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Structural plasticity and dynamic selectivity of acid sensing ion channel–toxin complexes

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Abstract

Acid sensing ion channels (ASICs) are voltage-independent, amiloride-sensitive channels implicated in diverse physiological processes ranging from nociception to taste. Despite the importance of ASICs in physiology, we know little about the mechanism of channel activation. Here we show that psalmotoxin activates non- and sodium-selective currents in chicken ASIC1a at pH 7.25 and 5.5, respectively. Crystal structures of ASIC1a – psalmotoxin complexes map the toxin binding site to the extracellular domain and illuminate how toxin binding triggers an expansion of the extracellular vestibule and stabilization of the open channel pore. At pH 7.25 the pore is ~10 Å in diameter whereas at pH 5.5 the pore is largely hydrophobic and elliptical in cross section with dimensions of ~5 by ~7 Å, consistent with a barrier mechanism for ion selectivity. These studies define mechanisms for activation of ASICs, illuminate the basis for dynamic ion selectivity and provide the blueprints for new therapeutic agents.

Acid-sensing ion channels (ASICs)¹, members of the epithelial sodium channel/degenerin (ENaC/DEG) superfamily of cation channels^{2, 3}, open a transmembrane pore upon exposure to low pH⁴. Primarily found in the central and peripheral nervous systems^{5–7}, ASICs occupy diverse physiological roles that include nociception^{8, 9}, mechanosensation⁸, synaptic plasticity, learning and memory⁷, and fear conditioning¹⁰. The ASIC subfamily is coded by four genes which give rise to seven isoforms¹¹, of which ASIC1a is permeable to Na⁺ and Ca²⁺ and is implicated in ischemic neuronal injury^{12, 13}. The ENaC channel³, found throughout the human body, is crucial to the regulation of blood pressure¹⁴ and is directly involved in Liddle's syndrome¹⁵ and pseudohypoaldosteronism¹⁶.

ASICs and ENaCs are trimeric¹⁷, voltage-independent and sodium-selective ion channels sensitive to the classic ENaC blocker amiloride^{1, 3}. Whereas ASICs display a selectivity of Na⁺:K⁺ ranging from 3 to 30:1 and are inhibited by micromolar concentrations of amiloride, ENaCs harbor a preference for Na⁺:K⁺ of >100:1 and are blocked by nanomolar concentrations of amiloride^{2, 18}. For both ASICs and ENaCs, Li⁺ permeability is similar to that of Na⁺ and monovalent ions larger than K⁺, such as Cs⁺, are generally impermeable¹⁹. However, the 'peak' and 'sustained' or 'steady state' ionic currents carried by ASICs display variable ion selectivity and blocker sensitivity^{20–25}, properties reminiscent of the dynamic ion selectivity of trimeric P2X receptors²⁶. At present there is no understanding of

Author Contributions

I.B. and E.G. designed the project. I.B. performed protein purification, crystallography, and electrophysiology. I.B. and E.G. wrote the manuscript.

how ASICs adopt sodium-selective and non-selective conformations with differential sensitivity to the blocker amiloride.

Activation of the ion channel pore in ASICs is classically conditioned by drops in extracellular pH from ~7.5 to pH 4–6⁴ with the currents of ASIC1a exhibiting rapid and nearly complete desensitization²⁷. Psalmotoxin (PcTx1), classified as an inhibitor cystine knot toxin from a South American tarantula^{28, 29}, acts potently on ASIC1a, increasing the channel's affinity for protons³⁰ and, contingent on the species and splice variant of the channel, acts as an agonist, eliciting steady state current or as an antagonist, diminishing ion channel activation^{31, 32}. The action of PcTx1 as an antagonist confers both analgesic³³ and neuroprotective¹² properties.

Here we report crystallographic and electrophysiological studies of the action of PcTx1 on chicken ASIC1a, showing the determinants of toxin binding, the mechanism by which toxin binding opens the ion channel, and the architecture of non- and Na⁺-selective conformations of the ion channel pore. Our studies inform mechanisms of gating and permeation in ASICs and ENaC/DEG channels and lay a foundation for development of new molecules for modulation of ion channel activity.

Function and architecture of ASIC1a – PcTx1 complex

PcTx1 slows desensitization of ASIC and yields substantial steady state current when applied to ASIC1a/1b chimeras, and thus we asked whether PcTx1 stabilizes open channel states of cASIC1a³¹. We generated a cASIC1a construct for structural studies by removing 13 and 63 residues from the N- and C-termini respectively, yielding a channel with wild type-like electrophysiological properties ($\Delta 13$; Supplementary Figs. 1 and 2). Application of a pH 5.5 solution to $\Delta 13$ gives rapidly activating and desensitizing inward current while perfusion of a saturating PcTx1 solution at pH 7.25 elicits a current that activates and decays over a time scale of seconds to yield a steady state current (Fig. 1a and Supplementary Fig. 3). Subsequent application of saturating PcTx1 at pH 5.5 further activates peak current and steady state currents.

The $\Delta 13$ -PcTx1 complex forms crystals at pH 7.25 (high pH) that belong to the *R*3 space group, diffract to ~3.3 Å resolution, and have a single ASIC subunit-toxin complex positioned on the 3-fold axis of crystallographic symmetry. Crystals grown at pH 5.5 (low pH) belong to the *C*2 space group with an ASIC trimer and three toxin molecules in the asymmetric unit and a diffraction limit of ~2.8 Å resolution (Figs. 1b, c, d, Supplementary Fig. 4). Structures of both crystal forms were solved by molecular replacement and refined to good crystallographic statistics (Supplementary Table 1).

PcTx1 binds at subunit interfaces

The high and low pH channel-toxin complexes show three toxin molecules bound to the extracellular domain of each trimer at similar subunit interfaces ~45 Å from the transmembrane domain (Fig. 1). PcTx1 molecules bury ~900 Å² of solvent accessible surface area and make multiple ionic, polar and hydrophobic contacts consistent with studies mapping sites of channel-toxin interaction³⁴ (Supplementary Fig. 5) yet distinct from computational modeling of the channel-toxin complex^{35, 36}. Docked on the thumb domain, toxin molecules form nonpolar interactions mediated by aromatic residues, and they nestle an arginine-rich hairpin between adjacent subunits, making polar interactions in the acidic pocket (Fig. 2). Together, these interactions bridge the finger, β -ball, and thumb domains of one subunit and the palm domain of the adjacent subunit. Arg 26 of PcTx1 tunnels under the toxin β -sheet to hydrogen bond with the side chain carboxylate of Asp 350 of the thumb domain, which in turn interacts with Asp 238 and Arg 191 of the finger domain. In contrast,

Arg 27 is oriented in the opposite direction, forming hydrogen bonds with backbone oxygens of Thr 215, Gly 216, and Gly 218 and also lying near the Glu 220-Asp 408 acidic pair, all on the palm domain of an adjacent subunit. Like Arg 27, Arg 28 interacts with backbone oxygens of Asp 238 and Thr 240 in the finger domain.

PcTx1 is further anchored on the thumb domain by an aromatic 'embrace' between Trp 7 and Trp 24 of PcTx1 and Phe 351 (Fig. 2), a residue that when mutated in human ASIC1a to leucine renders the channel insensitive to PcTx1³⁷. Phe 351 is conserved in ASIC1 orthologs¹⁷ and appears crucial to the specificity of PcTx1 to ASIC1 channels. Site-directed mutagenesis of PcTx1³⁵ previously implicated Trp 24, Arg 26, and Arg 27 as central to toxin specificity, consistent with our structures of the channel-toxin complex. In addition, a recent structure of PcTx1 in complex with a non functional construct of cASIC1a supports our definition of the toxin binding site³⁸.

Conformational changes in the extracellular domain

Structural comparisons of the high and low pH PcTx1-bound states with the desensitized state (PDB code: 3HGC) show that the upper palm β -strands and knuckle domains define a structurally conserved scaffold (Supplementary Fig. 6), reminiscent of the scaffold of P2X receptors³⁹. Relative to the desensitized state, the lower palm domain and the wrist of the high pH Δ 13-PcTx1 complex rotate by as much as $\sim 13^\circ$ around an axis positioned below the scaffold (Fig. 3a). This rotation shifts subunit-subunit interfaces compared to those of the desensitized state structure, separating the thumb and palm domains of adjacent subunits by 2–3 Å and displacing the finger domains of the high and low pH complexes (Fig. 3a).

The consequences of toxin binding are manifested in the flexing of a blanket of β -sheets encapsulating the negatively charged central vestibule⁴⁰, a cavity composed of the lower palm domains, poised between the toxin binding site and the wrist (Fig. 3a and Supplementary Fig. 7). With the C α atom of Val 75 as a landmark, the distances between adjacent subunits are ~ 11 Å and ~ 12 Å in the low and high pH structures, respectively; upon formation of the desensitized state, the distance diminishes to ~ 7 Å. Chemical modification of the E79C mutant of ASIC3⁴¹, a residue equivalent to Glu 80 in cASIC1a and predicted to face the interior of the central vestibule, slows the rate and extent of channel desensitization, consistent with the notion that the central vestibule contracts upon transition from the PcTx1-bound states to the desensitized state. Small molecules that activate²³ or potentiate the steady state current of ASIC3^{22, 42}, respectively, bind within the central vestibule²³, or at the subunit interface near Ala 82, and stabilize the central vestibule in an expanded conformation, recalling the ATP-dependent expansion of the extracellular vestibule of P2X receptors³⁹.

A striking conformational change in the palm domain, common to both PcTx1 complexes and located within the β 1- β 2 linker, is a $\sim 180^\circ$ flip of the Thr 84-Arg 85 peptide bond (Supplementary Fig. 8), inducing a shift of β 1 away from the equivalent β -sheet of the adjacent subunit. In addition, Ala 413, Leu 414, and Asn 415 in the β 11- β 12 linker in the high pH structure adopt conformations in which the side chains of Leu 414 and Asn 415 have effectively swapped positions (Fig. 3b and Supplementary Fig. 9). Indeed, the C α atoms of residues Ala 82 and Ala 413 are farther apart in the high pH state (8.1 Å), in comparison to the low pH state (6.4 Å), consistent with the notion that a disulfide can form between the equivalent residues in shark ASIC1b, stabilizing the channel in the desensitized state²⁵ (Supplementary Fig. 10). Studies at sites equivalent to Leu 86, a residue that interacts with Leu414 in the high pH Δ 13-PcTx1 state, and Asn 415 reinforce the conclusion that the β 1- β 2 and β 11- β 12 linkers are crucial to gating^{42–44}.

The structures of the high and low pH $\Delta 13$ -PcTx1 complexes suggest molecular mechanisms underlying the pH dependent conformations of the $\beta 1$ - $\beta 2$ and $\beta 11$ - $\beta 12$ linkers. We speculate that at high pH, the carboxyl group of Glu 80 is ionized, favoring an expanded conformation of the central cavity (Supplementary Fig. 7). As the pH drops, acidic residues in the vestibule bind protons, allowing Glu 80 and adjacent residues to adopt a 'contracted' central vestibule conformation. In fact, the distance between C α of Glu 80 and Glu 412, residues that flank both linkers, diminishes from 14.1 Å to 11.3 Å in the high and low pH states, respectively. Neutralization of Glu 80 by mutation to Ala results in a channel that desensitizes ~40-fold more rapidly than the parent construct and that does not yield measureable steady state current upon application of PcTx1 at pH 7.25 (Supplementary Fig. 11), thus underscoring the role that titratable residues play in pH dependent conformational changes of the central vestibule.

A highly conserved non polar residue in the $\beta 11$ - $\beta 12$ linker, Leu 414, is also important to the conformational transitions of the central vestibule. In the high pH $\Delta 13$ -PcTx1 complex, Leu 414 interacts with Leu 86 of the $\beta 1$ - $\beta 2$ linker. Upon transition to the low pH $\Delta 13$ -PcTx1 complex and to the desensitized state, however, Leu 414 forms multiple hydrophobic contacts involving Leu 281, Ile 306, and Val 368 of the adjacent subunit. In addition, Asn 415 hydrogen bonds with Tyr 416 and the backbone nitrogen of Ala 83 in the $\beta 1$ - $\beta 2$ linker (Supplementary Fig. 9c, d). Together, these rearrangements highlight the importance of the $\beta 1$ - $\beta 2$ and $\beta 11$ - $\beta 12$ linkers and nearby subunit interfaces to conformational transitions of the central vestibule.

Ion channel at high pH

The high pH $\Delta 13$ -PcTx1 complex harbors a cavernous, 3-fold symmetric pore in which TM2 resides on the periphery of the pore and lines the ion channel together with TM1 (Fig. 4a, b). In comparison to the desensitized state, a kink in TM2 at Asp 433 results in an effective rotation of the cytoplasmic end of TM2 by ~90° around the 3-fold axis and a tilting of TM2 by ~50°; movements in TM1 are smaller, characterized by a ~20° rotation around the 3-fold axis. Collectively, these movements rupture intrasubunit interactions between TM1 and TM2 and completely disrupt intersubunit contacts between TM2 segments that define the closed, desensitized channel gate, yielding sparse intersubunit and hydrophobic contacts between TM1 and TM2 mediated primarily by Ile 66 and Ile 434, and Val 46 and Ile 446 in the high pH complex (Supplementary Fig. 12).

The large TM pore of the pH 7.25 PcTx1 complex, with a diameter of ~10 Å near Asp 433 (Supplementary Fig. 13), led us to probe the selectivity of the $\Delta 13$ -PcTx1 complex using bi-ionic patch clamp electrophysiology (Fig. 4c, d). At neutral pH, the channel-toxin complex does not discriminate between Li⁺, Na⁺, K⁺, and Cs⁺, and we suggest that these ions permeate through the pore in a fully hydrated state. Furthermore, *N*-methyl-D-glucamine (NMDG), with a radius of ~4.0 Å, permeates through the ion channel and application of 500 μM amiloride only blocks 10% of the steady-state current (Supplementary Fig. 14). This non selective behavior of the $\Delta 13$ -PcTx1 complex is reminiscent of the non selective behavior previously observed in steady state currents of wild type ASIC3 and in the degenerin mutants of ASICs^{20, 21}, and of the pore-dilated behavior of trimeric P2X receptors²⁶.

Architecture of low pH ion channel pore

The TM helices of each subunit in the low pH $\Delta 13$ -PcTx1 complex not only adopt different conformations but they also occupy distinct positions relative to the pore axis (Fig. 4e, f). TM2 segments of subunits A and B bend and 'stretch' at residues 433–435, similar to conformations observed in the high pH structure. In comparison to the desensitized state, the TM1 helices of the low pH complex rotate by 9–12° around the pore axis and the TM2

helices of subunits A, B and C tilt by $\sim 47^\circ$, 65° and 30° , respectively. Thus the TM2 helices adopt an orientation nearly perpendicular to the membrane plane, reminiscent of the TM conformation of the low pH Δ ASIC1 structure¹⁷.

TM2 of the C subunit adopts a straight α -helix, resulting in a ~ 4 residue displacement along the pore axis relative to subunits A and B, shifting subunit C toward the extracellular side of the membrane, thus conferring axial asymmetry onto the pore. This asymmetry has precedent in chemical modification studies of ENaC which were interpreted in terms of a model in which the β subunit is displaced along the pore axis by about 1 turn of an α -helix⁴⁵. The axial displacement of the TM's gives rise to a striking constellation of hydrophobic contacts mediated by the staggered arrangement of Leu 440 and Leu 447 of subunits B and C that resembles a leucine zipper motif (Supplementary Fig. 15a, b). The extensive contacts between the TM2 domains of the B and C subunits give rise to TM1 of subunit B residing on the periphery of the ion channel domain, while the proximity of TM2 of subunits A and C shields TM1 of subunit C from exposure to the pore. The remaining 4 TM segments line the pore and participate in intersubunit interactions that include contacts between Val 46 (C) and Ile 446 (A), as observed in the high pH structure (Supplementary Fig. 15c, d). The exposure of portions of TM1 from subunit A to the pore concurs with the results from accessibility studies of TM1 residues of FaNaC⁴⁶, a peptide-gated channel, but stands in contrast to cysteine-directed chemical modification studies of lamprey ASIC⁴⁷, which suggest that TM2 primarily lines the pore. Determining how the conformation of the asymmetric pore in the low pH Δ 13-PcTx1 complex is related to the conductive pore of the Δ 13 construct upon activation by protons will require additional studies. Nevertheless, we note that ENaCs likely harbor an asymmetric pore based on variable reactivity of cysteine residues at equivalent sites on different subunits⁴⁵ and that the extensive intra- and intersubunit interactions between TM1 and TM2 seen in the low pH Δ 13-PcTx1 complex renders asymmetric pore formation favorable.

Low pH pore is sodium selective

The low pH complex harbors an elliptical pore with a region of constriction spanning a helical turn and located approximately halfway along the transmembrane domain, primarily lined by hydrophobic side chains of Leu 440 (Supplementary Fig. 16). The position of the constriction exposes the putative amiloride binding site, Gly 439 and the 'GAS' selectivity tract, to the extracellular side of the membrane, consistent with the inhibitory mechanism of amiloride as an open channel blocker⁴⁸. Strikingly, ion channel blockers of ASICs and ENaCs, including amiloride, are characteristically planar, non-symmetric molecules that roughly mirror the shape of the extracellular portion of the low pH pore (Supplementary Fig. 16).

To assess the properties of the Δ 13-PcTx1 ion channel pore at low pH, we determined its ion selectivity properties and found that the complex remains selective for Na^+ and Li^+ with a selectivity for Na^+ over K^+ of 10:1 (Fig. 4g). Because the pore is primarily lined by hydrophobic residues at the constriction point (Fig. 4h), we assume that Na^+ ions are hydrated when traversing the pore. We suggest that the pore discriminates ions by the size of a fully or partially hydrated ion and that the mechanism underlying ion selectivity is best described by a barrier model. Indeed, Hille proposed that sodium selective pores have a rectangular cross section with dimensions of 3.2 Å by ~ 5.2 Å, large enough to allow one sodium ion and one water molecule to percolate but too small to allow passage of hydrated potassium ions⁴⁹. The elliptical outline of the constriction in the low pH Δ 13-PcTx1 structure is in general accord with the proposed geometry of a Na^+ -selective pore albeit larger, with constrictions of ~ 5 Å by ~ 7 Å at Leu 440 of subunit C and of ~ 4 Å by 10 Å at Leu 440 of subunits A and B (Supplementary Fig. 16). The extent to which the mechanism

of Na⁺-selectivity upon proton activation, in the absence of PcTx1, hinges on Leu 440 and on the low pH Δ 13-PcTx1 conformation requires further experimentation. Nevertheless, mutation of Leu 440 to Ala or Ser (Supplementary Fig. 17) demonstrates that Leu 440 is crucial to the formation and function of the ion channel pore based on the reduced activity of the mutants compared to the parent construct.

Ion binding sites

To map ion binding sites we soaked crystals in solutions containing Cs⁺, a voltage-dependent, open channel blocker of the Δ 13 construct (Supplementary Fig. 18). Inspection of anomalous difference electron density maps revealed a site (6–12 σ) in each subunit, common to the high and low pH Δ 13-PcTx1 crystal forms and located at the interface between the wrist and the extracellular end of TM1 (Fig. 5a and Supplementary Fig. 19). In both crystal forms, the backbone carbonyl oxygens of Leu 71, Tyr 72, Pro 287, and Trp 288 coordinate Cs⁺ (Fig. 5b). Interestingly, the three crystallographically independent Cs⁺ sites in the *C2* structure vary in strength, with subunit C having the weakest signal, thus suggesting that the binding of ions to these sites is influenced by the variation in TM1/wrist conformation between subunits. Because Cs⁺ soaking experiments with crystals of the desensitized state failed to reveal ion binding to this site, and TM1 (C) in low pH Δ 13-PcTx1 state harbors the weakest Cs⁺ site, we suggest the occupancy of the site is state dependent, with ion binding favored when the pore is in an open state, augmenting interactions between Tyr 72 and Trp 288.

We identified two Cs⁺ sites in the electrostatically negative mouth of the pore, near Asp 433 in the *C2* structure (Fig 5c, d), a residue implicated in stabilization of the open state yet not crucial to ion selectivity⁵⁰. Ions at these sites are ~5 Å from the closest protein residues consistent with the notion that they are low affinity, transiently occupied cation sites bound by water-mediated contacts. We did not observe anomalous difference density features deeper into the pore, perhaps because the hydrophobic pore is devoid of favorable binding sites and the structural analysis was carried out in the absence of a membrane potential.

Mechanism

We used PcTx1 to stabilize open states of the Δ 13 construct at high and low pH. Comparison of the open state and desensitized state structures defines the upper palm and knuckle as a structural scaffold and the lower palm as a conformationally flexible, proton-sensitive domain at the core of ion channel gating (Supplementary Fig. 7). The finger and thumb domains flank the palm domain, harbor binding sites for protons and PcTx1 and modulate movements of the lower palm domain by alterations in intersubunit contacts. In the open conformations, the subunit interface between the thumb and the palm domain separates while the extracellular and transmembrane domain interface forming the extracellular vestibule expands. The presence of a modulator, such as PcTx1, precludes ‘collapse’ of the thumb – palm subunit interface to a desensitized state conformation. The motions of the lower palm domain converge at the wrist region, inducing radial and rotational movements of the transmembrane domains, gating the ion channel (Fig. 6 and Supplementary Fig. 20).

Conclusion

Our functional and structural studies of the chicken ASIC1a complex with PcTx1 at two proton concentrations provide new insights into how the movements of the multiple domains of ASICs are coupled to ligand and pH dependent gating. Channel opening is correlated to the expansion of the extracellular vestibule and to rearrangements at subunit interfaces, movements coupled to the ion channel pore by the direct connections of extracellular β -

strands to the transmembrane α -helices and also by the non covalent contacts between the thumb and wrist region, a previously unrecognized site of cation binding in the open state. The non selective and sodium selective states of the ion channel pore illustrate how transmembrane helices can rearrange to form a large, non selective pore at high pH and a small, asymmetric and sodium selective channel at low pH. Together, these studies illuminate mechanisms of gating and selectivity in ASIC/ENaC/DEG channels.

Methods Summary

The $\Delta 13$ construct was expressed in insect or mammalian cells using baculovirus mediated expression and purified by metal ion affinity and size-exclusion chromatography. The high and low pH crystal forms were obtained in conditions containing 20 mM Tris (pH 7.25) and 14–18% PEG 550 MME, and 100 mM sodium acetate (pH 5.5) and 9–12% PEG 2000 MME, respectively. Data processing, model building, and refinement were performed using the HKL2000, COOT, and PHENIX computer programs. The structures were solved by molecular replacement and subjected to crystallographic refinement. Whole-cell recordings were performed using CHO-K1 cells transfected with plasmid DNA encoding the $\Delta 13$ construct.

Methods

Expression and purification

The construct $\Delta 13$ was derived from the chicken ASIC1 gene and was expressed as N-terminal fusion with octa-histidine-tagged GFP using baculovirus expression systems in insect cells and mammalian cells⁵¹ with two thrombin sites and one thrombin site, respectively, encoded upstream of $\Delta 13$ generating a final protein sequence containing residues Gly 14 to Arg 463, verified by N-terminal amino acid sequencing. Protein expressed in insect cells was purified as described⁴⁰, while the $\Delta 13$ expressed in mammalian cells were collected by centrifugation (1000*g*) and sonicated in the presence of 150 mM NaCl, 20 mM Tris (pH 8.0) and protease inhibitors, then subsequently solubilized in 40 mM *n*-dodecyl β -D-maltoside (DDM) for 1 hour at 4°C. The solubilized material was clarified by centrifugation (19,000*g*) for 1 hour at 4°C and the supernatant was incubated with TALON resin for 1.5 hours at 4°C. Bound protein was then eluted with 150 mM NaCl, 20 mM Tris (pH 8.0), 250 mM imidazole, and 1 mM DDM. Cleavage of the histidine-tagged GFP was achieved by thrombin. The resulting $\Delta 13$ protein was further purified by size-exclusion chromatography using a mobile phase containing 150 mM NaCl, 20 mM Tris (pH 7.4), 1 mM DDM, 1 mM DTT, and 1 mM EDTA. Peak fractions were collected and concentrated to 1.60 mg/mL based on A280 measurement. Synthetic psalmotoxin (Peptides International) was immediately added in a 6:1 molar ratio of toxin to channel prior to crystallization.

Crystallization

Crystals of the high pH form of the $\Delta 13$ -PcTx1 complex were grown with a protein solution supplemented with 100 μ M 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) by way of vapor diffusion using a reservoir solution composed of 20 mM Tris (pH 7.25), 14–18% PEG 550 MME and drops composed of a ratio of 1.5:1 and 2:1, reservoir to protein, respectively, at 4 °C. For cryoprotection, crystals were soaked in reservoir solution supplemented with increasing concentrations of glycerol (15% v/v final concentration), 1 mM DDM, and 1 mM DMPC. For Cs⁺ anomalous diffraction experiments, crystals were soaked in reservoir solutions supplemented with 300 mM CsCl, 500 μ M DMPC and 1 mM DDM.

Crystals of the low pH form of the $\Delta 13$ -PcTx1 complex were obtained by vapor diffusion in hanging drop configuration using a 1:2 reservoir to protein ratio at 4°C. The reservoir solution typically contained 100 mM sodium acetate (pH 5.5) and 9–12% PEG 2000 MME. Cryoprotection was accomplished using reservoir solution supplemented with increasing concentrations of glycerol (20% v/v final concentration). For Cs⁺ anomalous diffraction experiments, a modified reservoir solution was employed in which the sodium acetate was replaced by cesium acetate, and the solution was supplemented with 500 mM CsCl and 1 mM DDM.

Structure determination

X-ray diffraction data sets from crystals grown at pH 7.25 and belonging to the *R3* space group were collected at the Advanced Light Source (beamline 5.0.2) and diffraction was measured to ~3.35 Å resolution. The best x-ray diffraction data sets from the *C2* form, pH 5.5 crystals, were collected at the Advanced Photon Source (beamline 24ID-C) and scaled to ~2.80 Å resolution. Diffraction data for crystals soaked in cesium containing solutions were measured using low energy x-rays (9000 eV) at the Advanced Light Source. Diffraction data were indexed, integrated, and scaled using the HKL2000 software⁵². The structures were solved by molecular replacement using the program PHASER⁵³, with the extracellular domain coordinates of the Δ ASIC1 structure¹⁷ (PDB code: 2QTS) as a search probe. Resulting maps after molecular replacement showed strong density of psalmotoxin, and thus the toxin was fitted into the density at the subunit interface using the solution structure²⁹. Iterative model building and refinement were performed using COOT⁵⁴ and PHENIX⁵⁵ package. Crystals in the *R3* space group contained one subunit and one toxin in the asymmetric unit; consequently, the channel is built using the subunits and toxins related by crystallographic symmetry. Alternatively, crystals in the *C2* space group contained one trimer and three toxins in the asymmetric unit. Manual building of the transmembrane domains was guided by electron density maps calculated using the crystallographically refined coordinates of the extracellular domain - toxin complex. “Omit” electron density maps were calculated to validate residue registration, particularly with the transmembrane domains. Subsequent iterative cycles of model building and refinement were performed after building the transmembrane regions. Non-crystallographic symmetry (NCS) restraints were implemented in the extracellular domain and toxin for the low pH structure containing regions defined automatically by the PHENIX package to improve maps. These regions consist of residues 72–135, 138–153, 155–291, 303–331, 333–360, 362–386, and 388–427 in the extracellular domain and residues 2–38 in the toxin. As for the high pH structure, TLS parameters were refined with 7 TLS groups defined by PHENIX, which comprised of 5 groups in the ASIC subunit and 2 groups in the toxin. Structure validation was performed using MolProbity⁵⁶. The low pH final model contains channel subunits with residues 50–454, 45–450 and 42–454 of A, B, and C chains, respectively and toxins with residues 2–38, 2–37, and 1–38 of M, N, and O chains, respectively. The high pH model contains residues 41–450 of the ASIC subunit, and 2–13 and 16–38 of the toxin. A region in the thumb domain of the high pH structure lacked electron density consisting of residues 298–301 and was omitted in the final structure. Pore surface and dimension were determined using the software HOLE⁵⁷, while the rotation axis shown in Figure 3a was analyzed using Dyndom⁵⁸.

Electrophysiology

Whole-cell recordings were carried out with CHO-K1 cells 24–48 hours after transfection by plasmid DNA encoding the $\Delta 13$ construct and GFP expressed from an internal ribosome entry site. Pipettes were pulled and polished to 2–3 M Ω resistance and filled with internal solution containing (in mM): 150 KCl, 2 MgCl₂, 5 EGTA and 10 HEPES (pH 7.35). External solution contained (in mM): 150 NaCl, 2 MgCl₂, 2 CaCl₂, 8 Tris and 4 MES. For

ion selectivity experiments, NaCl was substituted with equimolar concentrations of LiCl, KCl, CsCl, or NMDG in the external solution. Proton concentration-response profiles were recorded and normalized to maximal current generated by application of external solution buffered at pH 5.8, while the PcTx1 concentration-response curve was generated by normalizing to maximal current generated by application of 1 μ M of toxin. All concentration-response curves were fitted to the Hill equation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PcTx1 activates the chicken ASIC1a $\Delta 13$ construct

a, Whole-cell, patch-clamp current traces of activation by steps into pH 5.5 (1), pH 7.25 and 1 μ M PcTx1 (2), and pH 5.5 and 1 μ M PcTx1 (3). Inset, current trace of step into pH 5.5 and 1 μ M PcTx1. **b**, Structure of low pH $\Delta 13$ -PcTx1 complex viewed from the extracellular side. **c**, **d**, High pH (**c**) and low pH (**d**) complexes viewed parallel to the membrane. Each subunit is in a different color and toxin is in solvent-accessible surface representation.

Figure 2. Extensive interactions adhere PcTx1 to the $\Delta 13$ ion channel

Close-up views of toxin-binding site of the low pH complex. Dashed lines indicate possible hydrogen bonds.

Figure 3. Conformational changes in the extracellular domain

a, Low pH $\Delta 13$ -PcTx1 structure is in cartoon representation and colored as in Fig. 2. Black line indicates the axis around which the lower palm domain and the wrist region rotate following superpositions of the desensitized and open state structures. Close-up views of selected regions are boxed. The low pH complex is colored by domain, the high pH complex is orange, and the desensitized state (PDB code: 3HGC) is gray. Approximate position of PcTx1 is indicated by blue dashed lines and the boundaries between adjacent subunits is shown by solid gray lines. Measured C α distances are between residues Asn 357 (A) and Arg 85 (B) in box 1. In box 2 the distances are between Val 75 C α atoms on adjacent

subunits. **b**, Close-up view of strands $\beta 1$ and $\beta 12$, the $\beta 1$ - $\beta 2/\beta 11$ - $\beta 12$ linkers and the extracellular boundary of the TM domains from two subunits of the high pH $\Delta 13$ -PcTx1 complex (orange) and the desensitized state (gray) following superposition of the respective scaffold domains. Inset shows location of close-up view in the context of the entire channel.

Figure 4. Structural rearrangements and ion selectivity of the transmembrane pores
a, b, Comparison of transmembrane domains from the high pH (orange) and desensitized (gray) state structures with TM1 in ribbon and TM2 in cylinder representation. Transmembrane domains are viewed from the extracellular side (**a**) and parallel to the membrane (**b**). **c,** Current/voltage experiment showing that at neutral pH the $\Delta 13\text{-PcTx1}$ complex forms a non selective cation channel. **d,** Mapping of solvent accessible pathway along the 3-fold axis shows that the high pH complex has a large, transmembrane pore. The occluded pathway along the 3-fold in the extracellular domain suggests that ions access the pore by way of lateral fenestrations. **e, f.** A comparison of the transmembrane domains from

the low pH complex, where each subunit is in a different color. The desensitized state is gray. TM1 and TM2 segments are in ribbon and cylinder representations, respectively. Transmembrane domains are viewed from the extracellular side (**e**) and parallel to the membrane (**f**). **g**, Current/voltage experiment demonstrating that the ion channel of the low pH $\Delta 13$ -PcTx1 complex is sodium selective. **h**, Mapping of a solvent accessible pathway along the pseudo 3-fold axis of the low pH complex shows that it has an asymmetric ion channel pore and a constriction (opposing arrows) halfway across the bilayer. Maps of solvent-accessible pathways (d and h) were generated using the HOLE software (red < 1.4 Å < green < 2.3 Å < purple).

Figure 5. Cs^+ binding sites

a, Anomalous difference electron density map contoured at 3.5σ . The low pH complex is in ribbon representation. Cesium sites in the outer pore region are labeled Cs1 and Cs2. **b**, Close-up view of the Cs^+ site in the wrist region. Top, electrostatic potential contoured from -20kT (red) to $+20\text{kT}$ (blue). Bottom, Cs^+ coordination by backbone carbonyl oxygens. The main chain is drawn as sticks. **c,d**, Close-up view of Cs^+ sites near Asp 433 shown in ribbon and stick representation (**c**) and solvent accessible surface colored by electrostatic potential (**d**) viewed from the extracellular side.

Figure 6. Schematic representation of gating

a, The extracellular vestibule in the closed, desensitized state structure adopts a contracted conformation. **b**, In the open pore conformation the vestibule occupies an expanded conformation, stabilized by the thumb domain. The wrist region, in turn, couples the conformational changes of the extracellular domain to the transmembrane domains (red cylinder) of the ion channel pore.