Conformational Changes Associated with Proton-dependent Gating of ASIC1a* 

Christopher J. Passero1, Sora Okumura, and Marcelo D. Carattino2

From the Renal-Electrolyte Division, Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Acid-sensing ion channels are proton-gated Na+ channels expressed predominantly in neurons. How channel structure translates an environmental stimulus into changes in pore permeability remains largely undefined. The pore of ASIC1 is defined by residues in the second transmembrane domain (TM2), although a segment of the outer vestibule is formed by residues of TM1. We used the voltage clamp fluorometry technique to define the role of the region preceding TM2 (pre-TM2) in activation and desensitization of mouse ASIC1a. Oocytes expressing E425C channels labeled with Alexa Fluor 488 C5-maleimide showed a change in the emission of the fluorescent probe in response to extracellular acidification. The time course of the change in fluorescence correlated with activation but not desensitization of E425C channels. The fluorescence emission did not change following extracellular acidification in oocytes carrying an inactivating mutation (W287G/E425C), although these channels were labeled and expressed at the plasma membrane. Our data indicate that pore opening occurs in conjunction with a conformational rearrangement of the pre-TM2. We observed a change in the emission of the fluorescent probe when labeled E425C channels transition from the desensitized to the resting state. The substituted-cysteine-accessibility method was used to determine whether the pre-TM2 has different conformations in the resting and desensitized states. State-dependent changes in accessibility to 2-[(trimethylammonium)ethyl]methanethiosulfonate bromide modification were observed in oocytes expressing K421C, K422C, Y424C, and E425C channels. Our results suggest that the pre-TM2 of ASIC1a undergoes dynamic conformational rearrangements during proton-dependent gating.

Acid-sensing ion channels (ASICs)3 are members of the epithelial sodium channel (ENaC)/degenerin family expressed in neurons of the peripheral and central nervous system. To date, seven ASIC subunits have been cloned, including ASIC1a (1), ASIC1b (2, 3), ASIC1b2 (4), ASIC2a (5–7), ASIC2b (8), ASIC3 (9–12), and ASIC4 (13, 14). In the peripheral nervous system, ASICs are expressed in sensory nerve endings where they participate in normal touch sensation and pain perception (15–24). In the central nervous system, ASIC1a contributes to synaptic plasticity in the hippocampus, hippocampus-dependent spatial memory, spatial learning, and neural mechanisms of fear conditioning (25–27). Recently, ASICs have been implicated in the pathogenesis and migration of malignant glioma cells (28–30).

The first high resolution structure of the extracellular and membrane-spanning domains of Gallus gallus (chicken) acid-sensing ion channel 1 (cASIC1) was recently reported (31). cASIC1 is organized as a homotrimer. Each subunit has a short intracellular N terminus and C terminus and two transmembrane domains (TM) connected by a large extracellular region protruding from the plane of the membrane. The extracellular region is organized in discrete domains and, using the analogy of a hand, has been named the "palm, knuckle, β-ball, finger, and thumb" (31) (Fig. 1). Residues within TM2 constitute the pore, although TM1 defines a segment in the extracellular vestibule of the pore (31).

Proton-sensitive ASICs are activated by extracellular acidification, although the continued presence of protons desensitizes them. These channels reside in at least three different states as follows: (i) a nonconducting resting state where the channel can be activated by extracellular acidification; (ii) a conducting open state; and (iii) a desensitized nonconducting state where the channel is insensitive to extracellular protons. Several independent studies suggest that proton(s) interact with sites located in the extracellular region (31–33). At present, how conformational rearrangements initiated in the ectodomain of the channel by extracellular acidification are translated into opening and closing of the channel pore remains undefined. A rotation and/or bending of the α-helices in the pore is likely required to allow ion flux. Because the ion conduction pathway is formed by residues within TM2 in cASIC1, we hypothesized that reorganization of the region preceding TM2 (pre-TM2) mediates opening of the channel pore in response to extracellular acidification. Using voltage clamp fluorometry (VCF), we revealed that extracellular acidification induces a conformational rearrangement in the pre-TM2 region that is associated with activation but not desensitization of mouse ASIC1a (mASIC1a). A change in the emission of the fluorescent probe was observed when E425C channels transition from the desensitized to the resting state. We therefore

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3 Supported by a Carl W. Gottschalk award from the American Society of Nephrology. To whom correspondence should be addressed: Renal-Electrolyte Division, University of Pittsburgh, 5834 Scafe Hall, 3550 Terrace St., Pittsburgh, PA 15261. Tel.: 412-648-9277; Fax: 412-383-8956; E-mail: mdc4@pitt.edu.
4 The abbreviations used are: ASIC(s), acid-sensing ion channel(s); ENaC, epithelial sodium channel; VCF, voltage clamp fluorometry; TM, transmembrane domain; MTSET, 2-[(trimethylammonium)ethyl]methanethiosulfonate bromide; HA, hemagglutinin; MES, 4-morpholineethanesulfonic acid.
asked if the structural organization of the pre-TM2 was similar in the desensitized and resting states using the substituted–cysteine-accessibility method (34, 35). We observed changes in accessibility to covalent modification by MTSET of cysteine residues introduced at positions 421, 422, 424, and 425 in the desensitized state, compared with the resting state. Our data suggest that the pre-TM2 undergoes a conformational rearrangement following extracellular acidosis that is associated with activation of mASIC1a.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology and Expression—**mASIC1a (GenBank™ accession number BC067025) was obtained from Open Biosystems and subcloned into the vector pSP64 poly(A) (Promega Corp., Madison, WI). Site-directed mutagenesis was performed using QuikChange XL (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Constructs bearing mutations in the tract Lys421–Ala427 and Trp287 were generated in a mASIC1a Cys mutant (mASIC1a C70L). Mutations were confirmed by direct sequencing. *Xenopus laevis* oocytes stage 5–6 were injected with 0.3–15 ng of cRNA encoding wild type or mutant channels. Injected oocytes were maintained in modified Barth’s solution (MBS) at 18 °C for 24–72 h to allow channel expression as described previously (36).

**Electrophysiology—**Experiments were performed at room temperature (20–23 °C). Oocytes were placed in a recording chamber RC-1Z (Warner Instruments, Hamden, CT) and perfused by gravity at a rate of 5–6 ml/min. Ag/AgCl reference electrodes were localized in close proximity to the oocyte. The resistance of the glass electrodes was less than 2 megohms when filled with 3 M KCl. Two-electrode voltage clamp was performed using a TEV-200A amplifier (Dagan Corp., Minneapolis, MN), and data were acquired through Clampex 10.0 (Molecular Devices, Sunnyvale, CA) with a Digidata 1322A (Molecular Devices). Oocytes were clamped at a holding potential of —60 mV. The recording solution contained (in mM) 110 NaCl, 2 KCl, 1 CaCl2, 10 Hesper, pH 7.4. Acidic test solutions were buffered with MES. 2−[(Trimethylammonium)ethyl]-methanethiosulfonate bromide (MTSET) (Toronto Research Chemicals, Ontario, Canada) was directly dissolved in the recording solution immediately before the experiments.

**Oocyte Labeling—**To reduce the background in VCF experiments, oocytes were treated with 10 mM 3-maleimidopropionic acid in MBS for 1 h at 18 °C the day before the experiments (37). A 10 mM stock solution of Alexa Fluor 488 C5-maleimide (Invitrogen) was prepared in DMSO. Oocytes expressing mASIC1a channels were covalently labeled in the dark for 60–90 min with 5 μM Alexa Fluor 488 C5-maleimide dissolved in MBS. Following fluorophore labeling, the oocytes were washed with MBS.

**Voltage Clamp Fluorometry—**The optical setup for VCF consisted of an inverted Olympus microscope model IX-70 equipped with a mercury lamp (U-ULS100HG, Olympus), filter cube turret (IX-RFAC, Olympus), and a filter cube (Chroma 41001 (HQ480/40X, Q505LP, HQ535/50m)). A neutral density filter ND 0.1 (Omega Optical, Brattleboro, VT) was used to attenuate the intensity of the light and to reduce photobleaching. Emitted light was measured using a silicon photodiode S1336-18BQ (Hamamatsu Corp., Bridgewater, NJ) connected directly to the input of the integrating head stage of a patch clamp amplifier Axopatch 200B (Molecular Devices). The photodiode was mounted in the SLR port of the IX-70 microscope. The amplified signal was low pass filtered at 100 Hz with an eight-pole Bessel filter (LPF-8, Warner Instruments) and digitized at 2 kHz. Whole-cell currents were measured by two-electrode voltage clamp as described above. Fluorescence and current measurements were acquired simultaneously with a Digidata 1322A (Molecular Devices). Oocytes were placed on a RC-25F recording chamber (Warner Instruments) with the animal pole facing down and were perfused by gravity at a flow rate of 5–6 ml/min with a tube localized in close proximity to them. For VCF, oocytes were focused with a ×20 objective, although for background fluorescence measurements a ×10 objective was used.

**Detection of the Surface Pool of mASIC1a in Oocytes—**Twenty-four h after injection, 9–16 oocytes expressing untagged (C70L) or mutants with C-terminal HA epitope tags (E425C-HA and E425C/W287G-HA) were incubated on ice. Oocyte surface proteins were biotinylated and recovered with streptavidin beads (Pierce). Five percent of total cell lysate as well as streptavidin-precipitated proteins were immunoblotted with a mouse anti-HA antibody (Covance) as described previously (38, 39). The protein bands were quantified using Quantity One software (Bio-Rad).

**Data and Statistical Analysis—**Data are expressed as means ± S.E. (n), where n equals the number of independent experiments analyzed. Data were analyzed with Clampfit 10.2 (Molecular Devices) and SigmaPlot 8.02 (SPSS, Chicago). Statistical analysis was performed with GraphPad InStat version 3.05 (GraphPad Software Inc., La Jolla, CA). A p value of less than 0.05 was considered statistically significant.

**RESULTS**

VCF has been used to study conformational rearrangements in restricted areas during gating of ion channels and transporters. This method provides information about local protein motion associated with specific gating steps, where structural rearrangements are reported by a fluorophore attached in a site-specific manner (40–48). The quantum yield of a fluorescent probe depends on the microenvironment where the probe resides, and therefore it may change during conformational movements. In essence, cysteine mutations are introduced in areas of the protein expected to be accessible and thus suitable for covalent modification. Channels with cysteine mutations are expressed in *Xenopus* oocytes and covalently labeled with an environment-sensitive fluorophore. Ion channels are activated under voltage clamp conditions, and ionic currents are monitored in parallel with fluorescence. A change in fluorescence (ΔF) is detected if a conjugated cysteine is localized in a segment of the protein that undergoes a conformational rearrangement, and that rearrangement causes a change in the environment of the fluorophore. VCF has not been used previously to investigate conformational changes in ENaC/degenerin channels. A disadvantage of some fluorophores is that the fluorescence emission varies with the pH of the environment, as is the case of fluorescein and rhodamine. Alexa
Fluor 488 is suitable for studies of mASIC1a activation because its emission is pH-insensitive between pH 4 and 10. We confirmed the pH insensitivity of Alexa Fluor 488 in our recording solutions by measuring fluorescence intensity at various pH values using excitation/emission at 492/520 nm (data not shown).

Amino acid sequences of mASIC1a and cASIC1 share 89.8% identity with conservation of most residues in \( \alpha \)-helices and \( \beta \)-strands within the extracellular region. There are 15 cysteines in the extracellular region of cASIC1, 14 of them forming disulfide bonds. Alignment of cASIC1 and mASIC1a suggests that disulfide bonds are conserved between both proteins. There are two unpaired cysteines in the extracellular region of mASIC1a. Cys\(^{70} \) is localized in the region connecting TM1 and \( \beta \)1. Cys\(^{775} \) is in \( \beta \)9 within the palm domain. Although we have not identified accessible cysteine residues in the resolved structure of cASIC1 in the desensitized state using Deepview/Swiss-Pdb Viewer software, our studies indicate that Cys\(^{70} \) in mASIC1a is accessible from the extracellular region (see Fig. 7). Therefore, we mutated Cys\(^{70} \) to Leu, which is the homologous residue in cASIC1. This C70L mutant was used to generate constructs with cysteine substitutions in the pre-TM2 region of mASIC1a. Fig. 1A shows the structure of a single cASIC1 subunit superimposed onto the solvent-accessible surface. Key interactions between Trp\(^{288} \), in the base of the thumb, and residues in the pre-TM2 are highlighted. Based on the high degree of homology between cASIC1 and mASIC1a, we assumed that the structural organization of this area is conserved in mASIC1a.

Role of the Pre-TM2 in Gating of ASIC1a

VCF Reveals Conformational Rearrangements in the Pre-TM2 of mASIC1a—Oocytes expressing C70L channels were labeled with Alexa Fluor 488 C5-maleimide, and VCF experiments were performed as described under “Experimental Procedures.” Oocytes expressing C70L channels responded to a change in extracellular pH with a rapid increase in conductance but without a change in fluorescence (Fig. 2A). Although channels carrying cysteine substitutions in the tract Lys\(^{421} \)–Ala\(^{427} \) were functional, a change in fluorescence in response to extracellular acidification was observed only in oocytes expressing E425C channels (Fig. 2A). The localization of Glu\(^{426} \), a homolog of Glu\(^{425} \) in mASIC1a, is shown in the resolved structure of cASIC1 (Fig. 1). Some of the mutant channels in the pre-TM2 displayed reduced activity such as A423C and Y424C. For these mutant channels experiments were performed 48–72 h after injection. The linearity of fluorescence detection was examined in oocytes injected with various amounts of cRNA encoding the E425C mutation. We found that the intensity of the background fluorescence increased linearly with the number of expressed proteins, assuming that the expression of channels is directly proportional to the amount of cRNA injected (Fig. 2B). Covalent labeling by Alexa Fluor 488 C5-maleimide of the cysteine residue(s) at position 425 shifted the pH of half-maximal activation (pH\(_{50} \)) from a pH of 6.28 (CI 6.17 to 6.39) to 6.04 (CI 5.92 to 6.15) (n = 5–12) (data not shown), suggesting that proton-dependent activation of mASIC1a is slightly affected by the fluorescent probe covalently attached at position 425.

A Conformational Change in the Pre-TM2 Is Associated with Channel Opening—The effect of the pH of activation on the time course of the change in fluorescence and current was investigated in oocytes expressing labeled E425C channels. Oocytes expressing E425C channels were activated by a change in extracellular pH from 7.4 to 7.0, 6.5, 6.0, and 5.0 (Fig. 3A). The time course of change in current and fluorescence following extracellular acidification to pH 7.0, 6.5, and 6.0 was fitted to a single-exponential function, with one phase describing the activation and the other the desensitization of the channel (Fig. 3B). Following extracellular acidification to pH 5.0, the fluorescence signal was fitted to a single exponential function. The relationship between the time constants (\( \tau \)) of the exponential change in current and fluorescence was plotted for each individual experiment for activation and desensitization (Fig. 3, C and D). A correlation analysis suggests that the two variables tend to increase together during activation (\( r = 0.79, n = 42 \)) and desensitization (\( r = 0.82, n = 25 \)) (Spearman rank correlation).
Role of the Pre-TM2 in Gating of ASIC1α

A W287G Mutation at the Base of the Thumb Disrupts Conformational Changes in the Pre-TM2 and 425

We also used linear regression to analyze the relationship between the two variables. The slopes of the lines during activation and desensitization were 0.85 ± 0.09 (r = 0.84) and 0.16 ± 0.03 (r = 0.69), respectively. Because the time course of the increase in current and fluorescence was similar following a change in extracellular pH during activation, we assume that the pre-TM2 undergoes a conformational rearrangement that is associated with opening of the channel pore. Conversely, during desensitization changes in fluorescence become slower as the pH of activation decreases, suggesting that channel desensitization is not dependent on structural rearrangements in the pre-TM2 region.

We observed a change in the fluorescence signal in labeled oocytes expressing E425C channels when extracellular pH was increased from acidic to 7.4. The time course of the fluorescence change was best described by a dual-exponential function. The time constants of the exponential function were 28.8 ± 5.0 and 0.8 ± 0.1 s (n = 7) (transition from pH 6.5 to 7.4), 33.8 ± 6.6 and 1.1 ± 0.2 s (n = 9) (transition from pH 6.0 to 7.4), and 31.0 ± 4.6 and 1.1 ± 0.1 s (n = 16) (transition from pH 5.0 to 7.4) (not statistically significant). Because the time course of the change in fluorescence is described by a dual-exponential function, we assume that the pre-TM2 undergoes at least two consecutive conformational changes during the transition of the channel from the desensitized to the resting state.

A W287G Mutation at the Base of the Thumb Disrupts Conformational Changes in the Pre-TM2 of mASIC1α—At present, it is unknown how conformational rearrangements initiated in the ectodomain of the channel by extracellular acidification are translated into opening and closing of the pore. Our studies suggest that proton binding to the ectodomain induces a conformational change in the pre-TM2 that is associated with opening of the pore. Li et al. (49) found that nonconservative mutations at Tyr71, located immediately after TM1, and Trp287, located in the base of the thumb, disrupt proton-dependent activation of rat ASIC1α. We hypothesized that if conformational rearrangements occur sequentially, first in the base of the thumb and then in the pre-TM2, a W287G mutation should disrupt proton-dependent changes in fluorescence in channels bearing E425C mutations. We found that extracellular acidification does not elicit a change in either fluorescence or current in oocytes expressing channels bearing W287G/E425C mutations (Fig. 4A). To determine whether the W287G mutation affects the trafficking of the channel to the membrane, we introduced a hemagglutinin (HA) epitope in the C terminus of channels bearing E425C and W287G/E425C mutations. Oocytes expressing control (C70L), E425C-HA, and W287G/E425C-HA channels were biotinylated at 4°C. Surface proteins were precipitated with streptavidin beads and immunoblotted with an anti-HA antibody. The expression at the plasma membrane of E425C-HA and W287G/E425C-HA channels as a percentage of the total was 7.5 ± 1.1 and 6.4 ± 0.4 (p > 0.05, n = 6), indicating that the W287G mutation does not affect the trafficking of the channel to the membrane. In addition, we determined whether the W287G mutation affects labeling by Alexa Fluor 488 C5-maleimide of the cysteine residue at position 425. We found that the intensity of the background fluorescence was similar in oocytes expressing E425C-HA and W287G/E425C-HA channels labeled with Alexa Fluor 488 C5-maleimide (p > 0.05). Oocytes expressing channels carrying E425C mutations had significantly higher background fluorescence compared with oocytes expressing C70L channels (p < 0.001, n = 56–59) (Fig. 4B). Our results indicate that opening of the pore occurs in conjunction with a conformational rearrangement in the pre-TM2.

Pre-TM2 Has Different Conformations in the Resting and Desensitized State—Our data suggest that conformational changes in the pre-TM2 associated with the transition from the open to the desensitized state are not simply the reverse of those that occur when the channel transitions from the resting to the open state. VCF experiments indicate that the pre-TM2 experiences a minor reorganization during channel desensitization, suggesting that the conformation of this region is similar in the open and desensitized states. We observed a change in the fluorescence signal in oocytes expressing E425C channels when extracellular pH is increased from 5.0 to 7.4, which likely

![FIGURE 2. Conformational changes reported by Alexa Fluor 488 labeling at position 425. VCF was performed on oocytes expressing mutant mASIC1a channels that were covalently labeled with Alexa Fluor 488 C5-maleimide as described under “Experimental Procedures.” A, representative tracings of VCF experiments performed in oocytes expressing control (C70L) and E425C channels. The holding potential was −25 mV. Channels were activated by a change in extracellular pH from 7.4 to 5.0. Recordings are representative of at least 23 independent experiments. B, linearity of fluorescence detection. Oocytes were injected with various amounts of cRNA encoding the E425C mutation and labeled with Alexa Fluor 488 C5-maleimide. The intensity of the fluorescence signal measured in oocytes expressing labeled E425C channels was plotted as a function of the injected amount of cRNA. The fluorescence signal was directly proportional to the amount of injected cRNA (departures from linearity are not significant, p = 0.87, n = 29–30).](image-url)
Role of the Pre-TM2 in Gating of ASIC1a

The substituted-cysteine-accessibility method had been used to characterize channel structure (34, 35). The relative reactivity of substituted cysteines can be used to determine the accessibility of specific residues in the resting and desensitized states. ASIC1a is activated by a change in extracellular pH to 5.0 from a preconditioning pH of 7.4, but not from a preconditioning pH of 7.0 (Fig. 5). This indicates that at pH 7.4 channels are mainly in the resting state, whereas at pH 7.0 they are in the desensitized state. To determine whether the pre-TM2 has a different conformation in the resting and desensitized states, we looked for state-dependent changes in accessibility to covalent modification by MTSET in channels carrying cysteine mutations in the tract Lys421–Glu425. If the structural organization of the pre-TM2 region has distinctive conformations when the channel is in either the resting or desensitized state.

DISCUSSION

ENaC/degenerin family members share a similar topology, although they are gated by a broad variety of stimuli. Attempts to characterize the mechanism of activation of ASICs by protons has involved mainly site-directed mutagenesis (31, 33) and analysis of chimeras of proton-sensitive and proton-insensitive ASIC1 channels (32). We used VCF to investigate conformational rearrangements that take place in the pre-TM2 region of mASIC1a following extracellular acidification. In this study, we established the feasibility of this method to study conformational changes that occur in restricted areas of mASIC1a during gating and desensitization. We anticipate that VCF may provide critical information regarding the mechanism of action of inhibitors and modulators of ASIC function.

Our studies suggest that conformational changes that occur in the pre-TM2 following extracellular acidification are associated with activation but not desensitization of the channel. As only a slight change in the emission of the fluorescent probe is observed during desensitization of the channel, we hypothesize that the pre-TM2 has a similar organization in the open and performed in oocytes expressing Y424C channels covalently modified in the resting and desensitized states. Proton-gated currents were normalized to the response elicited by extracellular acidification before treatment. Wild type mASIC1a was irreversibly inhibited by the positively charged cysteine-modifying reagent MTSET at pH 7.0 (Fig. 7). However, a C70L mutation prevented covalent modification by MTSET of wild type channels (Fig. 7). The magnitude of the response to extracellular acidification was significantly different when channels carrying K421C, K422C, Y424C, and E425C mutations were covalently modified by MTSET at pH 7.0 or 7.4 (Fig. 7). Desensitization at pH 7.0 of channels carrying cysteine mutations in the pre-TM2 was similar to controls (C70L) (supplemental Table I). We found that introduction of cysteine mutations at position 422 and 423 in C70L channels significantly affected the response to repeated stimulations (p < 0.001) (Kruskal-Wallis test (nonparametric analysis of variance) followed by Dunn’s multiple comparisons test). Our data indicate that the pre-TM2 region has distinctive conformational changes when the channel is in either the resting or desensitized state.

E425C channels covalently modified by MTSET at pH 7.0 or 7.4. Whole-cell currents were elicited by a change in extracellular pH from 7.4 to 5.0 before and after treatment for 30 s with MTSET (100 μM) at pH 7.0 (i.e. the desensitized state) or 7.4 (i.e. the resting state). Fig. 6 shows representative tracings from experiments
desensitized states. Coric et al. (50) used chimeras of channels that display different rates of desensitization in response to extracellular acidification to identify residues that contribute to channel desensitization. They mapped a tract of three residues in the region connecting Ser83–Gln84–Leu85 within the palm domain responsible for most of the difference in the desensitization of rat and fish ASIC1. Cushman et al. (51) identified a region in ASIC3 where mutations affect channel desensitization without modifying channel activation. The palm domain directly connects to the TM1 and TM2 through residues 1 and 12, respectively (Fig. 1). Multiple hydrogen bonds between -strands likely provide structural stability and inte-

FIGURE 4. Mutation of Trp287 at the base of the thumb disrupts gating-associated conformational changes in the pre-TM2. VCF was performed as described under “Experimental Procedures.” A, representative recordings of desensitized states. Coric et al. (50) used chimeras of channels that display different rates of desensitization in response to extracellular acidification to identify residues that contribute to channel desensitization. They mapped a tract of three residues in the region connecting β1 to β2 within the palm domain (Ser83–Gln84–Leu85) responsible for most of the difference in the desensitization of rat and fish ASIC1. Cushman et al. (51) identified a region in β1 of ASIC3 where mutations affect channel desensitization without modifying channel activation. The palm domain directly connects to the TM1 and TM2 through β1 and β12, respectively (Fig. 1). Multiple hydrogen bonds between -strands likely provide structural stability and inte-

FIGURE 5. pH dependence of mASIC1a desensitization. Two-electrode voltage clamp was performed in oocytes expressing wild type mASIC1a channels as described under “Experimental Procedures.” The holding potential was −60 mV. A, representative recording of proton-activated currents elicited from various preconditioning pH values. B, effect of the preconditioning pH on activation of mASIC1a. Whole-cell currents were elicited by extracellular acidification to pH 5.0 from solutions of pH 8.0, 7.8, 7.4, 7.2, or 7.0. Currents were normalized to the signal obtained at pH 8.0 (n = 11).
griety to the palm domain. Therefore, conformational changes that occur in a specific area of the palm domain will likely affect the surrounding area. Our data suggest that Cys70, in the region connecting TM1 to β1, experiences a conformational reorganization during desensitization. We hypothesize that closing of the pore during desensitization may be accomplished by a reorganization of the palm domain.

It was proposed that the thumb undergoes conformational changes following proton binding/unbinding to a putative proton-binding site in the ectodomain and that these conformational rearrangements are transmitted to the pore through non-covalent interactions mediated by residues at the base of the thumb (31). Functional interaction between a residue at the base of the thumb (Trp287) and a residue in the region connecting TM1 to β1 (Tyr71) was recently confirmed by Li et al. (49). Paukert et al. (33) identified four residues in the same region (Glu63, His72, His73, and Asp78), highly conserved among proton-sensitive ASICs, where substitutions strongly affected pH sensitivity. His72 and His73 are located in the wrist within the region connecting TM1 to β1. Rat residues Glu63 and Asp78 are in TM1 and β1, respectively. Because Tyr71 and Trp287 are in close proximity in the crystal structure of cASIC1 in the desen-
Role of the Pre-TM2 in Gating of ASIC1a

sitized state, it was proposed that \( \pi-\pi \) interactions between these residues provide functional coupling between the extracellular domain and the pore of the channel (49). We found that channels carrying W287G/E425C mutations labeled with Alexa Fluor 488 do not display a change in either fluorescence or current in response to extracellular acidification, although these channels were labeled and expressed at the plasma membrane. We cannot define whether the W287G mutation alters the coupling of conformational rearrangements between the base of the thumb and the pore or, alternatively, if this mutation locks the channel in a specific conformation (i.e. desensitized state). However, our data indicate that pore opening occurs in conjunction with a conformational rearrangement in the pre-TM2. The interface between the binding and pore domains plays a critical role in gating of Cys loop ligand-activated receptors (52). Acetylcholine binding triggers opening of the pore in chimeric channels composed of an acetylcholine-binding protein and the pore domain of the serotonin type 3A receptor, suggesting that gating involves functional coupling between the ligand binding domain and the pore in this family of ion channels (52). We found that channels carrying A423C and Y424C mutations display reduced activity, although we did not define whether these mutations affect the trafficking and/or folding of the channel. Mutations introduced in the region connecting TM1 to TM2 has a different structural organization in the desensitized state. However, our data indicate that pore opening occurs in conjunction with a conformational rearrangement in the pre-TM2. Acetylcholine binding triggers opening of the pore in the extracellular transmembrane domain junction that leads to activation and desensitization of ASIC1a.

We observed a change in the fluorescence signal in labeled oocytes expressing E425C channels following a change in extracellular pH from acidic to neutral, which suggests that the pre-TM2 has a different structural organization in the desensitized and resting states. This finding was confirmed using the substituted-cysteine-accessibility method. State-dependent differences in accessibility to covalent modification by MTSET of residues in the tract 421–425 could be accomplished by a rotation and/or tilting of the pre-TM2. Clockwise rotational movements of the pre-TM2 will be significantly restricted by residues in the base of the thumb (Fig. 1C). We found that cysteine mutations introduced at position 422 and 423 significantly affected the response to repeated stimulation indicating that this region undergoes a considerable reorganization during channel gating and desensitization.

Imkme and McCleskey (53) proposed that the underlying mechanism of activation of ASIC involves removal of Ca\(^{2+}\) from a high affinity binding site. These investigators suggested that Ca\(^{2+}\) binds near the vestibule of the pore of ASIC3 blocking the permeation pathway (53). In their proposed model, during channel activation, protons bind to the Ca\(^{2+}\) site promoting Ca\(^{2+}\) release and allowing Na\(^{+}\) flux. This model argues that the channel opens through relief of Ca\(^{2+}\) blockade, rather than by an H\(^+\)/Ca\(^{2+}\)-triggered conformational change. Recently, Zhang et al. (54) suggested that an allosteric conformational change, rather than unblocking of the pore, modulates the gating of fish ASIC1 by Ca\(^{2+}\). Substitution of two residues (Glu\(^{425}\) and Asp\(^{425}\)) in the vestibule of the pore of rat ASIC1a completely abolished block by Ca\(^{2+}\) (55). We found that Glu\(^{425}\) undergoes a significant reorganization following extracellular acidification. Whether this conformational change is associated with relief of Ca\(^{2+}\) blockade remains undefined.

In summary, our studies suggest that proton binding to the ectodomain of mASIC1a induces a conformational change in the pre-TM2 region that is associated with opening of the channel pore. However, the pre-TM2 is not likely involved in channel desensitization. How structural rearrangements that occur in the ectodomain following extracellular acidification are communicated to the pore is unknown. We hypothesize that the extracellular-transmembrane domain junction plays a key role communicating conformational changes to the pore during activation and desensitization.

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Role of the Pre-TM2 in Gating of ASIC1α

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