Calcium regulates acid-sensing ion channel 3 activation by competing with protons in the channel pore and at an allosteric binding site

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The extracellular Ca2+ concentration changes locally under certain physiological and pathological conditions. Such variations affect the function of ion channels of the nervous system and consequently also neuronal signaling. We investigated here the mechanisms by which Ca2+ controls the activity of acid-sensing ion channel (ASIC) 3. ASICs are neuronal, H+-gated Na+ channels involved in several physiological and pathological processes, including the expression of fear, learning, pain sensation and neurodegeneration after ischaemic stroke. It was previously shown that Ca2+ negatively modulates the ASIC pH dependence. While protons are default activators of ASIC3, this channel can also be activated at pH7.4 by the removal of the extracellular Ca2+. Two previous studies concluded that low pH opens ASIC3 by displacing Ca2+ ions that block the channel pore at physiological pH. We show here that an acidic residue, distant from the pore, together with pore residues, controls the modulation of ASIC3 by Ca2+. Our study identifies a new regulatory site in ASIC3 and demonstrates that ASIC3 activation involves an allosteric mechanism together with Ca2+ unbinding from the channel pore. We provide a molecular analysis of a regulatory mechanism found in many ion channels.

1. Introduction

Intracellular Ca2+ is an established secondary messenger. The regulatory role of extracellular Ca2+ is less widely known. Extracellular free Ca2+ concentrations are locally decreased during high neuronal activity or seizures and in ischaemia [1–3]. These changes can have a strong effect on neuronal excitability [4], since the extracellular Ca2+ concentration affects the properties of many neuronal ion channels [5–8]. In the present study, we investigated the molecular mechanism by which the activity of one such Ca2+-sensitive channel, the acid-sensing ion channel (ASIC) 3, is controlled by extracellular Ca2+. ASICs are H+-gated, Na+-conducting ion channels of the nervous system [9–11]. Extracellular acidification leads to a rapid activation of the ASIC current. This current is transient in the continued presence of a low pH solution, because the channels enter a non-conducting, desensitized state after opening [9,10,12]. Functional ASIC channels are formed by the trimeric assembly of identical or homologous subunits. In rodents, six ASIC subtypes encoded by four genes are known [9,10]. Of these, ASIC1a contributes the high pH sensitivity of ASICs of the CNS [13,14], while ASIC3 is the most important ASIC subtype in the PNS [15].
The shape of an ASIC subunit is comparable to that of a hand holding a small ball, with the forearm being the transmembrane segment; the extracellular domains have accordingly been named as finger, knuckle, palm and thumb [16] (figure 1a). The ectodomain is composed of a central scaffold made up by the palm and the knuckle. The thumb and finger contain several α helices and are oriented towards the outside of the protein. The ASIC trimer contains two vestibules located along the central vertical axis between the three subunits, the ‘extracellular vestibule’, situated directly above the pore entry, and the ‘central vestibule’, enclosed by the β sheets of the lower palm domains (figure 1a). The ‘acidic pocket’, present three times in the trimer, is located at about 40 Å above the pore, enclosed by the finger, thumb and β-ball domains of one subunit, and the palm of a neighbouring subunit [16–19].

The ASIC pH dependence is inversely correlated with the extracellular Ca²⁺ concentration, suggesting that there is a competition between H⁺ and Ca²⁺ ions for common binding sites [8,20]. Changing of the extracellular medium at pH7.4 to a nominally Ca²⁺-free solution at the same pH induced an inward current in ASIC3 [8,21]. Based on the dependence of the macroscopic currents on the Ca²⁺ and H⁺ concentrations and the observation that unitary current amplitudes were decreased by high Ca²⁺ concentrations, it was concluded that acidification activates ASIC3 by a displacement of Ca²⁺ ions bound to the pore entry at neutral and alkaline pH, without conformational changes [8]. A recent study with ASIC3 showed that if the residue Glu435 in the pore region of ASIC3, which is not conserved in ASIC1a, was mutated, the current induced by Ca²⁺ removal at pH7.4 was strongly decreased, further supporting the hypothesis that the competition between H⁺ and Ca²⁺ occurs in the ASIC3 pore. The E435A mutation did, however, only partially suppress the low Ca²⁺-induced current. In the present work, we used several complementary approaches to determine the contribution of sites in and outside the ASIC3 pore to the gating control by Ca²⁺. We show that in addition to the previously

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**Figure 1.** Competition between H⁺ and Ca²⁺ on ASIC3. (a) Structural image of an ASIC3 trimer, showing one subunit with domain-specific colouring and the other two subunits in grey. The domains are labelled on the coloured subunit. The ASIC3 model is based on the open structure of chicken ASIC1a [17]. (b–h) Data are from Xenopus oocytes expressing ASIC3 WT, obtained by two-electrode voltage clamp to −60 mV. The conditioning solution at pH7.4 (= the solution perfused between stimulations) contained 2 mM Ca²⁺ except where noted; the Ca²⁺ concentration in the stimulation solutions was as indicated. (b) Representative current traces showing ASIC3 activation by lowering of the pH (i) and by lowering of the Ca²⁺ concentration (ii). (c) Ratio of the current induced by the two approaches described in (b). Ratios were calculated in individual cells, n = 13. (d) pH dependence of activation (filled circles) and of SSD (open squares). The protocols are described in the methods. The Ca²⁺ concentration was generally 2 mM. For the activation curves, the (free) Ca²⁺ concentration of the stimulation was as indicated in the figure; for SSD curves, the Ca²⁺ concentration of the conditioning solution was as indicated in the figure; n = 12–16. The inset shows a kinetic scheme of ASIC3 with the three functional states closed (C), open (O) and desensitized (D). The transitions corresponding to activation and SSD are indicated by arrows. (e) Current ratios measured in individual cells, of I_{pH5.0}/I_{pH7.4} obtained at 80 nM free Ca²⁺ (red circles), and of the I_{Ca²⁺-free}/I_{pH7.4} Ca²⁺ at pH5 (red-black squares), n = 10. (f) Activation pH dependence measured at different free Ca²⁺ concentration in the stimulation solution, as indicated; n = 8–14. (g) Plot of the pH50 as a function of the free Ca²⁺ concentration in the stimulation solution, based on (f). (h) Inhibition of pH6.6-induced current by Ca²⁺. The conditioning pH was 7.4 at a Ca²⁺ concentration of 2 mM. Channel activation was induced by changing to a solution at pH6.6 at the indicated free Ca²⁺ concentration for 10 s once every min, n = 10.
identified Glu435 in the pore entry, a residue in the acidic pocket and an acidic residue deeper down in the channel pore contribute to the control of ASIC activation by Ca\(^{2+}\). Protons activate ASIC3 therefore by binding to H\(^+\)-sensing residues that lead, via conformational changes, to the opening of the pore and by displacing Ca\(^{2+}\) bound into the pore entry.

2. Results

2.1. Lowering of the Ca\(^{2+}\) concentration shifts the pH dependence of acid-sensing ion channel 3

When ASIC3 was expressed in *Xenopus laevis* oocytes and its function was measured by two-electrode voltage clamp, extracellular acidification to pH5 resulted in large, transient inward currents (figure 1a). If the pH was kept at 7.4, and the free Ca\(^{2+}\) concentration was lowered from 2 mM to 80 nM, a transient current of similar amplitude as that induced by the acidification was measured (figure 1b; measurement from the same oocyte), as shown previously [8,21]. In this series of experiments, the peak current amplitudes induced with pH5/2 mM Ca\(^{2+}\) and pH7.4/80 nM Ca\(^{2+}\) were roughly equal (figure 1c). *Xenopus laevis* oocytes express endogenous connexin hemichannels that are activated by lowering of the extracellular Ca\(^{2+}\) concentration [22]. Control experiments showed that under our measuring conditions, the endogenous low Ca\(^{2+}\)-activated currents had a much smaller amplitude (less than or equal to 93 ± 50 nA, n = 29) and slower kinetics than the ASIC currents and did therefore not interfere with the ASIC current measurements (see Methods; electronic supplementary material, figure S1). To examine whether the low Ca\(^{2+}\)-induced current can be explained by a changed pH sensitivity of ASIC3, the pH dependence of activation was determined at the two Ca\(^{2+}\) concentrations 2 mM and 80 nM. The pH dependence of ASIC activation was measured by stimulating the channels with a series of solutions of increasingly acidic pH. Fitting the pH dependence curves obtained at 80 mM free Ca\(^{2+}\) and at 2 mM Ca\(^{2+}\) yielded pH values of half-maximal activation (pH\(_{50}\)) of 7.48 ± 0.14 (n = 62) and 6.14 ± 0.11 (n = 42), respectively, for the two Ca\(^{2+}\) concentrations, confirming therefore a strong alkaline shift of the ASIC3 pH dependence upon lowering of the Ca\(^{2+}\) concentration (figure 1d). According to the activation curve, pH7.4 induces in the 80 nM Ca\(^{2+}\) condition 64 ± 0.8% of the maximal current achieved at this Ca\(^{2+}\) concentration (n = 10, figure 1c, red circles). By contrast, in the 2 mM Ca\(^{2+}\) condition, no current was measured at pH7.4 (figure 1d). In addition to its effects on pH dependence, Ca\(^{2+}\) can, at millimolar concentrations, inhibit ASICS by a pore block mechanism [8,23]. When pH5-induced current amplitudes were compared between the two Ca\(^{2+}\) concentrations, the current ratio I\(_{pH5}/2\) mM Ca\(^{2+}\)/I\(_{pH5}\) (80 nM Ca\(^{2+}\)) was 0.65 ± 0.15 (n = 10, figure 1c, red-black squares), indicating that there is an approximately 35% inhibition of the maximal peak current amplitude at pH5 by 2 mM Ca\(^{2+}\). The similar amplitudes of the low Ca\(^{2+}\) (pH7.4/80 nM Ca\(^{2+}\))- and low pH (pH5/2 mM Ca\(^{2+}\))-induced currents (figure 1b,c) are therefore due (i) to the shift in pH dependence of activation leading to an increase of the current amplitude in the low Ca\(^{2+}\) condition of approximately 65% of the maximal amplitude (figure 1d) and (ii) to the 35% pore block by 2 mM Ca\(^{2+}\) at pH5. Lowering of the extracellular Ca\(^{2+}\) concentration also shifts the pH dependence of the transition from the closed to the desensitized state, termed steady-state desensitization (SSD), to more alkaline values (SSD in the inset of figure 1d). To determine the pH dependence of SSD, ASIC3-expressing oocytes were perfused with conditioning pH solution for 55 s, before the fraction of not-desensitized channels was measured by a step to pH5; this protocol was repeated with increasingly acidic conditioning solutions. In these experiments, the Ca\(^{2+}\) concentration in the conditioning solution was 2 mM in one series, and 0.1 mM in the second series, while the pH of the stimulation solution was 5, and it contained in both series 2 mM Ca\(^{2+}\). The lowering of the Ca\(^{2+}\) concentration in the conditioning solution induced a substantial alkaline shift of the pH dependence of SSD. In the 2 mM Ca\(^{2+}\) condition, the SSD and activation curves did not overlap, while there was a large overlap in the low Ca\(^{2+}\) condition (figure 1d). To avoid channel activation by the conditioning solution, a concentration of 0.1 mM had been chosen for the low Ca\(^{2+}\) condition of the SSD, thus much higher than the 80 nM of the activation experiments. At a Ca\(^{2+}\) concentration of 80 nM this curve would be shifted to more alkaline values, and the overlap would be smaller. To estimate the affinity of the Ca\(^{2+}\) binding site, activation curves at different free Ca\(^{2+}\) concentrations were recorded (figure 1f). The plot of the pH\(_{50}\) values as a function of the Ca\(^{2+}\) concentration (figure 1g) indicates an IC\(_{50}\) of 41 µM. As another measure of Ca\(^{2+}\) binding affinity, the ASIC3 current was determined at pH6.6 at different free Ca\(^{2+}\) concentrations, showing smaller current amplitudes with increasing Ca\(^{2+}\) concentration. The IC\(_{50}\) measured under these conditions was 69 ± 52 µM (n = 11, figure 1b). This IC\(_{50}\) depends on the pH, since there is a competition between Ca\(^{2+}\) and H\(^+\). At pH 7.0 the Ca\(^{2+}\) IC\(_{50}\) was 6.9 ± 6.8 µM (n = 11, electronic supplementary material, figure S2).

2.2. A chimera approach identifies channel domains important for the Ca\(^{2+}\) sensitivity

The observed shift of pH activation curves by changes of the Ca\(^{2+}\) concentration is likely due to a competition for binding sites between H\(^+\) and Ca\(^{2+}\) ions. The identification of the amino acid residues of ASIC3 to which Ca\(^{2+}\) binds would indicate where the competition occurs. As a first approach towards this aim, subdomain-based chimeras between ASIC3 and ASIC1a, which is much less Ca\(^{2+}\)-sensitive, were constructed (figure 2, table 1). Constructs named ‘CHB#’ (where ‘#’ is a number) are based on ASIC3 (blue in figure 2a, left column), and the subdomains indicated in table 1 are replaced by the corresponding sequence of ASIC1a (red in figure 2a). The inverse chimeras (ASIC3 in ASIC1a) are named ‘ICH#’. Most of the constructed chimeras were functional and expressed transient currents (figure 2a; electronic supplementary material, figure S3). The pH\(_{50}\) values of ASIC1a and ASIC3 WT are quite similar at 2 mM Ca\(^{2+}\) but different at 80 mM Ca\(^{2+}\) (figure 2ii,ii). Lowering the Ca\(^{2+}\) concentration from 2 mM to 80 mM Ca\(^{2+}\) induced an alkaline shift of the pH\(_{50}\) values (∆pH\(_{50}\)) of 1.19 ± 0.30 and 0.23 ± 0.22 (mean ± sum of s.d.; n = 12–21), respectively, in ASIC3 and ASIC1a (figure 2ii). Of the ASIC1a-in-ASIC3 chimeras, CHB1, CHB2, CHB4 and CHB8 decreased the Ca\(^{2+}\)-induced ∆pH\(_{50}\) (figure 2bc), suggesting a possible
Figure 2. ASIC3-ASIC1a chimeras identify channel domains important for Ca$^{2+}$ sensitivity. Data are from Xenopus oocytes expressing the indicated ASIC constructs, obtained by two-electrode voltage clamp to −60 mV. (a) Schematic view and representative current traces of chimeras, induced by a pH close to the $p_{H50}$. Left column, chimeras based on ASIC3, in which the parts highlighted in red (table 1) were replaced by the corresponding ASIC1a sequence. On the right of the structural images, representative current traces measured in a stimulation solution containing 2 mM or 80 nM free Ca$^{2+}$ are shown. Right column, chimeras based on ASIC1a, in which the parts highlighted in blue (table 1) were replaced by the corresponding ASIC3 sequence; representative current traces on the right. Empty fields indicate that for a given chimera/condition no current was recorded. (b) Activation curves of ASIC3 WT and the chimera CHB4; $n=10–15$. (c) (ii), $p_{H50}$ values obtained at 2 mM and 80 nM free Ca$^{2+}$ in the stimulation solution for ASIC1a, ASIC3 and the indicated ASIC3-based chimeras; $n=2–21$; (i), the $\Delta p_{H50}$ ($p_{H50,80nM} - p_{H50,2mM}$ Ca$^{2+}$, mean ± s.d.) values are plotted for the indicated constructs. CHB3 and CH3 (in (e)) showed with 2 mM Ca$^{2+}$ irreversible rundown at more acidic pH. Their complete $pH$ dependence is therefore assembled from data from different cells, and one single $p_{H50}$ from the pooled data was determined. (d) Activation curves of ASIC1a WT and the chimera CH8; $n=8–11$. (e) (ii), $p_{H50}$ values obtained at 2 mM and 80 nM free Ca$^{2+}$ in the stimulation solution for ASIC1a, ASIC3 and the indicated ASIC1a-based chimeras; $n=5–24$. (i), the $\Delta p_{H50}$ ($p_{H50,80nM} - p_{H50,2mM}$ Ca$^{2+}$, mean ± s.d.) values are plotted for the indicated constructs. (f) Representative current traces of oocytes expressing the indicated constructs, in response to a lowering of the extracellular Ca$^{2+}$ concentration to 80 nM at pH 7.4. (g) Ratio of the current induced by lowering the Ca$^{2+}$ concentration at pH 7.4 from 2 mM to 80 nM / current induced by acidification from pH 7.4 to pH 5.0 at 2 mM Ca$^{2+}$; $n=7–23$. (c,e,g) *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ’’, $p<0.0001$; ’’, $p=0.089$; different from the corresponding value obtained with WT ASIC1a or WT ASIC3; (g) and (ii) of (c) and (e), as analysed by one-way ANOVA followed by Dunnett’s test; (i) of (c) and (e), based on permutation analysis (see Methods).

Role of the ASIC1a intracellular and transmembrane segments and lower palm (CHB1, CHB2), the palm and knuckle (CHB4), and the upper transmembrane and lower palm parts together with the palm-thumb loops and the thumb (CHB8) in reducing this Ca$^{2+}$ sensitivity. Of the ASIC3-in-ASIC1a chimeras, CH8 showed an increased, and CH3 showed a tendency of increased $\Delta p_{H50}$ ($p=0.089$) relative to ASIC1a WT (figure 2d,e). CH3 contains the upper transmembrane and lower palm parts of ASIC3, whereas CH8 contains the ASIC3 upper transmembrane and lower palm parts together with the palm-thumb loops and the thumb. The currents at 80 nM Ca$^{2+}$ of both CH3 and CH8 were sustained. These currents activated rapidly upon the pH change; their kinetics and amplitude were thus different from the
Ca²⁺ was disrupted. The low Ca²⁺-induced current, normal-
indicating that in these two chimeras, the activation by low
CHB8 was not different from that of non-injected oocytes,
oocytes. Of the ASIC3-based chimeras, CHB2 generated a
Figure 2

endogenous currents (electronic supplementary material,
figure S1). The disruption of the desensitization in these chi-
meras may be due to the sequence changes in the palm,
which controls desensitization. For selected chimeras, it was
tested whether lowering of the Ca²⁺ concentration from
2 mM to 80 nM free Ca²⁺ at pH7.4 induced a current. Figure 2f compares representative current traces of these chi-
meras to those of non-injected or ASIC3 WT-expressing oocytes. Of the ASIC3-based chimeras, CHB2 generated a
slowly increasing current, while the current of CHB4 and
CHB8 was not different from that of non-injected oocytes,
indicating that in these two chimeras, the activation by low
Ca²⁺ was disrupted. The low Ca²⁺-induced current, normal-
ized to the low pH-induced current in the same oocyte (the
IpH7.4(80 nM Ca²⁺) / IpH5(2 mM Ca²⁺) ratio) was high for
CH3 and CH8, as expected, even higher than in ASIC3 (figure 2g). Taken together, the chimera analysis highlights
the importance of the pore and pore entry for the gating by
Ca²⁺, as well as that of the palm, thumb and knuckle.

2.3. MD simulations predict candidate Ca²⁺ binding
sites
To identify candidate Ca²⁺ binding sites, MD simulations
with a structural model of ASIC3 were carried out in the pres-
ence of a high concentration of Ca²⁺ (100 mM). Analyses were
performed to identify the Glu, Asp, Asn, His and Thr amino
acid residues of ASIC3, in whose proximity (i.e. at a distance
of less than 2.5 Å), a Ca²⁺ ion was found during a part of the
simulation. Figure 3a plots the fraction of time a Ca²⁺ was
close to the indicated residues of the transmembrane and
extracellular parts of ASIC3 in two conditions, either without
protonation of acidic side chains (Blue squares), or with a
chosen protonation corresponding approximately to pH5.5
(red circles, Methods). We expect that acidification displaces
Ca²⁺ from sites where Ca²⁺ and H⁺ compete, thus that at
such sites, Ca²⁺ would not be found in the pH5.5 condition.
In MD simulations, Ca²⁺ ions tend to stick to a site once
they have bound. Residues to which Ca²⁺ bound in most of
the experiments are therefore more likely than others to
bind Ca²⁺. The computational analysis led together with
inspection of the ASIC3 structural model to the identification
of several clusters of residues in whose proximity Ca²⁺ ions
were found. In the lower palm domain, at a subunit inter-
action site, Glu81, which had during most of the simulation
time a Ca²⁺ ion bound, is close to Asn421 of the same sub-
unit, and to ‘Asn370 of a neighbouring subunit (figure 3h; residue names of the neighbouring subunit are preceded by
the prefix ‘x). Asp282, located further down just above the β-
turn, had also a Ca²⁺ ion bound during most of the simu-
lation time. Asp78 and Glu426 that also scored high are
farther away. Glu79 and Glu423 of the lower palm are
oriented towards the inside of the central vestibule where
these residues of the three subunits may form a Ca²⁺ binding
site (figure 3c). Glu423 did not score in our analysis but may
be of interest because it has been suggested to bind 2-guani-
dine-4-methylquinazoline, which is known to compete with
Ca²⁺ for binding sites [24,25]. Further up in the central vesti-
bule, Glu380 and Glu418, identified in our screening, may
form an additional binding site in the central cavity. Several
residues were identified in our screening that are part of
the acidic pocket, which has been identified as Ca²⁺ binding
site in ASIC1a [26] (figure 3d). Of these, ‘Glu212, Asp228,
Glu230 and Asp358 all had frequently a Ca²⁺ in their prox-
imity (figure 3d). The structural image shows that Glu230
and Asp358 are oriented towards each other, while ‘Glu121
of a neighbouring subunit is oriented towards Glu235. ‘Asp414 has a similar orientation as ‘Glu212 and is located on an adjacent, antiparallel strand of the same palm β-sheet
(figure 3d). A potentially interesting cluster was identified
at the extracellular pore entry (figure 3e). Glu432 and
Asp439 are homologous to the two Ca²⁺ pore-blocking bind-
ing residues identified in ASIC1a [23]. Glu435 is unique to
ASIC3; ASIC1a contains a Gly residue at the homologous
position. Some residues that scored in our analysis appeared
not to be of interest after inspection of the structural model
since they are located in a loop region that is not well con-
served between ASIC3 and ASIC1a, on which the structural
model is based (electronic supplementary material, figure S4A), or they appeared to be too distant from other acidic
residues (electronic supplementary material, figure S4B,C).

2.4. Functional analysis of mutants identifies the pore
entry and the acidic pocket as likely Ca²⁺ binding
sites for activation
Based on the predictions of the MD simulations and of visual
inspection of the ASIC3 structural model, approximately 30
residues—most of them acidic residues—were mutated to
Ala, and the pH dependence of activation was determined
at the two Ca²⁺ concentrations. Representative current
traces of mutants that showed a difference to WT ASIC3 are
shown at the two Ca²⁺ concentrations, at a pH close to the
pH150 (figure 4b). The pH150 values measured at 2 mM Ca²⁺
were remarkably constant across mutants (black open circles in the figure 4ii). The largest shifts were observed with the
mutations E423A and E426A. E79A produced only a very
small current in these conditions, precluding the analysis of
the pH dependence. The pH150 obtained at 80 nM free Ca²⁺
was more affected by the mutations. Mutation of Glu212,
located in the palm and facing the acidic pocket (figure 3d),
induced an acidic shift of the pH150 (figure 4u,b) and decreased

Table 1. Chimera constructs.

<table>
<thead>
<tr>
<th>Name</th>
<th>introduced subdomains</th>
</tr>
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<tbody>
<tr>
<td>CH(B)1</td>
<td>intracellular termini, transmembrane part</td>
</tr>
<tr>
<td>CH(B)2</td>
<td>lower palm, transmembrane part</td>
</tr>
<tr>
<td>CH(B)3</td>
<td>upper transmembrane, lower palm, palm side of palm-thumb linkers</td>
</tr>
<tr>
<td>CH(B)4</td>
<td>palm, knuckle</td>
</tr>
<tr>
<td>CH(B)5</td>
<td>β-ball</td>
</tr>
<tr>
<td>CH(B)6</td>
<td>thumb, thumb part of palm-thumb linkers</td>
</tr>
<tr>
<td>CH(B)7</td>
<td>finger</td>
</tr>
<tr>
<td>CH(B)8</td>
<td>upper transmembrane part, lower palm, palm-thumb linkers, thumb</td>
</tr>
<tr>
<td>Chx</td>
<td>the listed domains of ASIC3 were introduced in ASIC1a; ChBx, the listed domains of ASIC1a were introduced in ASIC3</td>
</tr>
</tbody>
</table>
the ΔpH50 to 0.84 ± 0.29 (n = 9–12, figure 4ii), compared to 1.34 ± 0.25 in ASIC3 WT (n = 42–62). Mutation of the close residue Asp414 resulted in non-functional channels. If the E212A mutation was combined with the mutation of Glu235, which is oriented towards the β-turn, the ΔpH50 was not further decreased (figure 4ii). The E435A mutation (figure 3c), which was previously shown to decrease the low Ca2+-induced current [21], lowered the ΔpH50 value to 0.77 ± 0.35 (figure 4a,c, n = 11–19), thus similarly to E212A. In ASIC1α, the residues corresponding to Glu432 and Asp439 were identified as binding sites for pore block by Ca2+ [23]. In the E432A mutant, the ΔpH50 was similar to the WT value (figure 4a). The D439A mutant did not produce any transient currents. Combination of the mutations of Glu435 of the pore and of Glu212 of the palm, with or without mutation of Glu235, resulted in a strong reduction of the pH50 shift upon lowering of the Ca2+ concentration, with ΔpH50 values of 0.24 ± 0.20 (E212A/E435A, n = 9–10) and 0.27 ± 0.27 (E212A/E235A/E435A, n = 9–14; figure 4a,c). Comparison with the WT pH dependence (dashed lines in figure 4c) shows that these mutations affected mostly the channel properties in the 80 nM Ca2+ condition, and much less in the 2 mM Ca2+ condition. Other mutants with a significantly lower ΔpH50 than ASIC3 WT, but with smaller effects, were E230A/D358A, E322A and E426A (figure 4a, b). E230 and D358 are located in the acidic pocket (figure 3d), E322 just below, in the α4 helix of the thumb, while E426 is in the wrist (figure 3b). Measurement of the low Ca2+-induced current at pH 7.4 demonstrated a decrease of the IpvH7.4 (80 nM Ca2+) / IpvH52 mM Ca2+) ratio by the single mutations (figure 4d), confirming further the importance of these two sites for modulation by Ca2+. The low Ca2+-induced currents of the mutant E212A/E435A showed a slowly developing sustained current without a clear peak that could not be reliably quantified. The mutational analysis indicates therefore that two distinct Ca2+ binding sites, one in the pore entry and one in the palm/acidic pocket, contribute to the Ca2+-induced modulation of ASIC3 activation, consistent with the analysis of the chimeras.

2.5. Acid-sensing ion channel 3 concatamers highlight the importance of the pore residue Asp439 for modulation by Ca2+

Several mutations resulted in channels that did not express transient currents, among them mutations of Asp439, the residue homologous to a pore-blocking site in ASIC1α. To allow the study of such sites, concatamers were constructed in which residues of interest were mutated only in one or two subunits of the channel trimer. All tested concatamers carrying mutations in one or two subunits, except the one containing the double mutation E435A/D439A in two subunits of the trimer, produced measurable, transient currents. The pH dependence of concatamers carrying the mutations D282A or N421A of the wrist (figure 3e) or D414A of the acidic pocket (figure 3d; ‘A’, ‘AB’ at the end of the name indicates in which subunits of the trimer [A, B, C] a given mutation is present) in one or two of the subunits was not different from that of WT (figure 5a), thus it is unclear why

![Figure 3. Molecular dynamics simulations predict candidate Ca2+ binding sites. (a) Plot of the fraction of time, a Ca2+ ion is closer than 2.5 Å from the indicated residue. Simulations were carried out without the protonation of side chains (blue squares) or at the protonation of side chains estimated at pH5.5 (red circles, using the program propka; see Methods; values are from 3 to 6 subunits). (b–e) Structural images made from a model of ASIC3, based on the open structure of chicken ASIC1α [17]. Focused views of specific regions. One subunit is shown with domain-specific colouring, the other two subunits are shown in grey. (b) Lower palm with intersubunit interaction site above the β-turn; (c) Central vestibule enclosed by the lower palm β-sheets; (d) acidic pocket; (e) lower palm and upper part of transmembrane domains. Residues identified in (a) as potentially involved in Ca2+ binding are shown in (b–e).]
the conventional mutants D282A, N421A and D414A, carrying these mutations in the three subunits, were not functional. The analysis of the concatamers provided the most interesting results in the pore region, where the presence of the mutation D439A or the double mutation E435A/D439A in one subunit of the trimer led to a $\Delta pH_{50}$ of 0.88 ± 0.39 ($n=11–12$, D439A-A) and 0.69 ± 0.40 ($n=9–14$, E435A/D439A-A), respectively, compared to 1.20 ± 0.22 of the WT concatamer ABC (figure 5a–c). In the construct containing the D439A mutation in two of the three subunits, the $\Delta pH_{50}$ was 0.26 ± 0.38 (figure 5a, $n=12–13$), highlighting the importance of Asp439 for the Ca$^{2+}$ modulation of the ASIC3 pH dependence. Representative current traces of concatamers with different activation pH dependence from WT are shown in figure 5b. Consistent with the above observations, the $I_{pH7.4}(80 \text{ nM Ca}^{2+})/I_{pH5.0}(2 \text{ mM Ca}^{2+})$ ratio of the current induced by lowering the Ca$^{2+}$ concentration at pH7.4 from 2 mM to 80 nM / current induced by acidification from pH7.4 to pH5.0 at 2 mM Ca$^{2+}$, $n=9–23$. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; #, $p<0.0001$; different from the corresponding value obtained with WT ASIC3; (d) and (ii) of (a), as analysed by one-way ANOVA followed by Dunnett’s test; (i) of (a), based on permutation analysis (see Methods).
Ca\(^{2+}\)) ratio was low in the concatamers E435A/D439A-A and D439A-AB (figure 5d). This indicates that residues Glu435 and Asp439 contribute together to the Ca\(^{2+}\) binding site in the pore entry.

To test whether the modulation of the pH dependence by Ca\(^{2+}\) depended on the membrane potential, complete pH dependence curves were carried out with ASIC3 WT at either −100 or 0 mV. The pH\(_{50}\) values obtained were not different between these two conditions (figure 5e). To have a more direct comparison than activation curves obtained in different cells, the ratio of the current induced by a pH of the steep range of the pH-current curve, divided by the current induced by a pH generating maximal activation, was measured at −100 and 0 mV at different Ca\(^{2+}\) concentrations in paired experiments in the same cell (figure 5f). This \(I_{pH16.5}/I_{pH4.5}\) ratio is sensitive to changes in the pH dependence. If Ca\(^{2+}\) binding to sites in the electrical field of the pore competes with pH-dependent activation, the \(I_{pH16.5}/I_{pH4.5}\) ratio would be lower at the membrane potential of −100 mV. In contrast with these expectations, the \(I_{pH16.5}/I_{pH4.5}\) ratio was significantly lower at 0 mV in the condition with 5 mM Ca\(^{2+}\) and showed no voltage dependence with 2 and 10 mM Ca\(^{2+}\) (figure 5f). This suggests that the Ca\(^{2+}\) binding site at residues Glu435 and Asp439 is located outside the electrical field of the pore, unlike the binding site of diminazene, which comprises residues corresponding to Gly442, Gly445 and Leu446 in ASIC3, and is closer to the selectivity filter [27,28].

### 2.6. The Ca\(^{2+}\) modulation of steady-state desensitization depends on residues in the acidic pocket and the lower palm

The SSD of the chimeras that generated a transient current were measured at two different Ca\(^{2+}\) concentrations in the conditioning solutions, 2 mM and 0.1 mM. The pH\(_{50}\) shift
induced by the lowering of the Ca²⁺ concentration from 2 to 0.1 mM was 0.27 ± 0.07 with ASIC1a, and 0.40 ± 0.07 with ASIC3 (n = 10–24 (figure 6b–c). The SSD curves showed some shifts in the pH dependence of SSD (figure 6d); however, the ΔpH50 was in all cases except for CH6 close to the parent channel type value (figure 6c). Due to the high variability in the CH6 ΔpH50 values, the CH6 ΔpH50 was not significantly different from that of ASIC1a WT. In the chimera CH6, the thumb and part of the thumb-palm linkers is replaced by the corresponding sequence of ASIC3.

Functional analysis of ASIC3 mutants showed low ΔpH50 values in the mutants E212A, E212A/E235A and E212A/E235A/E435A (figure 6d–f). Mutation of the pore residue Glu435 did not affect Ca²⁺ modulation of the SSD. This analysis identifies therefore E212 and E235 as important for SSD. These residues are located in the acidic pocket and are part of the palm and finger, respectively (figure 3d).

Measurement of concatameric channels showed low ΔpH50 values with N421A-AB and N421A/E423A-AB (figure 6g–i). Because the mutation E423A induced an alkaline shift of the pH50 at both Ca²⁺ concentrations but did not decrease the ΔpH50 (figure 6c,f), the observed decrease of the ΔpH50 with N421A/E423A-AB identifies Asn421 as important for the Ca²⁺ modulation of the SSD. Asn421 is located in the lower palm (figure 3c). To combine the SSD-affecting mutations in the acidic pocket and wrist, we constructed a concatamer containing the mutations E212A and E235A in all three, and N421A in two subunits. This construct expressed only very small currents that were mostly sustained and did therefore not allow us to determine the Ca²⁺ dependence of SSD. Taken together, the analysis with chimeras and two mutagenic approaches identified Glu residues 212 and 235 of the acidic pocket, and Asn421 of the palm as contributing residues to Ca²⁺ modulation of SSD.

**Figure 6.** The modulation of SSD by Ca²⁺ depends on residues in the acidic pocket and the lower palm. Data are from Xenopus oocytes expressing the indicated ASIC constructs, obtained by two-electrode voltage clamp to −60 mV. (a–c) Data from chimeras; (d–f) data from ASIC3 with point mutations; (g–i) data from concatamers. (a,d,g) Plot of pH dependence of SSD for the indicated constructs, obtained with conditioning solutions at a Ca²⁺ concentration of 0.1 or 2 mM; n = 6–19. (b, e,h) Plot of pH50 (pH of half-maximal SSD) values for the indicated constructs, n = 4–26. (c,f,i) ΔpH50 (pH50,0.1mM − pH50,2mM Ca²⁺, mean ± s.d.), n = 4–26. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; different from the corresponding value obtained with the chimera basis (b,c), ASIC3 WT (e,f) and ASIC3 WT-ABC (h,i); (b,e,h) as analysed by one-way ANOVA followed by Dunnett’s test; (c,f,i) based on permutation analysis (see Methods).
Figure 7. Domains and residues involved in the $\text{Ca}^{2+}$ modulation of activation and of SSD. The images were made from a homology model of ASIC3, based on the crystal structure of the open chicken ASIC1a, 4NTW [17]. (a) Domains that were shown to be involved in $\text{Ca}^{2+}$ modulation of activation with one chimera are highlighted. The SSD analysis further showed that the acidic pocket residues are between 1 and 2 mM. During high neuronal activity, this concentration decreases to approximately 0.8 mM or lower [1,29,30]. Following ischaemic stroke, a stronger decrease in $\text{Ca}^{2+}$ concentration (to approx. 0.1 mM) occurs [2]. A heart attack and muscle stress or ischaemia are generally associated with an increased production of lactate. Since lactate is a weak $\text{Ca}^{2+}$ chelator, this results in a lower concentration of free $\text{Ca}^{2+}$ [3]. Thus, free extracellular $\text{Ca}^{2+}$ concentrations change under some conditions, and since $\text{Ca}^{2+}$ and other ligands compete for ion channel binding, this can thereby affect the activity of ASICs and other ion channels.

Lowering of the extracellular $\text{Ca}^{2+}$ concentration shifts the voltage dependence of voltage-gated $\text{Na}^{+}$ channels to more hyperpolarized potentials [5,31]. It was concluded that this effect was due to reduced surface charge screening at lower $\text{Ca}^{2+}$ concentrations [5]. The sodium leak channel (NALCN) is modulated by voltage and by the $\text{Ca}^{2+}$ concentration. It is inhibited by extracellular $\text{Ca}^{2+}$ with an $\text{IC}_{50}$ of 320 $\mu$M at $-80 \text{ mV}$; this inhibition is likely due to a pore block, since it is strongly suppressed by mutations of selectivity filter residues [6]. The calcium homeostasis modulator 1 (CALHM1) is, similarly to NALCN, regulated by voltage and by the extracellular $\text{Ca}^{2+}$ concentration. Reducing the $\text{Ca}^{2+}$ concentration increases the channel open probability [7]. The $\text{IC}_{50}$ for CALHM1 inhibition by $\text{Ca}^{2+}$ is approximately 200 $\mu$M at $-60 \text{ mV}$. Neutralization of an extracellular Asp residue that is not in the permeation pathway was shown to strongly alter the regulation by $\text{Ca}^{2+}$ [7].

Our mutagenesis approach showed that the most important residues for $\text{Ca}^{2+}$ modulation of ASIC3 activation are located in the acidic pocket and the pore entry (figure 7a). This is consistent with the analysis of chimeras indicating a contribution of the transmembrane segments, the thumb and the palm. Mutation of Glu435 in ASIC3 suppressed only partially the low $\text{Ca}^{2+}$-induced current at pH7.4 in the study by Zuo et al. [21] and in our hands. The E435A and E212A mutations inhibited the low $\text{Ca}^{2+}$-induced shift in the activation curve to a similar degree. In concatamers, the presence of the D439A mutation in two of three subunits inhibited the $\text{Ca}^{2+}$ modulation to a higher degree than the single E212A and E435A mutation in the three subunits (i.e. in conventional mutants), underlining the importance of Asp439 for $\text{Ca}^{2+}$ modulation of ASIC3. Of these three residues, both Glu212 and Asp439 are conserved between ASIC3 and ASIC1a; only Glu435 is unique to ASIC3. The different roles of these identical residues in ASIC1a and ASIC3 is therefore likely due to (i) differences in the amino acid residues or conformation in the proximity of these residues that may affect the $\text{Ca}^{2+}$ affinity and/or (ii) a different coupling of these $\text{Ca}^{2+}$ binding sites to ASIC activity.

The mutations that decreased the $\Delta \text{pH}_{50}$ did this mostly by lowering the $\text{pH}_{50}$ at the 80 $\text{nM}$ $\text{Ca}^{2+}$ condition, and with no, or only minor changes of the $\text{pH}_{50}$ at 2 mM $\text{Ca}^{2+}$. Since these mutations disrupt the binding of $\text{Ca}^{2+}$, it would seem more logical that they have a stronger effect at the higher $\text{Ca}^{2+}$ concentration. As a possible explanation for this apparent discrepancy, we hypothesize that the charge of the acidic side chains at the $\text{Ca}^{2+}$ binding sites may allow the high pH sensitivity in the absence of $\text{Ca}^{2+}$. If such acidic side chains are removed by mutagenesis, the channels may lose the possibility of gating with high pH sensitivity.

The analysis of SSD showed that the acidic pocket residues Glu212 and Glu225 are important for the competition between $\text{Ca}^{2+}$ and $\text{H}^{+}$ (figure 7b). The SSD analysis further highlighted the contribution of Asn421 located in the lower
palm, which may form a Ca$^{2+}$ binding site together with Glu79 and Glu423. These residues are also conserved between ASIC3 and ASIC1a.

For ASIC1a, it was shown that mutation of the two residues in the ASIC1a pore that contribute to Ca$^{2+}$ block, corresponding to ASIC3-Glu432 and -Asp439, suppressed the pore block but not the Ca$^{2+}$ modulation of the pH dependence, indicating that the Ca$^{2+}$ binding site for gating is different from that for pore block in ASIC1a [23]. A study with toadfish ASIC1 also found a competition between H$^+$ and Ca$^{2+}$ and concluded, based on a detailed functional analysis, that this channel is activated by an allosteric mechanism and not by a release of Ca$^{2+}$ block [32]. A further indication that in ASIC1a, activation involves conformational changes comes from voltage clamp fluorometry studies that detected conformational changes associated with channel opening [33,34].

At the homologous position to ASIC3-Glu435, ASIC1a contains a Gly residue (Gly430). When the homologous Gly residue in chicken ASIC1a (cASIC1a) was mutated to Glu—in order to create a site favourable for Ca$^{2+}$ binding—the shift of the activation curve induced by lowering of the Ca$^{2+}$ concentration was increased relative to that seen in WT cASIC1a [21]. In this mutant, the removal of extracellular Ca$^{2+}$ activated the channel at pH7.4 [21], indicating therefore that an acidic side chain at this position of ASIC1a has a similar role as does Glu435 in ASIC3. A recent study showed that if ASIC1a-Gly430 was mutated to Cys, exposure to Cys-reactive reagents shifted the pH dependence of activation towards alkaline values, indicating that large side chains at this position facilitate ASIC1a opening [35] in a similar way as lowering the Ca$^{2+}$ concentration shifts the pH dependence of ASIC3.

The original study describing the competition between H$^+$ and Ca$^{2+}$ for the activation of ASIC3 concluded that this competition occurs in the pore entry, based on the good fit by a kinetic model of the H$^+$ and Ca$^{2+}$ dependence of the currents, and the dependence of the unitary current amplitude on the Ca$^{2+}$ concentration [8]. It was proposed that ASIC3 opening is due to unbinding of Ca$^{2+}$ bound to the pore entry, without any conformational changes involved. This conclusion was later supported by the demonstration of the involvement of Glu435 in the Ca$^{2+}$ modulation [21]. A structural analysis of closed and desensitized ASIC1a identified divergent ion binding sites in the acidic pocket and the central vestibule, but not in the pore [26]. In the structural model of closed hASIC1a, the O–O distances of the D439 side chains around the pore were approximately 4 Å [19], thus compatible with a Ca$^{2+}$ binding site [36,37]. In binding sites containing multiple coordinating ligands, Ca$^{2+}$ can bind with reasonable affinity even if it is hydrated. In such cases, the ion is placed 4–5 Å from the interacting side chains [38]. According to the original hypothesized mechanism [8], Ca$^{2+}$ binds into the open ASIC3 pore. The ASIC3 structural model is based on the chicken ASIC1a structure [17]. ASIC1a and ASIC3 have similar Na$^+$/K$^+$ permeability ratios, suggesting that at least at the selectivity filter they have similar pore radii. Assuming that the pore dimensions are correct in the ASIC3 open channel model, the three Asp439 side chains would not be able to coordinate binding of a Ca$^{2+}$ ion between them, because of side chain O–O distances of greater than 12 Å. At the level of Glu435, the intersubunit carboxylic O–O distances are also close to 12 Å. The closest distance between side chain oxygens of Glu435 and Asp439 of the same subunit is 6.9 Å; therefore, it seems also unlikely that Glu435 and Asp439 would together coordinate a Ca$^{2+}$ ion in the open ASIC3 pore. This indicates that in a pore conformation compatible with ion flow through the ASIC3 pore, the Glu435 or Asp439 residues of the three subunits cannot coordinate a Ca$^{2+}$ ion, strongly suggesting that conformational changes in the pore accompany ASIC3 activation.

Based on our functional analysis and the comparison with the ASIC1a open pore dimensions, we conclude that ASIC3 activity is controlled by a hybrid mechanism involving on one hand conformational changes induced by protonation of an allosteric site, and on the other hand a release of Ca$^{2+}$ bound to the pore entry. At binding sites distant from the pore, Ca$^{2+}$ interferes with allosteric gating by competing with H$^+$. The competition for binding sites in the pore leads to release of Ca$^{2+}$ from the pore when the pH is lowered.

4. Methods

4.1. Molecular biology

The rat ASIC3 clone [39] and the human ASIC1a clone [40] and derived mutants and chimeras were subcloned into a pSP65-derived vector containing 5′ and 3′ non-translated sequences of β globin to improve the stability in Xenopus laevis oocytes. The ASIC1a clone used in this study contains a G212D substitution [41]. Mutations were generated by site-directed mutagenesis using the Quikchange approach, with KAPA HiFi HotStart PCR polymerase (KAPA Biosystems). Mutations were verified by sequencing (Synergen Biotech or Microsynth). Chimeras between ASIC1a and ASIC3 were designed based on a sequence alignment and the attribution of subdomains based on [16] and were synthesized by Genscript or Eurofins. The protein sequences of these constructs are provided in the electronic supplementary material, table S1. The concatamer constructs contained three repeats of ASIC3 subunits, A, B and C. These repeats contained the mutation L529A that had been shown to increase the current by approximately threefold without changing other functional properties of the channel [42]. The linker between repeats A and B was composed of the sequence QQASQQ, while the linker between repeats B and C contained the sequence NNNNTSNNN. The presence of a HindIII and a SpeI restriction sites in the A-B and B-C linkers, respectively, allowed the subcloning of mutant repeats at the desired positions. The concatameric constructs were cloned in the oocyte expression vector pSDeasyBS with the EcoRI and XbaI sites. The concatamers were synthesized in part or completely by Genscript. The complete coding sequence of chimeras and concatamers was verified by sequencing (Synergen Biotech or Microsynth).

4.2. Oocytes and electrophysiological measurements

Female Xenopus laevis frogs were anaesthetized with 1.3 g L$^{-1}$ MS-222 (Sigma-Aldrich). For the extraction of oocytes, a small incision (approx. 1 cm) was made on the abdominal wall. The procedures with the Xenopus laevis frogs were approved by the veterinary office of the canton de Vaud. Healthy stage V and VI oocytes were isolated and incubated with
collagenase to isolate and defolliculate the oocytes. Oocytes were injected with 50 nL of cRNA at a concentration of 20–500 ng μL−1. During the protein expression phase, the oocytes were stored in modified Barth’s saline containing (in mM) 85 NaCl, 1 KCl, 2.4 NaHCO3, 0.33 Ca (NO3)2, 0.82 MgSO4, 0.41 CaCl2, 10 HEPES and 4.08 NaOH at 19°C.

It has been reported that Xenopus oocytes express endogenous connexin hemichannels, and that lowering of the extracellular Ca2+ concentration can induce inward currents [22]. Control experiments with non-injected oocytes showed that switching at pH 7.4 from a solution containing 2 mM Ca2+ to a solution containing 100 nM free Ca2+ induced a slowly developing inward current that still increased at the end of the 10 s solution change (electronic supplementary material, figure S1A). Ca2+ was previously shown to inhibit these endogenous currents with an IC50 of approximately 100 μM at ~60 mV [43]; therefore, the inhibition is not different between the 100 nM used in these control experiments and the 80 nM used with ASIC3.

Interestingly, the amplitude of the endogenous currents was smaller at pH 6 and was inhibited with a pH50 of 5.41 ± 0.41 (n = 6); electronic supplementary material, figure S1A, B). The peak of ASIC currents appears normally within the first second after solution change (see e.g. figure 1b). The amplitude of the current induced in non-injected oocytes by lowering of the Ca2+ concentration to 100 nM at pH 7.4, measured at 3.5 s after the solution change (which would be after the ASIC peak in ASIC-expressing oocytes) was −93 ± 50 nA (n = 29, from three different batches of oocytes; electronic supplementary material, figure S1C). To estimate the consequences of the presence of endogenous low Ca2+-activated currents for the analysis of the ASIC3 pH dependence, pH dependence curves were generated in silico with Hill equations describing the pH dependence of the ASIC3 WT and of endogenous low Ca2+-activated currents (I = Imax/(1+(IHC50/10−pH/nH)^nH)), where Imax is the maximal current amplitude, pH50 is the pH inducing 50% of the maximal current amplitude and nH is the Hill coefficient). For ASIC3 WT, the pH50 and nH were 7.48 and 2.08, respectively, as determined in figure 1d. For the endogenous currents, the fit parameters determined in the electronic supplementary material, figure S1B were used. Activation curves were generated for pure ASIC3 and for conditions in which the endogenous current contributed 5, 10, 15 or 20% to the total maximal peak current amplitude (electronic supplementary material, figure S1D,E). As expected from the opposite pH dependence of the two current types, contamination by endogenous currents affected the resulting pH dependence mostly at pH > 7.3. An endogenous current amplitude corresponding to 10% of the maximal total peak current induced for example an alkaline shift of the pH50 value of 0.07 pH units and a decrease of nH from 2.08 to 1.57. With the measured amplitude of the endogenous current of 93 nA, such an error would be expected for ASIC currents with a maximal peak amplitude of 0.93 μA. Of the ASIC constructs used in this study, the maximal peak current in activation curves with 80 nM Ca2+ was 0.92 μA for the chimera CHB6, 1.84 μA in the concatamer N421A/E423A-AB, and greater than 2 μA for all other constructs (electronic supplementary material, table S2), indicating therefore that the endogenous currents did not affect the measurement of the ASIC pH dependence. The current traces of experiments with low ASIC current amplitudes at 80 nM Ca2+ were visually inspected and were only used if at pH conditions of greater than or equal to 7.3, the amplitude of the slowly activating endogenous current was less than 5% of the maximal low pH-induced current amplitude. For SSD experiments, the conditioning solution contained either 2 mM or 0.1 mM Ca2+. If at pH 7.4, the Ca2+ concentration was lowered during 10 s to 0.1 mM, the current amplitude relative to the baseline at pH 7.4/2 mM Ca2+ was 15 ± 9 nA at pH 7.4 and 12 ± 8 nA at pH 8.5, thus negligible (n = 8, electronic supplementary material, figure S1F).

Electrophysiological recordings were performed 1–3 days after cRNA injection as described [44]. The currents were recorded using the two-electrode voltage clamp technique at a holding potential of ~60 mV unless noted differently. Recordings were carried out with a Dagap TEV200 amplifier (Minneapolis, MN) equipped with two bath electrodes, using either the Clampfit or FitMaster software. The recording solution contained (in mM): 110 NaCl, 10 HEPES for pH ≥ pH 16.8 (MES for pH < 6.8) and the appropriate Ca2+ concentration. The pH was adjusted using NaOH or HCl. For Ca2+ concentrations greater than or equal to 0.1 mM, the Ca2+ was provided as CaCl2 and no Ca2+ chelator was added. For Ca2+ concentrations less than 0.1 mM, Ca2+ chelators at a concentration of generally 10 mM were used and total Ca2+ concentrations were chosen based on the MaxChelator program (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/webmaxc5.htm) to obtain the desired free Ca2+ concentration. Depending on the pH and the free Ca2+ concentration, EDTA, EGTA or citrate were used as chelators. Once placed in the recording chamber, the oocyte was impaled with two glass electrodes that had a resistance of less than 0.5 MΩ when filled with 1 M KCl. Oocytes were perfused with experimental solutions by gravity at a flow rate of 8–12 mL min−1. The cFlow 8 channel flow controller (CellMicroControls) together with an eightfold perfusion head was used to change solutions. Currents were filtered at 2 kHz and the sampling interval was 20 ms. The oocytes were exposed once per minute for 5–15 s (according to the specific protocol used) to the stimulation solution. The pH activation curves were fit to the Hill equation that is described above. An analogous equation was used to fit the SSD curves.

4.3. Computational analysis
The structural model of ASIC3 used here was created with SWISS-MODEL [46] based on the open chicken ASIC1 structure (4NTW [17]). Molecular simulation systems were composed of an ASIC3 protein trimer surrounded by about 360 POPC lipid molecules forming a bilayer. This protein/membrane complex is solvated by about 36 000 water molecules and 100 mM of Ca2+, to which Na+ and Cl− ions are added to neutralize the whole system and mimic a salt concentration of about 150 mM. Following an approach developed by Woolf & Roux [47], simulation systems were first equilibrated with a set of gradually decreasing restraints using the CHARMM software. Molecular dynamics simulations were then performed with the NAMD package [48].

As we were interested in potential Ca2+ binding sites which are in competition with protonation of side chains, we ran two simulations with all acidic side chains deprotonated
(all histidines were also neutral) for a total of 520 ns of simulation time. One of these two simulations additionally had a transmembrane potential of 200 mV to favour the entry of Ca$^{2+}$ ions into the pore to identify potential residues involved in Ca-dependent pore block. A third simulation was run for 200 ns with protonation states of Glu and Asp residues adjusted to mimic pH 5.3, thus with side chains with pKa ≥ 5.5 deprotonated; the pKa values were estimated by propKa [49,50]. In this system, residues D78, E79, E221, E231, E235, E247, E322, D351, D358, D414, E418, E423 and D439 were protonated and all His residues were neutral. Results with and without transmembrane potential were similar and are aggregated for clarity.

4.4. Quantification and statistical analysis

The fits and the statistical analyses were carried out with Graphpad Prism, Version 9. Statistical differences between two groups were analysed with Student’s t-test and differences between greater than two groups were analysed with ANOVA (or Kruskal–Wallis test for not normally distributed data) followed by a Dunnett’s post hoc test. Data are presented as mean ± s.d. or as individual data points. The significance of the difference of ΔpH30 or ΔpHΔH0 values between mutants and WT channels was assessed using a permutation test, with n = 10 000 permutations, using the R statistical language (version 4.2.1).

References

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