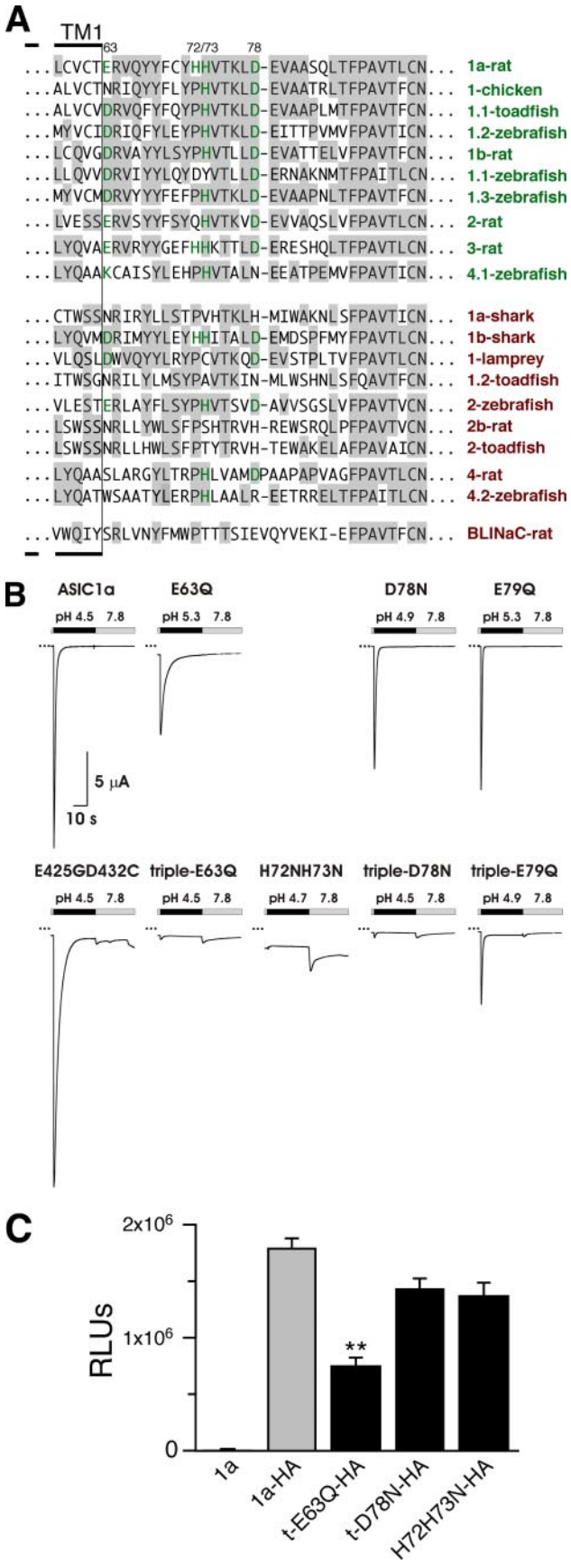

FIGURE 5. **Characteristics of channels with substitutions of residues Glu^{63}, His^{72}/His^{73}, and Asp^{78}.** *A*, alignment of the amino acid sequences of the proximal ectodomain of several ASICs. ASICs with their names in *green* are H^+-sensitive, and those with their names in *red* have been reported insensitive. The crucial amino acids identified in this study are highlighted in *bold green*. Conserved amino acids are written on a *gray background*. For comparison, the sequence of proton-insensitive BLINaC is shown at the *bottom*. *B*, representative current traces of ASIC1a, ASIC1a-E63Q, ASIC1a-D78N, and ASIC1a-E79Q (*upper row*) and, for comparison, of the respective channels carrying in addition the double substitution E425G/D432C and of ASIC1a H72N/H73N (*bottom row*); channels were activated with a saturating H^+ concentration. Note that, following prolonged activation, removal of the acidic solution induces a small transient inward current for all ion channels carrying the double substitution E425G/D432C. In contrast, the transient H^+ activated current was dramatically reduced or absent for triple E63Q, triple D78N, and ASIC1a H72N/H73N (amount of cRNA injected per oocyte was as indicated in Fig. 2 and 3). H^+ activation of triple E79Q was unaffected. The *dashed lines* represent zero current level. *C*, surface expression of HA-tagged ASIC1a, triple E63Q, triple D78N, and ASIC1a-H72N/H73N (mean ± S.E.). Untagged ASIC1a served as a control (*first column*). The results are expressed as RLUs/oocyte/s. $n = 14–16$; **, $p < 0.01$.

tion response curves at these low pH values, but heteromers containing the ASIC1a triple E63Q and triple D78N mutants still seemed to be more H^+-sensitive than homomeric ASIC2a, suggesting some contribution of the mutant subunits to H^+ gating in the heteromeric complexes. H^+ sensitivity of heteromers containing the H72N/H73N mutant was even more reduced, not being significantly different from homomeric ASIC2a channels (Table 1), suggesting that this mutant subunit did not contribute to H^+ gating. In summary, these results suggest that Glu^{63}, Asp^{78}, and His^{72}/His^{73} contribute to allosteric changes upon H^+ binding; they do not support a role in Ca^{2+} binding.

DISCUSSION

Amino Acids Contributing to the Second Ca^{2+}-binding Site—Several studies point to a critical role for extracellular Ca^{2+} in gating of ASICs (19, 20, 22, 27, 28); Ca^{2+} bound to the extracellular part of the channel is supposed to stabilize the closed state, and unbinding of Ca^{2+} is necessary for opening the channel.

In a previous study (21) two amino acids (Glu^{425} and Asp^{432}) were identified that might form a ring of negative charges around the outer entrance to the channel pore and contribute to a Ca^{2+}-binding site that mediates open channel block by Ca^{2+}. The first crystal structure of an ASIC confirms that Asp^{432}, which is located well within TM2, faces toward the ion pore (7) (Fig. 8); Glu^{425} resides at the N-terminal end of TM2 and points in the direction opposite of the ion pore (7). Because the channel has to be unblocked to open, these two amino acids may also contribute to H^+ gating. Combined substitution of Glu^{425} and Asp^{432} results in channels that are still gated by H^+, albeit with slightly changed characteristics (21). Hence, although this Ca^{2+}-binding site may contribute to H^+ gating, it is dispensable for H^+ gating of ASIC1a.

With the aim of identifying amino acids contributing to the postulated second Ca^{2+}-binding site, in the present study, we performed a comprehensive screen of conserved acidic amino acids in the extracellular loop of ASIC1a, which are candidates to contribute to a Ca^{2+}-binding site. We found that single substitutions of two amino acids (Glu^{63} and Asp^{78}) in ASIC1a, which already contains substitutions of Glu^{425} and Asp^{432} (triple mutant channels), render the channel largely H^+-insensitive (Figs. 2 and 5). This H^+ insensitivity was not due to reduced surface expression of the mutated channels (Fig. 5), suggesting

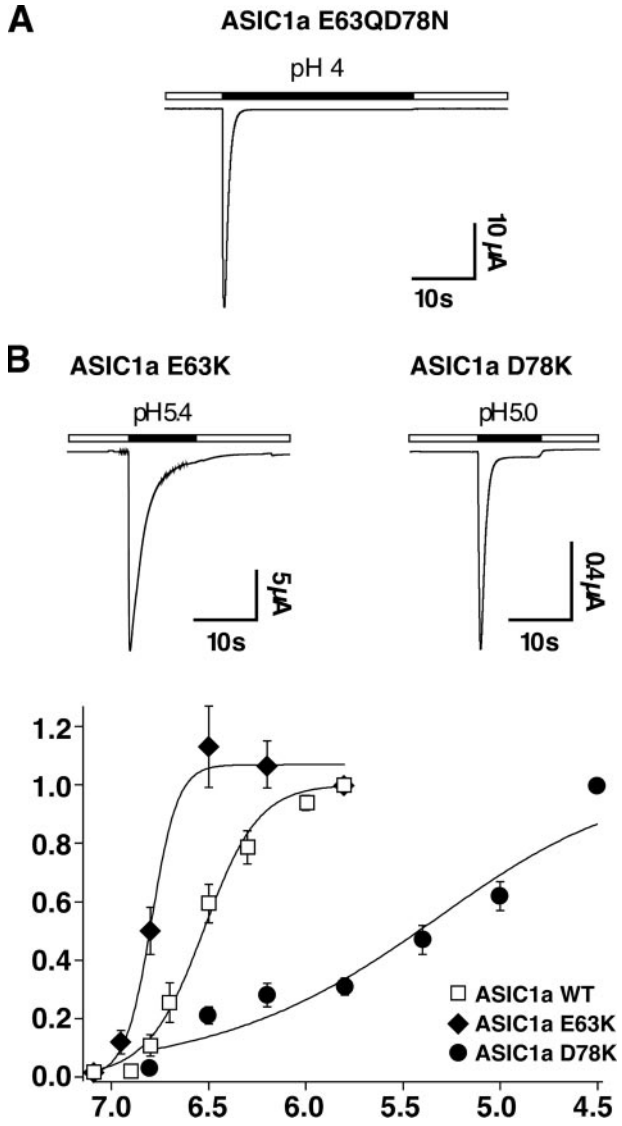

FIGURE 6. **Substitution of Glu^{63} or Asp^{78} by basic amino acids affected pH sensitivity of ASIC1a.** *A*, representative current trace of ASIC1a-E63Q/D78N double mutant channel. *B*, *top*, representative current trace of ASIC1a-E63K and ASIC1a-D78K mutants. *Bottom*, pH response curve for H^+ activation of ASIC1a, ASIC1a-E63K, and ASIC1a-D78K. Conditioning pH was 7.4. The pH_{50} of activation were 6.54 ± 0.03 for ASIC1a (n = 12), 6.69 ± 0.06 for ASIC1a-E63K (n = 7), and 5.43 ± 0.09 for ASIC1a-D78N (n = 9), respectively.

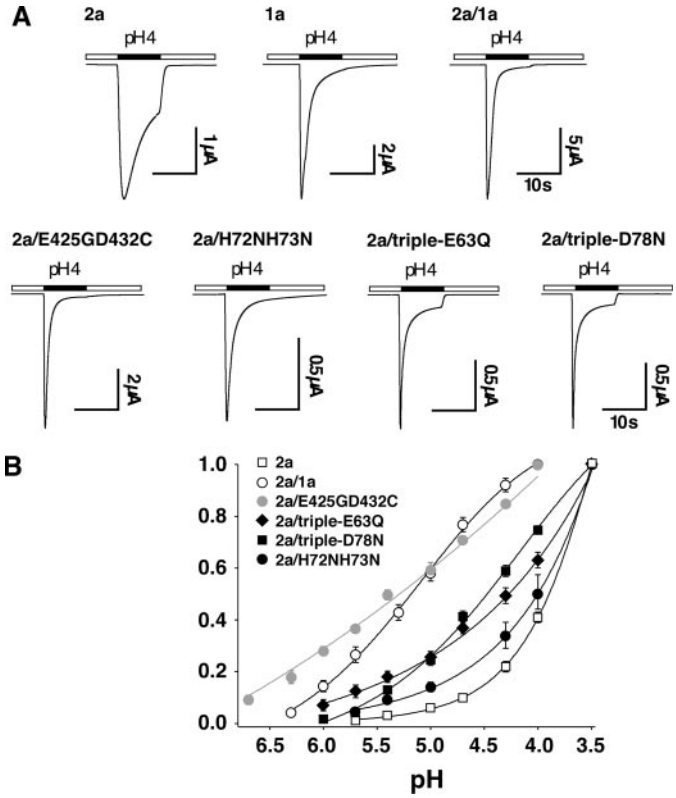

FIGURE 7. **Point mutations in ASIC1a change the pH sensitivity of heteromeric ASIC2a/1a.** *A*, *top*, representative current traces of homomeric ASIC2a, ASIC1a, and heteromeric ASIC2a/1a. *Bottom*, representative current traces of heteromeric ASIC2a/ASIC1a where the ASIC1a subunit contained the indicated amino acid substitutions. Conditioning pH was pH 7.4 and testing pH was pH 4.0. *B*, pH dependence of activation. Conditioning pH was pH 7.4.

TABLE 1

Properties of heteromers between ASIC2a and different ASIC1a variants

The data are the means ± S.E. for the number n of individual oocytes indicated in parentheses. pH values at which channels were half-maximally activated or desensitized (pH_{50}) were obtained from a fit to the Hill function. n.d., not determined. For statistical analysis of τ_{des}, mutants and 2a were compared; for statistical analysis of pH_{50}, 2a/1a-H72N/H73N was compared with 2a/1a and 2a/1a-t(riple)-E63Q(D78Q) with 2a/1a-E425G/D432C, respectively.

	τ_{des}/ms	pH_{50}	
		Activation	Desensitization
1a	720 ± 130 (8)	n.d.	n.d.
2a	2830 ± 280 (10)	3.8 ± 0.1 (10)	6.55 ± 0.03 (9)
2a/1a	730 ± 50 (9)	5.3 ± 0.1 (10)	6.68 ± 0.04 (10)
2a/1a-H72N/H73N	1660 ± 110 (14)[a]	4.0 ± 0.1 (10)[a]	6.68 ± 0.04 (5)
2a/1a-E425G/D432C	990 ± 100 (11)[a]	5.3 ± 0.1 (11)	6.93 ± 0.01 (14)
2a/1a-t-E63Q	920 ± 80 (12)[a]	4.3 ± 0.1 (12)[a]	6.82 ± 0.05 (7)[b]
2a/1a-t-D78N	880 ± 60 (11)[a]	4.5 ± 0.04 (11)[a]	6.58 ± 0.02 (10)[a]

[a] $p < 0.01$ (unpaired t test).
[b] $p < 0.05$.

a specific effect on gating. These results are consistent with the idea that Glu^{425} and Asp^{432} contribute to H^+ gating and suggest that also Glu^{63} and Asp^{78} are involved in H^+ gating. Glu^{63} and Asp^{78} are highly conserved among ASICs (Fig. 5*A*). Our study shows that individual substitution of these amino acids does not strongly affect H^+ gating. Similarly, substitution of Asp^{78} in ASIC3 has no effects on gating of this ASIC (29). But we show that combined substitution of Asp^{78} and Glu^{425}/Asp^{432} severely impairs H^+ gating. Substitution of the adjacent Glu^{79} did not have such an effect, demonstrating the specificity of the effect; Glu^{79} seems to be specifically involved in desensitization (29). Two recent studies confirm the importance of Glu^{63} and Asp^{78}: combined substitution of amino acids at the positions corresponding to Glu^{63} and Asp^{78} causes a dramatic reduction of peak current amplitude of H^+-sensitive zASIC4.1 (26), and substitution of Asp^{77} in rat ASIC2a (corresponding to Asp^{78} in rat ASIC1a) abolishes H^+ sensitivity of this ASIC (30).

Because individual substitution of Glu^{63} and Asp^{78} did not impair H^+ gating of ASIC1a, comparable with the individual substitution of Glu^{425} and Asp^{432}, it was possible that Glu^{63} and Asp^{78} contributed to a second Ca^{2+}-binding site that is redundant to the Glu^{425}-Asp^{432} site and that therefore is also dispensable for H^+ gating. Only the combined elimination of both Ca^{2+}-binding sites would render ASIC1a H^+-insensitive, as observed in our study. This conclusion is not supported by the crystal structure, however (7). In the three-dimensional struc-

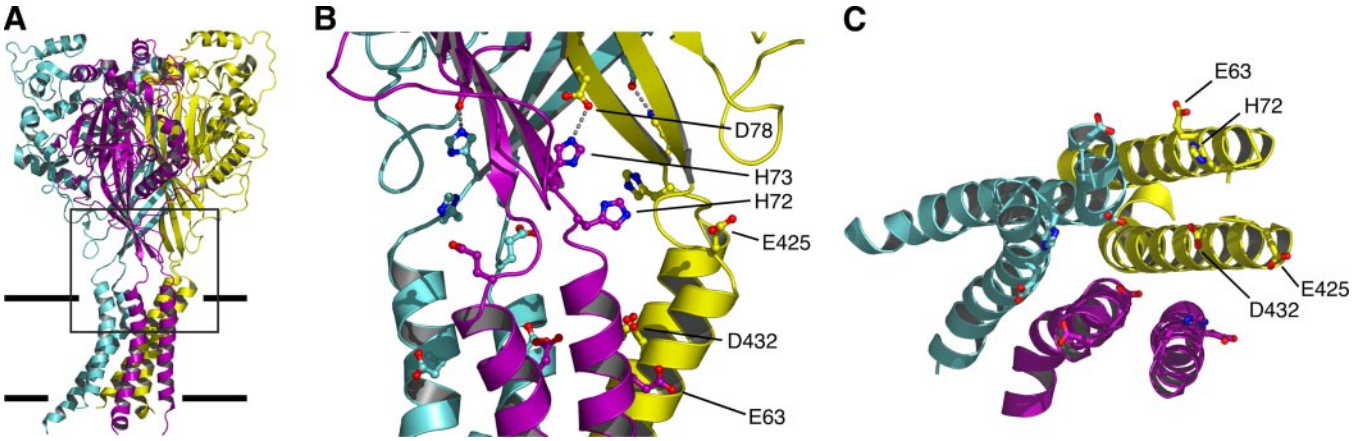

FIGURE 8. **Scheme illustrating the position of the identified amino acids within the three-dimensional structure of the channel.** *A*, ribbon diagram of the trimeric chicken ASIC1 structure (Protein Data Bank entry 2QTS) with subunits colored differently. The extent of the lipid bilayer is indicated by the *thick lines*. *B*, close-up view of the region that is highlighted in *A* by the *rectangular box*. Subunits are color coded as in *A*, and functionally important residues identified in this study are shown with their side chain in ball-and-stick representation and are labeled for one subunit with residue numbers corresponding to rat ASIC1a. The intersubunit contacts between the side chains of His^{73} and Asp^{78} are indicated by a *dashed line*. His^{72} has been modeled into the structure by replacing the Pro present at this position in chicken ASIC1 (7) and optimization of its side chain to avoid steric overlap. Similarly, Glu^{63} has been introduced at the position of Asn^{64}. *C*, view onto the TM domain of trimeric ASIC1 roughly perpendicular to the lipid bilayer. Polar residues within the helices together with the mutated His^{72} in the loop between TM1 and β1 are shown.

ture, the residues corresponding to Glu^{63} and Asp^{78} (Asn^{64} and Asp^{79}) are not in close contact with each other (7) (Fig. 8), making it unlikely that they contribute to a common Ca^{2+}-binding site. Asn^{64} is at a similar height as Asp^{433} (corresponding to Asp^{432} in ASIC1a) within the transmembrane domains (Figs. 1 and 8) with a variable spacing between the side chains ranging from under 7 Å to over 11 Å (7) because of the asymmetry in the TM region. It is not inconceivable that these residues directly interact with each other because only small rotations in the TM helices would be required.

Based on the presence of a negatively charged depression in the crystal structure of ASIC1, named the acidic pocket, it has been proposed that acidic amino acids that form three pairs of carboxylic acid-carboxylate groups within the acidic pocket are primary sites for H^+ sensing in ASICs (7). The negatively charged side chains of these residues could coordinate a Ca^{2+} ion in the closed state of the channel and upon protonation of one of the two carboxylates from each pair both side chains could come in close contact to form an acidic residue pair in the desensitized conformation (7). This is an attractive model; however, the crystal structure does provide only indirect evidence for it. Because our study eliminates the possibility that any individual pair in the acidic pocket is crucial for H^+ gating, it has to be assumed that these pairs are redundant and that only a combined substitution of more than one pair will render an ASIC H^+-insensitive. Future studies will show whether this is the case.

Amino Acids Involved in Allosteric Gating of ASIC1a—The most clear-cut effect in our study was the H^+ insensitivity of the H72N/H73N double mutant. The most parsimonious explanation is that His^{72} and His^{73} constitute the switch on which H^+ act to open ASIC1a; they may be the H^+ sensor. H^+ insensitivity of the H72N/H73N mutant, its presence on the cell surface, and its contribution to heteromeric channels without apparent contribution to H^+ gating of these channels are all characteristics consistent with this interpretation. Consistent with this

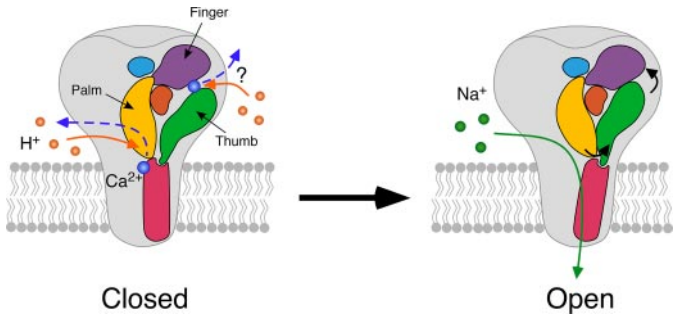

FIGURE 9. **Scheme illustrating the hypothetical gating mechanism of ASICs.** Only one subunit with its five subdomains is shown. The details are in the text. The scheme is based on a scheme proposed by Jasti and colleagues (7).

interpretation is also that His^{73} is essential for H^+ gating of ASIC2a (24).

In the desensitized state of the channel, the side chain of His^{73} is in close contact (average distance of ~3.2 Å, with individual values ranging from 2.9 to 3.5 Å) with Asp^{78} of an adjacent subunit (7). Taking into account the low pH of 5.6 during crystallization this interaction presumably represents an ion pair. Because the neighboring Glu^{79} undergoes strong conformational changes during desensitization (29), it is unlikely, however, that a similar contact is made also in the closed conformation. Perhaps His^{73} gets protonated upon channel opening and rotates during desensitization to electrostatically interact with Asp^{78}. Because His^{73} can be substituted without loss of channel function (Fig. 3), it seems that in ASIC1a His^{72} can largely take over its function, pointing to substantial structural flexibility in this region (Fig. 8).

These results suggest the following model for H^+ gating of ASICs that is illustrated in Fig. 9. A drop in the pH leads to the protonation of His^{73}, inducing a conformational change. This conformational change displaces Ca^{2+} ions from (at least) two different Ca^{2+}-binding sites and the channel opens. One Ca^{2+}-binding site is located at the outer mouth of the ion pore, and

the other one may be formed by amino acids from the acidic pocket. Two of the acidic pairs observed in the crystal structure mediate interactions between the thumb and the finger domains (7) (Fig. 9), and it has been proposed that the thumb domain moves during gating (7). His^{73} is located in the edge strand (β1) of one of the β-sheets at the base of the palm domain (7) (Fig. 8), which is adjacent to the thumb. Perhaps the palm moves relative to the thumb to assist or even to trigger the movement of the thumb (Fig. 9).

Some of the acidic amino acids involved in Ca^{2+} binding probably get titrated upon unbinding of Ca^{2+}, and displacing Ca^{2+} from its binding sites may be sufficient to open the channel (20, 21); this could explain H^+ gating of zASIC1.1, which lacks the critical His (Fig. 5*A*). For ASIC1a and ASIC3, currents induced by removal of Ca^{2+} are very tiny (20, 21), however, excluding that Ca^{2+} release alone is sufficient for full activation of these channels. Possibly, protonation of the critical His is necessary for the regular maximal activation of most ASICs. Finally, protonated His^{73} would rotate to make a salt bridge with Asp^{78}, stabilizing the desensitized state of the channel. In summary, whereas the role of Glu^{63} remains uncertain, our results suggest a crucial role for the intersubunit His^{73}-Asp^{78} pair in H^+ gating of ASICs.

Acknowledgments—We thank M. Siba and P. Seeberger for expert technical assistance and M. Pusch for comments on the manuscript.

REFERENCES

1. Waldmann, R., Champigny, G., Bassilana, F., Heurteaux, C., and Lazdunski, M. (1997) *Nature* **386,** 173–177
2. Waldmann, R., and Lazdunski, M. (1998) *Curr. Opin. Neurobiol.* **8,** 418–424
3. Coric, T., Zhang, P., Todorovic, N., and Canessa, C. M. (2003) *J. Biol. Chem.* **278,** 45240–45247
4. Paukert, M., Sidi, S., Russell, C., Siba, M., Wilson, S. W., Nicolson, T., and Gründer, S. (2004) *J. Biol. Chem.* **279,** 18783–18791
5. Bassilana, F., Champigny, G., Waldmann, R., de Weille, J. R., Heurteaux, C., and Lazdunski, M. (1997) *J. Biol. Chem.* **272,** 28819–28822
6. Saugstad, J. A., Roberts, J. A., Dong, J., Zeitouni, S., and Evans, R. J. (2004) *J. Biol. Chem.* **279,** 55514–55519
7. Jasti, J., Furukawa, H., Gonzales, E. B., and Gouaux, E. (2007) *Nature* **449,** 316–323
8. Firsov, D., Robert-Nicoud, M., Gründer, S., Schild, L., and Rossier, B. C. (1999) *J. Biol. Chem.* **274,** 2743–2749
9. Schild, L., Schneeberger, E., Gautschi, I., and Firsov, D. (1997) *J. Gen. Physiol.* **109,** 15–26
10. Kellenberger, S., Hoffmann-Pochon, N., Gautschi, I., Schneeberger, E., and Schild, L. (1999) *J. Gen. Physiol.* **114,** 13–30
11. Sheng, S., Li, J., McNulty, K. A., Avery, D., and Kleyman, T. R. (2000) *J. Biol. Chem.* **275,** 8572–8581
12. Coscoy, S., de Weille, J. R., Lingueglia, E., and Lazdunski, M. (1999) *J. Biol. Chem.* **274,** 10129–10132
13. Bässler, E. L., Ngo-Anh, T. J., Geisler, H. S., Ruppersberg, J. P., and Gründer, S. (2001) *J. Biol. Chem.* **276,** 33782–33787
14. Kellenberger, S., and Schild, L. (2002) *Physiol. Rev.* **82,** 735–767
15. Sutherland, S. P., Benson, C. J., Adelman, J. P., and McCleskey, E. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 711–716
16. Lingueglia, E., de Weille, J. R., Bassilana, F., Heurteaux, C., Sakai, H., Waldmann, R., and Lazdunski, M. (1997) *J. Biol. Chem.* **272,** 29778–29783
17. Gründer, S., Geissler, H. S., Bässler, E. L., and Ruppersberg, J. P. (2000) *Neuroreport* **11,** 1607–1611
18. Benson, C. J., Eckert, S. P., and McCleskey, E. W. (1999) *Circ. Res.* **84,** 921–928
19. Babini, E., Paukert, M., Geisler, H. S., and Gründer, S. (2002) *J. Biol. Chem.* **277,** 41597–41603
20. Immke, D. C., and McCleskey, E. W. (2003) *Neuron* **37,** 75–84
21. Paukert, M., Babini, E., Pusch, M., and Gründer, S. (2004) *J. Gen. Physiol.* **124,** 383–394
22. Zhang, P., Sigworth, F. J., and Canessa, C. M. (2006) *J. Gen. Physiol.* **127,** 109–117
23. Chen, X., and Gründer, S. (2007) *J. Physiol.* (*Lond.*) **579,** 657–670
24. Baron, A., Schaefer, L., Lingueglia, E., Champigny, G., and Lazdunski, M. (2001) *J. Biol. Chem.* **276,** 35361–35367
25. Sakai, H., Lingueglia, E., Champigny, G., Mattei, M. G., and Lazdunski, M. (1999) *J. Physiol.* (*Lond.*) **519,** 323–333
26. Chen, X., Polleichtner, G., Kadurin, I., and Gründer, S. (2007) *J. Biol. Chem.* **282,** 30406–30413
27. de Weille, J., and Bassilana, F. (2001) *Brain Res.* **900,** 277–281
28. Immke, D. C., and McCleskey, E. W. (2001) *Nat. Neurosci.* **4,** 869–870
29. Cushman, K. A., Marsh-Haffner, J., Adelman, J. P., and McCleskey, E. W. (2007) *J. Gen. Physiol.* **129,** 345–350
30. Smith, E. S., Zhang, X., Cadiou, H., and McNaughton, P. A. (2007) *Neurosci. Lett.* **426,** 12–17

Candidate Amino Acids Involved in H^+ Gating of Acid-sensing Ion Channel 1a

Martin Paukert, Xuanmao Chen, Georg Polleichtner, Hermann Schindelin and Stefan Gründer

J. Biol. Chem. 2008, 283:572-581.
doi: 10.1074/jbc.M706811200 originally published online November 1, 2007
