CANDIDATE AMINO ACIDS INVOLVED IN H⁺ GATING OF ACID-SENSING ION CHANNEL 1A

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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 Ca²⁺ ions compete with H⁺; probably Ca²⁺ 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. Such a scheme makes amino acids contributing to Ca²⁺ binding to candidates contributing also 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: E63, H72/H73, and D78. These amino acids are highly conserved among H⁺-sensitive ASICs and are candidates for the "H⁺ sensor" of ASICs.

Acid-sensing ion channels (ASICs) 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 msec 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 hexa-helical 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 Ca²⁺ (19,20): low Ca²⁺ concentrations increase apparent H^+ affinity, high Ca^{2+} concentrations decrease affinity. This behavior is readily explained by a competition between Ca²⁺ 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, though 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 Ca²⁺, yet neither constitutively opens channels nor abolishes H⁺ gating and Ca²⁺ 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 loop 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 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 Ca²⁺-binding proteins, it is very likely that acidic amino acids (Glu or Asp) contribute also to the second Ca²⁺ binding site. Regarding the mechanism of the Ca^{2+} dependence of ASIC activation it could be that H⁺ activate the channel by direct titration of the carboxylates of one or both Ca²⁺ binding sites. Furthermore, it is unclear whether such protonation/Ca²⁺ 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 Ca²⁺ 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 PCRderived fragments were entirely sequenced.

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Part of the ovaries of adult Xenopus laevis females were surgically removed under anesthesia. Anesthetized frogs were killed after the final oocvte 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 Xenopus laevis oocytes and recordings of whole cell currents were done as previously described (21). We injected amounts of cRNA as indicated in Fig. 2 and 3. For coexpression 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 (in mM): 140 NaCl, 10 HEPES; concentrations of divalent cations (CaCl₂, or MgCl₂) were as indicated in the figure legends. HEPES was replaced by MES buffer where appropriate. Since H⁺ affinity of ASIC1 is modulated by extracellular Ca²⁺, we kept the Ca²⁺ concentration always constant (1.8 mM) *between* low-pH activation and changed it only *during* lowpH activation. This may slightly affect the shape of the blocking curve and the IC₅₀. Holding potential was -70 mV. All 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). Oocytes were injected with 1 ng cRNA. Relative light units (RLUs) per sec 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. 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 - Data were analyzed with the software IgorPro (WaveMetrics, Lake Oswego, OR USA). For each experiment, 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:

 $I = r + (I_{max} - r) / (1 + (pH_{50}/[H^+])^a),$

where I_{max} is the maximal current, r is the residual current, pH₅₀ 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.

Results are reported as means \pm S.E.M. They represent the mean of *n* individual measurements on different oocytes. Statistical analysis was done with the unpaired *t*-test. For pHresponse 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 (N) and glutamine (Q), respectively, the chemically most similar non-titratable amino acids. We expressed mutant channels in Xenopus oocytes and tested their function by two-electrode voltage-clamp. As 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 $(0.1 \text{ mM Ca}^{2+} \text{ and no Mg}^{2+})$ in the acidic solution. This tested 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.1. All single mutant channels could be activated by H^+ and most had maximal current amplitudes that were comparable to 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 D355 by N 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 Ca²⁺ (p = 0.6; Fig. 2). The significance of these observations, however, is unclear because when we substituted D355 by A the mutant channel expressed more readily and was activated by H^+ in a Ca²⁺ 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 D237-D349, E238-D345, and E219-D407 (7). Out of these six amino acids four were included in our screen (Fig. 1); from each pair at least one. 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, apparent affinity was still shifted by Ca²⁺, although this shift was small for E238Q. Thus, this initial screen does 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 E425 and D432 might constitute a redundant Ca²⁺ 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. 1. In order to measure E425G/D432C currents of comparable size as wild-type currents, we needed to inject 5 to 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 mutants were, however, still H⁺-sensitive – with two exceptions: triple mutant channels carrying substitutions at residues E63 (category II; see legend to Fig. 2) or D78 (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-E63O and -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 determined for the triple-E79Q mutant also 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), similar to wild-type ASIC1a. Thus, evidence that E79 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-D407 had a slightly increased H^+ affinity, which is unexpected if D407 forms a pair with E219 that is important for H^+ gating. Moreover, the apparent affinity was shifted by Ca²⁺ 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 E63 and D78 in the E425G/D432C background, however, led to non-functional 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 are 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 (H) is a prime candidate to constitute a sensor for H^+ activation of ASIC1a under physiological conditions. We considered also lysine (K) and arginine (R) residues since their more basic pK values could be shifted towards the physiological range, depending on their local environment within the channel protein. We individually substituted 5 conserved histidines (Fig. 1) by asparagine. Furthermore alanine or 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 R175) 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 K211 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 to 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 predominant effect on gating rather than on assembly. To further investigate the role of K211, we replaced this residue also by a D, an E, an H, or an R. In contrast to K211M, all 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 K211 in gating of show ASIC1a, thev that а 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, though (24). His-73 of ASIC1a corresponds to His-72 of ASIC2a. Substitution of H73 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, H72, immediately adjacent to H73. Individual substitution of H72 by Ala resulted in functional channels; in contrast, combined substitution of H72 and H73 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 H72/H73 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 H72H73N double-mutant. All four amino acids cluster in a post-TM1 region, where they are highly conserved in ASICs. Figure 5A shows a sequence comparison of the post-TM1 region of 19 ASICs from different species; for comparison, also the sequence of proton-insensitive BLINaC (25) - a channel closely related to ASICs - is shown. Sequences were assigned to one of two groups: proton-sensitive (green) and protoninsensitive ASICs. Proton-sensitivity (red) 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 E63 nor D78; for zASIC4.1 we have, however, recently shown that the amino acids at these two positions are crucial for its activity (26).

In the top part of Fig. 5B, we show for comparison representative current traces of single

H+ sensor of ASIC1a

substitutions at the critical sites. Wild-type ASIC1a rapidly activated and completely desensitized within less than 10 sec (time constant $\tau = 1116 \pm 137$ msec, n = 9). Channels with individual substitutions at E63, D78, H72, or H73 behaved similarly, except that desensitization of D78N was faster ($\tau = 506 \pm 25$ msec, 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. 5B) (21). As shown in the bottom part of Fig. 5B, after long H^+ stimulation washout of the acidic solution often induced a small "off" current for E425G/D432Cchannels. Introduction of the substitution E63O 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 H72H73N mutant (Fig. 5B). Sometimes oocytes expressing these channels showed a tiny transient inward current at the beginning of the acidification, like in the example in Fig. 5B, 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 due to heavy over-expression of channels. In contrast to substitution of D78, substitution in the E425G/D432C background of the directly adjacent E79 revealed currents very similar to those through E425G/D432C-channels (Fig. 5B).

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. 5C); 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 (Fig. 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 H72H73N 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 Ca²⁺ binding. In order to find more direct evidence for an involvement in Ca²⁺ 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. 6A), showing that this mutant channel was H^+ sensitive. Second, we assessed whether an increased Ca²⁺ concentration in the conditioning solution could rescue triple-E63O and triple-D78N channels and render them H⁺-sensitive. This was not consistently the case (not shown). Third, we introduced positive charges at position 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. 6B). 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. 6B), a result expected for a channel with reduced Ca²⁺ 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 E63 and D78 shows that if E63 and D78 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. 7A 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 E425G/D432C was containing 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. 7B and table 1). It was difficult to obtain complete concentration 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 E63, D78, and H72/H73 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 (E425, D432) 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 D432, which is located well within TM2, faces towards the ion pore (7) (Fig. 8); E425 resides at the N-terminal end of TM2 and points in the direction opposite of the ion pore (7). Since the channel has to be unblocked to open, these two amino acids may also contribute to H^+ gating. Combined substitution of E425 and D432 results in channels that are still gated by H^+ , albeit with slightly changed characteristics (21). Hence, although this Ca²⁺ binding site may contribute to H^+ gating of ASIC1a.

With the aim of identifying amino acids contributing to the postulated second Ca²⁺ 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 Ca²⁺ binding site. We found that single substitutions of two amino acids (E63, D78) in ASIC1a, which already contains substitutions of E425 and D432 (triple mutant channels), render the channel largely H^+ insensitive (Fig. 2 and 5). This H^+ -insensitivity was not due to reduced surface expression of the mutated channels (Fig. 5), suggesting a specific effect on gating. These results are consistent with the idea that E425 and D432 contribute to H^+ gating and suggest that also E63 and D78 are involved in H^+ gating. E63 and D78 are highly conserved among ASICs (Fig. 5A). Our study shows that individual substitution of these amino acids does not strongly affect H^+ gating. Similarly, substitution of D78 in ASIC3 has no effects on gating of this ASIC (29). But we show that combined substitution of D78 and E425/D432 severely impairs H⁺ gating. Substitution of the adjacent E79 did not have such an effect, demonstrating the specificity of the effect; E79 seems to be specifically involved in desensitization (29). Two recent studies confirm the importance of E63 and D78: combined substitution of amino acids at the positions corresponding to E63 and D78 causes a dramatic reduction of peak current amplitude of H⁺sensitive zASIC4.1 (26) and substitution of D77 in rat ASIC2a (corresponding to D78 in rat ASIC1a) abolishes H^+ -sensitivity of this ASIC (30).

Since individual substitution of E63 and D78 did not impair H^+ gating of ASIC1a, comparable to the individual substitution of E425 and D432, it was possible that E63 and D78 contributed to a second Ca²⁺ binding site that is redundant to the E425-D432 site and that therefore is also dispensable for H^+ gating. Only the combined elimination of both Ca²⁺ 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 threedimensional structure, the residues corresponding to E63 and D78 (N64 and D79) are not in close contact with each other (7) (Fig. 8), making it unlikely that they contribute to a common Ca²⁺ binding site. N64 is at a similar height as D433 (corresponding to D432 in ASIC1a) within the transmembrane domains (Fig. 1 and 8) with a variable spacing between the side chains ranging from under 7 Å to over 11 Å (7) due to the asymmetry in the TM region. It is not inconceivable that these residues directly interact with each other since 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. Since 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 H72H73N double mutant. The most parsimonious explanation is that H72 and H73 constitute the switch on which H⁺ act to open ASIC1a – they may be the H⁺ sensor. H⁺ insensitivity of the H72H73N mutant, its presence on the cell surface, its contribution to heteromeric channels without apparent contribution to H⁺ gating of these channels are all characteristics consistent with the interpretation that H72H73 constitute the H⁺ sensor. Consistent with this interpretation, His-73 is also essential for H⁺ gating of ASIC2a (24).

In the desensitized state of the channel, the side chain of H73 is in close contact (average distance of ~ 3.2 Å, with individual values ranging from 2.9 to 3.5 Å) with D78 of an adjacent subunit (7). Taking into account the low pH of 5.6 during crystallization this interaction presumably represents an ion pair. Since the neighboring E79 undergoes strong conformational changes during desensitization (29), it is unlikely, however, that a similar contact is made also in the closed conformation. Perhaps H73 gets protonated upon channel opening and rotates during desensitization to electrostatically interact with D78. Since H73 can be substituted without loss of channel function (Fig. 3), it seems that in ASIC1a H72 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 Ca²⁺ ions from (at least) two different Ca²⁺ 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 (β 1) 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 trigger the movement of the thumb (Fig. 9).

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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. 5A). 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 D78 stabilizing the desensitized state of the channel.

In summary, whereas the role of E63 remains uncertain, our results suggest a crucial role for the intersubunit H73-D78 pair in H⁺ gating of ASICs.

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FOOTNOTES

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¹The abbreviations used are: ASIC, acid-sensing ion channel; HA, hemagglutinin; RLUs, relative light units; TM, transmembrane domain.

FIGURE LEGENDS

Fig. 1. Sequence alignment highlighting the amino acids substituted in this study. The amino acid sequences of the extracellular loops of rat ASIC1a, 2a, 3 and 4 are aligned. Numbering refers to ASIC1a. Amino acids that have been exchanged in this study are marked by a black asterisk; amino acids whose substitution impaired H^+ activation are highlighted by an orange arrow head and amino acids from the acidic pocket by a mauve arrow. The extracellular loop of ASIC1a contains 56 aspartate (D) or glutamate (E) residues (blue boxes); E425 and D432 (orange arrow head) mediate open channel block by Ca^{2+} (21). 20 of the remaining residues are completely conserved among ASIC1a, ASIC2a, ASIC3 and ASIC4 (category I). 5 are conserved among ASIC1a, ASIC2a and ASIC3 (category II) and further 7 are conserved only among ASIC1a and ASIC3 (category III). Finally, 15 D or E residues are present in ASIC1a but not in ASIC3 (category IV) and 7 exist exclusively in ASIC1 (category V). We engineered 31 ion channels carrying individual substitutions: we substituted all category I and II residues, 5 out of 7 category III residues, and from category IV we included D202 in our screen because ASIC3 also has a titratable amino acid, histidine (H), at this site. In addition, the extracellular loop of ASIC1a contains 7 H residues (green boxes), and 38 K or R residues (red boxes). 2 of the histidines are not conserved in ASIC3 and are therefore weak candidates for a conserved H⁺ sensor; we individually substituted the 5 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 R175).

Fig.2. Effect of substituting acidic candidate residues. Left, Bars represent mean \pm SEM of peak current amplitudes induced by saturating H⁺ concentrations. Test solutions contained 1.8 mM Ca²⁺ and 1.0 mM Mg²⁺; V_{hold} = -70 mV. Bars to the left represent wild-type channel or mutant channels carrying the indicated single substitutions. Bars to the right represent E425G/D432C double mutant channel or

triple mutant channels carrying E425G/D432C plus the indicated additional substitution. Labels [I] - [IV] indicate the degree of conservation of respective residue among ASIC subtypes. n.d., not determined. **Right**, Symbols and bars represent mean \pm SEM of pH values for half-maximal H⁺ activation. Numbers of recordings from different oocytes are indicated on the left side for 0.1 mM Ca²⁺ and on the right side for 1.8 mM Ca²⁺ / 1.0 mM Mg²⁺. The gray bars represent EC₅₀ values for the wild-type (light gray, 0.1 mM Ca²⁺; dark gray, 1.8 mM Ca²⁺ / 1.0 mM Mg²⁺). **Bottom**, Explanation of color-code and symbols.

Fig. 3. Effect of substituting basic candidate residues. Left, Bars represent mean \pm SEM 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 Ca²⁺ and 1.0 mM Mg²⁺; V_{hold} = -70 mV. Labels [I] - [III] indicate the degree of conservation of respective residue among ASIC subtypes. **Right**, Symbols and bars represent mean \pm SEM of pH values for half-maximal H⁺ activation. Numbers of recordings from different oocytes are indicated on the left side for 0.1 mM Ca²⁺ and on the right side for 1.8 mM Ca²⁺ / 1.0 mM Mg²⁺. The gray bars represent EC₅₀ values for the wild-type (light gray, 0.1 mM Ca²⁺; dark gray, 1.8 mM Ca²⁺ / 1.0 mM Mg²⁺). **Bottom,** Explanation of color-code and symbols.

Fig. 4. The K211M mutant is present on the oocyte surface. Surface expression of HA-tagged ASIC1a and ASIC1a-K211M (mean \pm SEM). Untagged ASIC1a served as a control (first column). Results are expressed as RLUs/oocyte/sec. n = 16; *, p < 0.05.

Fig. 5. Characteristics of channels with substitutions of residues E63, H72/H73, and D78. (A) Alignment of the amino acid sequences of the proximal ectodomain of several ASICs. ASICs with their names in green are H⁺-sensitive, 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 gray background. For comparison, also the sequence of proton-insensitive BLINaC is shown at the bottom. (B) Representative current traces of ASIC1a, ASIC1a-E63, ASIC1a-D78, and ASIC1a-E79 (upper row) and, for comparison, of the respective channels carrying in addition the double substitution E425G/D432C and of ASIC1a H72H73N (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 H72H73N (amount of cRNA injected per oocyte was as indicated in Fig. 2 and 3). H⁺ activation of the triple-E79 was unaffected. Dashed lines represent zero current level. (C) Surface expression of HA-tagged ASIC1a, triple-E63, triple-D78, and ASIC1a-H72H73N (mean ± SEM). Untagged ASIC1a served as a control (first column). Results are expressed as RLUs/oocyte/sec. n = 14-16; **, p < 0.01.

Fig. 6. Substitution of E63 or D78 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 pH₅₀ 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.

Fig. 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 acids 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).

Fig. 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 (PDB 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 which 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 H73 and D78 are indicated by a dashed line. H72 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, E63 has been introduced at the position of N64. (C) View onto the TM domain of trimeric ASIC1 roughly perpendicular to the lipid bilayer. Polar residues within the helices together with the mutated H72 in the loop between TM1 and β 1 are shown.

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

Table 1. Properties of heteromers between ASIC2a and different ASIC1a variants. Data are mean \pm SEM for the number *n* of individual oocytes indicated in parentheses. pH values at which channels were half-maximally activated or desensitized (pH₅₀) were obtained from a fit to the Hill function. n.d., not determined. *, *p* < 0.05; **, *p* < 0.01 (unpaired t-test); for statistical analysis of τ_{des} , mutants and 2a were compared; for statistical analysis of pH₅₀, 2a/1a-H72H73N was compared with 2a/1a and 2a/1a-t(riple)-E63Q(D78Q) with 2a/1a-E425G/D432C, respectively.

	τ_{des}/ms	pH ₅₀ (act.)	pH ₅₀ (desens.)
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-H72H73N	1660 ± 110 (14)**	4.0 ± 0.1 (10)**	6.68 ± 0.04 (5)
2a/1a-E425G/D432C	990 ± 100 (11)**	5.3 ± 0.1 (11)	6.93 ± 0.01 (14)
2a/1a-t-E63Q	920 ± 80 (12)**	4.3 ± 0.1 (12)**	6.82 ± 0.05 (7)*
2a/1a-t-D78N	880 ± 60 (11)**	4.5 ± 0.04 (11)**	6.58 ± 0.02 (10)**

	* *	<u> </u>	75 I **	85 I	95 *	105 I *	115	125 * I	135
ASIC1a	VCT <mark>ER</mark> Ý	QYYFCY <mark>H</mark> H	V T <mark>K</mark> LDEVA	ASQLT-FPAV	TLCNĹN <mark>E</mark> F <mark>R</mark> F	SQV S <mark>K</mark> NDLY <mark>I</mark>	<mark>I</mark> A G <mark>E</mark> LLALLNN	RYEIPDTQMA	DE <mark>k</mark> qĹ
ASIC2a	ESS <mark>ER</mark> V	SYYFSYQH	IV TKV <mark>DE</mark> VV	AQSLV-FPAV	TLCNLNGF <mark>R</mark> F	SRLTTNDLY	AGELLALLDV	NLQIPDPHLA	DPTVL
ASIC3	QVA <mark>ER</mark> V	RYYGEFHH	IKTTL DE RE	SHQLT-FPAV	TLCNINPL <mark>R</mark> R	SRLTPNDLHV	VAGTALLGLDP	'-AE	HAAYL
ASIC4	QAASLA	RGYLTRPH	LVAMDPAA	PAPVAGFPAV	TLCNINRF <mark>R</mark> H	SALSDADIF	LANLTGLPPK	.DRD	-GHRA
	1	45	155	165	175	185	195	205	
								GAHGAELLTT	
					KSCNESCHHC				
	AGER	11 21			KJCN Julie		Kernik	ADI QUULI DI	
	215 🍷 🛨	225	235	245	255 ±	265	275	285	
	GNGLEIMLD		WWGETDE T	SFEAGIKVOI	HSODEPPFID	OLGFGVAPG	OTFVSCOEOR	LIYLPSPWGT	CNAVT
	GNGLEIMLD	IQQDEYLF	PIWGETEE T	TF <mark>E</mark> AGV <mark>K</mark> VQI	HSQSEPPFIQ	ELGFGVAPG	OTEVATOEOR		CRSSE
	GNGLEIMLD	VQQEEYLF	YIWK <mark>DME</mark> ET	PFEVGIRVQI	HSQDEPPAID	QLGFGAAPGH	IQTFVSCQQQQ	LSFLPPPWGD	CNTAS
	GSGL <mark>E</mark> IMLD	IQQEEYLF	YIWRETNE T	SFEAGIRVQI	HSQEEPPYIH	QLGFGVSPGF	QTFVSCQKQR	LTYLPQPWGN	CRAES
	205			205	015	205	005	345	055
	* *			1 <u>*</u>	* *	* *	**	• •	**
	-MDSDFFD-		S	YSITACRIDO	ETRYLVENCN	CRMVHMPGD4	APYCTPEQY <mark>K</mark> E	CADPALDFLV	E <mark>KD</mark> QE
	-MGLDFFP-		V	YSITACRIDO	ETRYIVENCN	CRMVHMPGDA	APFCTPEQHKE	CAEPALGLLA	EKDSN
		EPSDPLGS	PRPRPSPP	YSLIGCRLAC	ESRYVARKCG	CRMMHMPGNS	SPVCSPQQY <mark>K</mark> D		R <mark>KD</mark> I-
	KLREPELQG		YSA	YSVSAC <mark>R</mark> LRC	EKEAVLQRCH	CKMVHMPGNE		CADHILDSLG	GGSEG
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									TG
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		TTRYAKEI		A SARYI ARKY	NRSESYTTEN				TG
	PCFCPTPCN	LTRYGKET	SMVKIPNR	GSARYLARKY	NRNETYIREN	FLVLDVFFEA		AYGLSALLGD	LG
	Category		ategory						
	Calegory		Jucyory						

Category I	Category I
Category II	Category II
Category III	Category IV/V
Category IV/V	

Figure 1

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н				E238Q [I]		F			6/8		Ю. Ю	H	9/
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Figure 2



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Figure 3

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Figure 4

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Α
<u>63</u> 72/73 78
LCVCTERVQYYFCYHHVTKLD-EVAASQLTFPAVTLCN 1a-rat
ALVCTNRIQYYFLYPHVTKLD-EVAATRLTFPAVTFCN 1-chicken
<pre>ALVCVDRVQFYFQYPHVTKLD-EVAAPLMTFPAVTFCN 1.1-toadfish</pre>
MYVCIDRIQFYLEYPHVTKLD-EITTPVMVFPAVTICN 1.2-zebrafish
<pre>LCQVGDRVAYYLSYPHVTLLD-EVATTELVFPAVTFCN 1b-rat</pre>
LLQVVDRVIYYLQYDYVTLLD-ERNAKNMTFPAITLCN 1.1-zebrafish
MYVCMDRVYYYFEFPHVTKLD-EVAAPNLTFPAVTFCN 1.3-zebrafish
LVESSERVSYYFSYQHVTKVD-EVVAQSLVFPAVTLCN 2-rat
LYQVAERVRYYGEFHHKTTLD-ERESHQLTFPAVTLCN 3-rat
LYQAAKCAISYLEHPHVTALN-EEATPEMVFPAVTICN 4.1-zebrafish
CIWODINKIRILLOIPVIIKLII-MIWAKNEORVEDAVIICN IG-SHORK
TTWSCNPTLVINSYDAVTKTN_MIWSHNISEOAVTECN 1 2-toadfish
ISWEENDLINW CEDENTRY DEWEDOL DEDAVTYON 26 not
ISWSSINGLINUSEPSOTRUGENEWSKULPEPAVIVON 20-Pac
LYVAADLAKGYLIKPHLVAMUPAAPAPVAGPPAVILCN 4-rat
LIVAIWSAAIILEKPHLAALK-ELIKKELIFPAIILON 4.2-Zebratish

....VWQIYSRLVNYFMWPTTTSIEVQYVEKI-EFPAVTFCN... BLINaC-cds







Figure 5

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Figure 6

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Figure 7

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Figure 8



Figure 9

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