Acid-sensing ion channels (ASICs) are trimeric cation channels that undergo activation and desensitization in response to extracellular acidification. The underlying mechanism coupling proton binding in the extracellular region to pore gating is unknown. Here we probed the reactivity toward methanethiosulfonate (MTS) reagents of channels with cysteine-substituted residues in the outer vestibule of the pore of ASIC1a. We found that positively-charged MTS reagents trigger pore opening of G428C. Scanning mutagenesis of residues in the region preceding the second transmembrane spanning domain indicated that the MTSET-modified side chain of Cys at position 428 interacts with Tyr-424, in the region preceding TM2 (pre-TM2). Western blot analysis of the double Cys position 428 interacted with Tyr-424, in the region preceding TM2 (pre-TM2). Western blot analysis of the double Cys mutant Y424C-G428C demonstrated that the second transmembrane spanning domain, associated by intersubunit disulfide bonds and were insensitive to MTSET. Despite the spatial constraints introduced by these intersubunit disulfide bonds in the outer vestibule of the pore, Y424C-G428C transitions between the resting, open, and desensitized states in response to extracellular acidification. This finding suggests that the opening of the ion conductive pathway involves coordinated rotation of the second transmembrane-spanning domains.

In this study, we explored the reactivity to methanethiosulfonate (MTS) reagents of channels bearing Cys mutations in the outer vestibule of the pore. Positively charged MTS reagents triggered pore opening of G428C. Double-mutant cycle analysis indicated that the MTSET-modified side chain of Cys at position 428 interacts with Tyr-424, in the region preceding TM2 (pre-TM2). Western blot analysis of the double Cys mutant Y424C-G428C established the presence of intersubunit disulfide bonds, though the apparent proton affinity for activation and desensitization of these channels was similar to controls. This finding implies a geometric constraint that supports a model in which ion permeation is accomplished by coordinated rotation of the TM2 segments.
**Experimental Procedures**

Reagents—[1-(Trimethylammonium)methyl] methanethiosulfonate bromide (MTSMT), [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET), [3-(trimethylammonium)propyl] methanethiosulfonate bromide (MTSPT), [2-aminoethyl] methanethiosulfonate hydrobromide (MTSEA), and sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES) were from Toronto Research Chemicals (North York, Ontario). Protease inhibitors mixture III was from EMD Chemicals Inc (Gibbstown, NJ). EZ-link-sulfo-NHS-LC-biotin, EZ-link-sulfo-NHS-SS-biotin, and streptavidin beads were from Pierce. CaptAvidin beads and D-biotin were from Invitrogen (Carlsbad, CA). Quikchange II XL site-directed mutagenesis kit was from Agilent Technologies, Inc. (Santa Clara, CA). Western Lightning Chemiluminescence Reagent was from PerkinElmer (Waltham, MA). Monoclonal mouse HA-specific antibody (HA.11) was from Covance, monoclonal mouse anti-specific antibody was from Sigma, peroxidase-labeled affinity purified goat antibody to mouse IgG was from KPL (Gaithersburg, MD). Reagents for electrophoresis were from Bio-Rad. All other reagents were from Sigma.

**Molecular Biology and Oocyte Expression**—Site-directed mutagenesis was performed using QuikChange II XL according to manufacturer’s instructions. Mutations were confirmed by direct sequencing. Constructs were generated in a mouse ASIC1a template bearing a C70L mutation as previously described (11). *Xenopus laevis* oocytes stages 5–6 were injected with 0.3–3 ng of cRNA encoding ASIC1a mutants. Oocytes were maintained at 18 °C in modified Barth’s solution (MBS) containing (mM) 88 NaCl, 1 KCl, 2.4 NaHCO₃, 15 HEPES, 0.3 Ca (NO₃)₂, 0.41 CaCl₂, 0.82 MgSO₄, 10 mM Tris, 3% Triton X-100, and 1% of protease inhibitors mixture III, pH 7.4. Oocytes were homogenized in a buffer containing 100 mM NaCl, 50 mM Tris, and 1% of protease inhibitor mixture III, pH 7.4. Oocytes were homogenized with an insulin syringe. To remove the yolk proteins and nuclei, the homogenate was centrifuged at 200 × g for 10 min. Then, solubilization buffer (100 mM NaCl, 50 mM Tris, 3% Triton X-100, and 1% of protease inhibitors mixture III, pH 7.4) was added at a 1:3 ratio to the supernatant, and the mix was rotated for 2 h at 4 °C. Insoluble material was sedimented by centrifugation for 15 min at 30,000 × g and 4 °C. The supernatant was used for biotinylated protein pull-down and Western blot (see below).

**Electrophysiology**—Two-electrode voltage clamp was performed with a TEV-200A amplifier (Dagan Corporation, Minneapolis, MN) as previously described (11). Experiments were performed 24–48 h after injection at room temperature 20–25 °C. The resistance of the glass electrodes was less than 2 megohms when filled with 3 M KCl. Oocytes were clamped at a holding potential of −60 mV. The recording solution contained (mM) 110 NaCl, 2 KCl, 1 CaCl₂, 10 HEPES, pH 7.4. Acidic test solutions were buffered with MES. MTS reagents were directly dissolved in the recording solution prior to the experiments. Solutions of pH 7.0 and 7.4 containing MTS reagents were used within 10 min of preparation. Solutions of pH 8.0 containing MTSET were prepared immediately before each experiment.

**Rates of MTSET Hydrolysis**—MTS reagents are relatively unstable in alkaline solutions, and the rate of hydrolysis depends of the pH of the solution (12). To estimate the rates of MTSET hydrolysis in the recording solutions at pH 7.0 and 7.4, we use a method previously described by Stauffer and Karlin (13, 14). 5-thio-2-nitrobenzoate (TNB) has maximum absorbance at 412 nm and the reaction with methanethiosulfonate produces a decrease in its absorbance (13). TNB was generated from the reaction of 2 mM 5,5’-dithiobis (2-nitrobenzoate) with 50 μM of dithiothreitol in NT50 buffer (50 mM NaCl, 10 mM Tris, 3 mM NaN₃, 1 mM EDTA, pH 8.1). For each experimental condition 100 μl of test solution (blank or MTSET solution) was combined with 900 μl of TNB solution. To estimate the effect of pH on the half-time of hydrolysis of MTSET, we dissolved the reagent to a final concentration of 50 μM in recording solutions of pH 7.0 or 7.4. A standard curve was generated with various concentrations of MTSET. The concentration of non-hydrolyzed MTSET remaining after incubation was calculated from the standard curve. MTSET solutions were incubated at room temperature. The half-time of hydrolysis of MTSET in the recording solution at pH 7.0 and 7.4 was 130 min and 36 min, respectively. NaN₃ catalyzes the hydrolysis of MTS reagents (14). In agreement with the finding of Vemana et al., the half-time of hydrolysis of MTSET in NT50 was 4.8 min.

**Biotinylation of Surface Proteins**—All the procedures were performed at 4 °C to minimize protein endocytosis. 24 h post-injection groups of 8–20 oocytes expressing untagged or mutants with C-terminal HA epitope tags were placed in individual wells of a cell culture plate and incubated in MBS without antibiotics on ice for 30 min. Each group of oocytes was washed twice with 6 ml of biotinylation buffer (10 mM triethanolamine, 150 mM NaCl, 1 mM CaCl₂, pH 8.0) and then incubated for 15 min with 3 ml of biotinyltion buffer containing either EZ-link-sulfo-NHS-SS-biotin (1 mg/ml) or EZ-link-sulfo-NHS-LC-biotin (1 mg/ml). To quench the reaction, each group of oocytes was washed twice with 6 ml of quench buffer (MBS + 192 mM glycine) and then incubated in quench buffer for 5 min with gentle agitation. Finally, oocytes were washed twice with MBS without antibiotics before performing the homogenization.

Oocytes were homogenized and membrane protein solubilization—Biotinylated oocytes were transferred to a 1.5 ml tube and resuspended in homogenization buffer (100 mM NaCl, 50 mM Tris, and 1% of protease inhibitor mixture III, pH 7.4). Oocytes were homogenized with an insulin syringe. To remove the yolk proteins and nuclei, the homogenate was centrifuged twice at 200 × g for 10 min. Then, solubilization buffer (100 mM NaCl, 50 mM Tris, 3% Triton X-100, and 1% of protease inhibitors mixture III, pH 7.4) was added at a 1:3 ratio to the supernatant, and the mix was rotated for 2 h at 4 °C. Insoluble material was sedimented by centrifugation for 15 min at 30,000 × g and 4 °C. The supernatant was used for biotinylated protein pull-down and Western blot (see below).

**Biotinylated Protein Pull-down**—To recover the biotinylated proteins, solubilized supernatants were rotated overnight at 4 °C with 50 μl of washed streptavidin or CaptAvidin beads.

**Detection of the Surface Pool of ASIC1a**—Proteins recovered with streptavidin beads were boiled in 30 μl of Laemmli sample buffer containing β-mercaptoethanol (β-ME) for 5 min. A fraction of the total cell lysate, as well as streptavidin-recovered proteins, was separated by SDS-PAGE. Western blots were performed as indicated below.

**Detection of Intersubunit Disulfide Bonds**—24 h postinjection, groups of 20 oocytes expressing untagged (G428C) or mutants with C-terminal HA epitope tags (G428C-HA and Y424C-G428C-HA) were incubated in MBS without antibiotics on ice for 30 min. Oocyte surface proteins were biotinylated with EZ-link-sulfo-NHS-LC-biotin (1 mg/ml) as indicated above. Oocytes were homogenized in a buffer containing N-ethyl-maleimide (NEM) to block free thiol groups and pre-
TABLE 1
Biochemical characterization of mASIC1a mutants

| pH dependence of activation | Fractional activation pH 7.0 | Fractional activation pH 7.4 |
|----------------------------|----------------------------|----------------------------|
| pH<sub>50</sub> | pH<sub>70</sub> | n | pH<sub>70</sub> | pH<sub>74</sub> |
|----------------- |----------------- |-----|----------------- |----------------- |
| C70L            | 6.34 ± 0.07     | 7.34 ± 0.01 | 8.49 ± 1.15 | 0.04 ± 0.01 | 0.76 ± 0.03 |
| G428C           | 5.99 ± 0.07     | 7.30 ± 0.02 | 10.91 ± 1.97 | 0.04 ± 0.01 | 1.00 ± 0.04 |
| K422A-G428C     | 6.03 ± 0.06     | 7.35 ± 0.02 | 10.96 ± 4.00 | 0.01 ± 0.01 | 0.70 ± 0.02 |
| Y424C-G428C     | 6.19 ± 0.09     | 7.38 ± 0.01 | 8.64 ± 1.62 | 0.04 ± 0.01 | 0.88 ± 0.05 |
| Y424L-G428C     | 5.40 ± 0.06     | 7.35 ± 0.01 | 25.72 ± 6.71 | 0.06 ± 0.01 | 1.09 ± 0.05 |
| Y424F-G428C     | N.D.            | N.D.         | N.D.         | N.D.          | N.D.          |
| Y424C-G428A     | 5.54 ± 0.02     | 7.31 ± 0.02 | 6.75 ± 1.32 | 0.10 ± 0.02 | 0.85 ± 0.03 |
| E425A-G428C     | 6.38 ± 0.09     | 7.34 ± 0.03 | 14.6 ± 7.54 | 0.02 ± 0.01 | 0.82 ± 0.03 |

vent intersubunit disulfide bond formation (30 mM NEM, 50 mM Tris, 100 mM NaCl, and 1% of protease inhibitors mixture III, pH 7.4) (15). Proteins were solubilized and recovered with CaptAvidin beads as described above. Proteins were eluted from CaptAvidin beads with a high pH solution (50 mM sodium CaptAvidin beads as described above. Proteins were eluted with a high pH solution from the 3HGC structure. In addition, we included symmetry restraints to minimize deviations from the 3-fold symmetry observed in the 3HGC structure.

RESULTS

Gating the Pore of mASIC1a—To define structural rearrangements associated with gating and desensitization of ASIC1a, we explored the reactivity toward MTS reagents of channels bearing Cys substitution in individual residues of the tract Ile-426 to Gln-436. Residues in this tract constitute the outer vestibule of the pore. Channels with the C70L mutation were used as background in our studies. These channels have parameters of activation and desensitization that are similar to wild-type channels (pH<sub>50</sub> 6.34 ± 0.07 and pH<sub>70</sub> 7.34 ± 0.01) (Table 1), but unlike wild type channels, C70L are insensitive to extracellular acidification. A modest change in the response to extracellular acidification following MTSET treatment was observed in oocytes expressing I426C and A427C (Fig. 1). MTSET activates quiescent Cys in the absence of a change in extracellular pH (Fig. 2). Glys-428 is located about one helical turn upstream of the “degenerin” site at Gly-431 in mASIC1a. ENaC/Deg channels have a conserved “degenerin” site within the TM2 segment where bulky substitutions cause gain of function (20–22). The current increase elicited by MTSET in oocytes expressing G428C, relative to the current increase elicited by a change in extracellular pH from 7.4 to 5.0, was dependent on the extracellular pH. The relative response elicited by MTSET at pH 7.0 and 7.4 was 0.87 ± 0.06 (n = 37) and 0.32 ± 0.04 (n = 37), respectively. Our studies suggest that covalent modification by MTSET of Cys at posi-

C<sub>α</sub>-C<sub>α</sub> restraint between G435 (436 in chicken numbering) from different subunits, which recapitulates the narrowest approach of the TM2 helices in the 3HGC structure. In addition, we included symmetry restraints to minimize deviations from the 3-fold symmetry observed in the 3HGC structure.

Data and Statistical Analysis—Data are expressed as means ± S.E. (n), where n equals the number of independent experiments analyzed. A p value of less than 0.05 was considered statistically significant. The time constants for the reaction of the MTS reagents with mutant channels were estimated by fitting experimental data to a single exponential function with a sloping baseline, I = a<sup>−t/τ</sup> + mt + c, where I is the macroscopic current, τ is the time constant, and c, a, and m are constants determined by curve fitting. The second-order rate constants for the reaction of MTS reagents were estimated as described by Wilson and Karlin (19).
tion 428 triggers a conformational change that opens the ion permeation pathway.

Extracellular acidification initiates a sequence of events that result in activation and desensitization of ASIC1a. Desensitization occurs in a narrow range of pH and exhibits positive cooperativity, as indicated by the positive value of the Hill coefficient (Table 1). Whereas activation occurs when extracellular pH drops below pH 7.0. Similar to wild-type channels, G428C resides in the resting and desensitized states at pH 7.4 and 7.0, respectively (Fig. 2, b and d, and Table 1). The response to extracellular acidification of MTSET-modified channels was similar at pH 7.0, 6.5, 6.0, 5.5, and 5.0 (Fig. 3, b and c). This pH dependence of activation, with a peak effect occurring at a pH as high as 7.0, is clearly distinct from the dose-dependent activation of unmodified G428C (Fig. 2). MTSET modification of G428C may shift the pH dose-response relationship to more basic pH values, where desensitization also occurs. In addition, G428C are active at pH 7.4. The facts mentioned above do not allow us to define properly the shift in the dose-response relationship for activation and desensitization. The modification of G428C by MTSET was irreversible, as suggested by the change in the response to extracellular acidification (Fig. 3, a and b).

To define structural requirements for activation of G428C, we evaluated the response to negatively and positively charged MTS reagents in the resting and desensitized states. Positively charged MTS reagents (MTSMT, MTSET, MTSPT, and MTSEA) robustly activated G428C (Fig. 4a). MTSES, a negatively-charged thiol-modifying reagent, only elicited a modest increase in current in oocytes expressing G428C at pH 7.0. The magnitude and time course of the change in current evoked by positively charged MTS reagents was dependent on the conformational state of the channel, i.e. resting or desensitized (Fig. 4, a and b). MTSMT, MTSET, and MTSPT are MTS reagents with a trimethylammonium group and different linkers (methyl, ethyl, and propyl) (Fig. 4c). The magnitude of the response elicited by MTSMT, MTSET, and MTSPT was similar at pH 7.0, i.e. channels in the desensitized state (Fig. 4a). However, we observed a dependence on the length of linker when MTS reagents were applied at pH 7.4, i.e. channels in the resting state (Fig. 4a). Our results suggest that the transferable moiety...
Opening the Pore of ASIC1a

Mechanism of Activation of G428C Channels by MTS Reagents—We generated a homology model of mouse ASIC1a-G428C using MODELLER (23) and the resolved structure of chicken ASIC1 (PDB code 3HGC) as a template. The ASIC1a-G428C model clearly shows the side chain of Cys-428 pointing toward residues in the pre-TM2 region (Fig. 5, a and b). To elucidate the mechanism of activation of G428C by MTS reagents, we mutated residues within the pre-TM2 region and investigated the response to MTSET. These substitutions were generated in G428C channels. The relative response to MTSET was substantially increased in Y424L-G428C and Y424F-G428C (Fig. 5). However, these mutations may have also affected the response to extracellular acidification. The peak current evoked by a drop in extracellular pH from 7.4 to 5.0 in oocytes expressing Y424F-G428C was $-362 \pm 137$ nA, while the peak current evoked by MTSET was $-2878 \pm 875$ nA ($n = 22$). The variability of the response to extracellular acidification impeded the generation of a pH dose-response curve for Y424F-G428C (Table 1). To overcome this limitation we performed double-mutant cycle analysis (see below). Y424C-G428C responded to extracellular acidification, but not to MTSET (Figs. 5 and 7). Channels bearing G428C-Y424A, G428C-Y424E, G428C-Y424Q, G428C-Y424K, G428C-Y424N mutations were neither activated by MTSET nor by extracellular acidification. The surface expression of G428C-Y424A, of the MTS reagents, when covalently attached to Cys at position 428, interacts with a residue in close proximity. Based on the results shown in Fig. 4a, we conclude that the distance between the interacting residues is shorter in the desensitized state than in the resting state. We fit the time course of modification of G428C by MTS reagents to an exponential function with a sloping baseline and estimated the second-order rate constants as previously described (19). The second-order rate constants of reaction for positively charged MTS reagents were different at pH 7.0 and 7.4 (Fig. 4b).

FIGURE 2. Activation of G428C channels by MTSET. a, sequence alignment of ENaC/Deg family members. Gly-428 in mASIC1a and the "degenerin" site are shown in gray and underlined gray, respectively. b, representative recording of proton-activated currents in oocytes expressing G428C (note that the magnitude of the response is determined by the preconditioning pH). c, pH-dependence of activation of G428C. Whole-cell currents were elicited by a change in extracellular pH from 7.4 to solutions of lower pH. Currents were normalized to the response obtained at pH 4.0 ($n = 12–27$). d, pH dependence of desensitization of G428C. Whole-cell currents were elicited by extracellular acidification to pH 5.0 from solution of pH 8.0, 7.8, 7.4, 7.2, or 7.0. Currents were normalized to the response elicited at pH 7.0 before and after treatment with MTSET (1 mM) at pH 8.0. Whole-cell currents were elicited by a change in extracellular pH from 7.4 to solutions of lower pH. Currents were normalized to the response elicited for a change in extracellular pH from 7.4 to 5.0 before covalent modification ($n = 12$).

FIGURE 3. Activation of MTSET-modified G428C by extracellular acidification. a, representative recording of proton-activated currents in oocytes expressing G428C. Whole-cell currents were elicited by a change in extracellular pH from 7.4 to 7.0 before and after treatment with MTSET (1 mM) at pH 8.0. Tracings are representative of 11 independent experiments. b, effect of extracellular pH on MTSET-modified G428C. Channels were covalently modified with MTSET (1 mM) at pH 8.0. Tracings are representative of 12 independent experiments. c, fractional activation of MTSET-modified G428C by extracellular acidification. Oocytes expressing G428C were treated with MTSET (1 mM) at pH 8.0. Whole-cell currents were elicited by a change in extracellular pH from 7.4 to solutions of lower pH. Currents were normalized to the response elicited for a change in extracellular pH from 7.4 to 5.0 before covalent modification ($n = 12$).

FIGURE 4. Activation of G428C channels by MTSET. a, fractional activation of MTSET-modified G428C by extracellular acidification. The relative response evoked for a change in extracellular pH from 7.4 to 5.0 before covalent modification ($n = 12$).
G428C-Y424E, and G428C-Y424C was markedly reduced compared with controls (G428C), suggesting that some substitutions at position 424 lead to improper folding or poor trafficking of the channel to the membrane (Fig. 6). We did not investigate the surface expression of other channels bearing mutations at position 424. We previously demonstrated that MTSET treatment modifies the response to extracellular acidification of Y424C, indicating that the thiol group of the Cys is reduced under basal conditions and accessible for covalent modification (see Ref. 11 and Fig. 8, a–d). However, MTSET treatment did not affect the response of Y424C-G428C to extracellular acidification (Fig. 7). We hypothesized that intrasubunit or intersubunit disulfide bonds may form between Cys residues at positions 424 and 428, preventing MTSET modification at position 428. We performed Western blot analysis under reducing and non-reducing conditions with the pool of Y424C-G428C expressed at the cell surface to determine the presence of intersubunit disulfide bonds. Oocytes were homogenized in the presence of NEM to prevent the formation of intersubunit disulfide bonds between intracellular Cys residues (15). A band of ~60 kDa, consistent with the molecular mass of a monomer, was immunodetected under reducing and non-reducing conditions in the surface pool recovered from oocytes expressing G428C-HA (Fig. 7e). In the recovered surface pool of oocytes expressing Y424C-G428C-HA we immunodetected bands of ~60 and 190 kDa under reducing and non-reducing conditions, respectively. The 190 kDa band is consistent with the molecular weight of a homo-trimer. A minor fraction of the Y424C-G428C-Y424E, and G428C-Y424C was markedly reduced compared with controls (G428C), suggesting that some substitutions at position 424 lead to improper folding or poor trafficking of the channel to the membrane (Fig. 6). We did not investigate the surface expression of other channels bearing mutations at position 424. We previously demonstrated that MTSET treatment modifies the response to extracellular acidification of Y424C, indicating that the thiol group of the Cys is reduced under basal conditions and accessible for covalent modification (see Ref. 11 and Fig. 8, a–d). However, MTSET treatment did not affect the response of Y424C-G428C to extracellular acidification (Fig. 7). We hypothesized that intrasubunit or intersubunit disulfide bonds may form between Cys residues at positions 424 and 428, preventing MTSET modification at position 428. We performed Western blot analysis under reducing and non-reducing conditions with the pool of Y424C-G428C expressed at the cell surface to determine the presence of intersubunit disulfide bonds. Oocytes were homogenized in the presence of NEM to prevent the formation of intersubunit disulfide bonds between intracellular Cys residues (15). A band of ~60 kDa, consistent with the molecular mass of a monomer, was immunodetected under reducing and non-reducing conditions in the surface pool recovered from oocytes expressing G428C-HA (Fig. 7e). In the recovered surface pool of oocytes expressing Y424C-G428C-HA we immunodetected bands of ~60 and 190 kDa under reducing and non-reducing conditions, respectively. The 190 kDa band is consistent with the molecular weight of a homo-trimer. A minor fraction of the Y424C-G428C subunits at the cell surface are associated forming dimers. This experiment indicates that Y424C-G428C monomers are covalently associated by intersubunit disulfide bonds formed between Cys residues at position 424 and 428 (Fig. 7e). Surprisingly, the apparent proton affinities for activation and desensitization of Y424C-G428C were similar to those from controls (G428C) (Fig. 7 and Table 1), suggesting that these intersubunit disulfide bonds in the outer vestibule of the pore do not impede conformational rearrangements associated with proton-dependent gating. Neither β-ME nor TCEP affected the proton-dependent activation of Y424C-G428C (data not shown). MTSET did not activate Y424C-G428C pre-treated with TCEP (data not shown). We speculate that disulfide bonds formed between Cys residues at position 424 and 428 may be inaccessible to these reducing reagents.

To confirm the interaction of the MTSET-modified side chain of Cys at position 428 and Tyr-424 we performed double mutant cycle analysis. In essence, we substituted the Gly residue at position 428 in channels bearing Y424C mutations. As mentioned above, MTSET modifies the response of Y424C to extracellular acidification in a state-dependent manner (see reference (11) and Fig. 8, a–d). We generated two constructs Y424C-G428L and Y424C-G428A. MTSET evoked an increase in current in oocytes expressing Y424C-G428A that was substantially larger in magnitude at pH 7.0 than 7.4 (Fig. 8, g–i). Y424C-G428L were insensitive to extracellular acidification and MTSET (data not shown).

Y424C-G428A was irreversibly modified by MTSET (Fig. 9). Moreover, we observed that a change in extracellular pH from 8.0 to 7.4 elicited a substantial increase in current in covalently modified Y424C-G428A (Fig. 9a). The response to a change in extracellular pH from 7.4 to 7.0, 6.5, 6.0, 5.5, and 5.0 could not be properly evaluated because of the baseline decay observed when extracellular pH was changed from 8.0 to 7.4. These experiments suggest that the transferable moiety of MTSET covalently attached to Cys at position 424 interacts with the side chain of Ala at position 428.

**DISCUSSION**

ASICs are ligand-gated ion channels that undergo activation and desensitization in response to extracellular acidification. Recent studies suggested that activation and desensitization of ASICs involve a broad reorganization of the extracellular domain rather than a restricted region (6, 8–10). Jasti et al. (6)
Opening the Pore of ASIC1α

![Diagram showing the pore of ASIC1α with various amino acids highlighted.](image)

### Figure C

| Condition | Current (μA) | Time (15 s) |
|-----------|-------------|-------------|
| G428C     | 2           |             |
| K422A-G428C | 4         |             |
| E425A-G428C | 4         |             |
| Y424L-G428C | 4         |             |
| Y424F-G428C | 2         |             |
| Y424C-G428C | 0.2       |             |

### Figure D

Bar graph showing relative response for different conditions:

- G428C
- K422A-G428C
- Y424L-G428C
- Y424F-G428C
- E425A-G428C

* indicates significant difference.
Opening the Pore of ASIC1a

Mutations at position 424 reduce surface expression of mASIC1a. a, surface expression of mASIC1a mutant channels. Oocytes expressing control (G428C) and C-terminal HA epitope-tagged mutant channels were biotinylated at 4°C and surface proteins were recovered with streptavidin beads. Surface proteins and a fraction of the total cell lysate were immunoblotted with a HA-specific antibody. Membranes were also immunoblotted with an antibody against β-actin to corroborate that only surface proteins were biotinylated (Data not shown). Numbers to the left of the image represent the mobility of Bio-Rad Precision Plus protein standards in kDa. b, relative expression of control (G428C-HA) and mutant channels at the cell surface, *p < 0.01 and **p < 0.001 (n = 3–6) (Kruskal-Wallis test following by Dunn’s multiple comparisons test). The intensity of the protein bands was quantified with Quantity One software.

FIGURE 6. Mutations at position 424 reduce surface expression of mASIC1a. a, surface expression of mASIC1a mutant channels. Oocytes expressing control (G428C) and C-terminal HA epitope-tagged mutant channels were biotinylated at 4°C and surface proteins were recovered with streptavidin beads. Surface proteins and a fraction of the total cell lysate were immunoblotted with a HA-specific antibody. Membranes were also immunoblotted with an antibody against β-actin to corroborate that only surface proteins were biotinylated (Data not shown). Numbers to the left of the image represent the mobility of Bio-Rad Precision Plus protein standards in kDa. b, relative expression of control (G428C-HA) and mutant channels at the cell surface, *p < 0.01 and **p < 0.001 (n = 3–6) (Kruskal-Wallis test following by Dunn’s multiple comparisons test). The intensity of the protein bands was quantified with Quantity One software.

proposed that residues at the base of the thumb communicate conformational changes initiated at the proton-binding sites to the pore. A recent study from Li et al. proposed that π-π interactions between Tyr-71, in the region connecting the TM1 segment to the extracellular region, and Trp-287, at the base of the thumb, provide functional coupling between the extracellular domain and the pore of the channel (9). Voltage clamp fluorometry studies indicated that extracellular acidification triggers a conformational rearrangement in the pre-TM2 region, which correlates with channel activation (11). Our studies extend this idea and provide insight into the molecular mechanism that mediates pore opening following extracellular acidification.

Activation of G428C by MTS Reagents—Wild-type ASIC1a and G428C reside in the resting and desensitized state at pH 7.4 and 7.0, respectively. The rate constant for the reaction of an MTS reagent and a Cys residue depends on the accessibility of the MTS reagent to the target Cys and the reactivity toward the Cys sulphydryl group. However, a Cys residue could be covalently modified by a MTS reagent without a noticeable effect on function. For instance, MTSET elicited only a modest increase in current at pH 8.0 in oocytes expressing Y424C-G428A, although the sensitivity of the channel to extracellular pH was significantly altered (Fig. 9). Besides the accessibility and reactivity of the target Cys residue toward a MTS reagent, the likelihood of observing a change in function will likely depend on structural factors. The magnitude of the response to a change in extracellular pH from 7.4 to 5.0 was similar when Y424C and Y424C-G428A were modified by MTSET at either pH 7.0 or pH 7.4 (Fig. 2), suggesting that the reactivity and accessibility of the thiol groups was independent of the conformational state of the channel. Moreover, the reactivity of the target Cys depends on the state of protonation of the Cys sulphydryl group, and it is preferential toward the thiolate anion. Independently of the pK_a of the sulphydryl group, the number of protonated groups should be higher at pH 7.0 than 7.4. The rate constants for the reaction of G428C and MTS reagents were faster at pH 7.0 than 7.4. Therefore, differences in the rate constant for the reaction of G428C and MTSET at pH 7.0 and 7.4 does not result from differences in the protonation state of the reactive sulphydryl group. The rate constants for the reaction of G428C with MTSSM, MTSET, or MTSSPT were similar to each other when performed at pH 7.0 or pH 7.4, suggesting that the accessibility to the target Cys is not dependent on the atomic volume of the MTS reagents. Our studies suggest that structural differences in the resting and desensitized states account for the observed changes in the reaction of MTS reagents with G428C.

Coupling Extracellular Acidification to Pore Opening—To gain insight into the mechanism of activation of G428C by positively charged MTS reagents we mutated residues in the pre-TM2 region and we studied the response evoked by MTSET. Substitutions at position 424 markedly affected the response of G428C to MTSET. Tyr-424 is localized in the extracellular-transmembrane domain interface with its side chain facing the interior of the outer vestibule of the pore. Our results showed that only channels with non-polar residues (Tyr, Phe, Leu, and Cys) at position 424 are electrically functional. Substitutions at this position with polar amino acids affected the surface expression of ASIC1a (Fig. 6). MTSET activated G428C and Y424F-G428C similarly (see Fig. 6c). However, the response to extracellular acidification was apparently reduced in Y424F-G428C compared with G428C, suggesting that Tyr-424 is important to couple proton binding in the extracellular region to pore opening. Phe and Tyr have a similar surface area, but they differ in their hydrophobic character and their ability to form hydrogen bonds. It is conceivable that during proton-dependent gating the hydrophobe of Tyr-424 participates in the formation of a transient hydrogen bond. Alternatively, Tyr-424 may partition into or interact with the lipid bilayer during proton-dependent activation. Therefore, the hydrophobic character of the residue at position 424 may affect these protein-lipid interactions, and consequently proton-dependent gating of the channel.

Mechanism of Activation of G428C by MTS Reagents—Our studies suggest that the transferable moiety of positively charged MTS reagents, when covalently attached to Cys-428, interacts with Tyr-424. Although a cation-π interaction between the positive charge of the transferable moiety and the benzene of Tyr may occur, it cannot explain the activation of Y424L-G428C by MTSET (24). The nature of the chemical entities involved in this interaction suggests that hydrogen

FIGURE 5. Mutations at position 424 affect the response to MTSET. a and b, homology model of mASIC1a-G428C in the desensitized state. The homology model was generated with Modeler (Sali Lab) using the resolved structure of chicken ASIC1 in the desensitized state (3HGC) as template. Mutagenesis and energy minimization were performed with oocytes expressing controls (G428C) and mutant channels treated with MTSET at pH 7.0. d, relative response to MTSET of controls (G428C) and mutant channels. The peak current elicited by MTSET was normalized to the peak current elicited by extracellular acidification. *, p < 0.001 (n = 10–22) (Kruskal-Wallis test following by Dunn’s multiple comparisons test).
bonds, salt bridges, and hydrophobic interactions do not play a substantial role in the activation of G428C channels by MTS reagents. We propose that steric repulsion between the transferable moiety of the MTS reagents and the side chain of Tyr-
424 initiates a conformational rearrangement in the pre-TM2 region that gates the pore.

MTSMT, MTSET, and MTSPPT evoked an increase in current of similar magnitude in oocytes expressing G428C channels at pH 7.0, i.e. channels in the desensitized state. The rate constants for reaction of MTSMT, MTSET, and MTSPPT were similar at pH 7.4. However, the magnitude of the response to positively charged MTS reagents in the resting state showed a strong dependence on the length of the transferable moiety of the MTS reagents. We propose that the magnitude of the response depends on the distance between the transferable moiety of the MTS attached at position 428 and the side chain of Tyr-424. The larger the moiety attached at position 428, the greater the steric repulsion generated with the side chain at position 424. Our data suggest that the distance between the interacting residues at positions 424 and 428 is different in the resting and desensitized states.

Desensitization entails structural changes that mediate pore closing when extracellular pH drops below 7.4. The mechanism that mediates channel desensitization is unknown. Previous studies have shown that selected residues in the palm domain within the extracellular region of ASICs contribute to channel desensitization (25–28). Then, desensitization-mediated pore closing must be necessarily accomplished through a reorganization of the extracellular transmembrane domain interface, since this region connects the ectodomain to the pore-forming transmembrane domains. The differential effects that positively-charged MTS reagents have on G428C and Y424C-G428A at pH 7.0 and 7.4 suggest that during desensitization the pre-TM2 region reorients. Desensitization-mediated pore closing may be accomplished by this conformational change in the pre-TM2 region, or alternatively this reorganization could be secondary to other structural rearrangements linked to desensitization.

Opening the Pore of mASIC1a—While MTS reagents covalently modify Y424C and G428C, Y424C-G428C are activated by extracellular acidification, but are insensitive to MTSET. Our data suggest that the reduced macroscopic activity of Y424C-G428C, compared with G428C, is due to a significant reduction in the expression of the channel at the cell surface (Fig. 6). Western blot analysis showed that Y424C-G428C migrates under non-reducing conditions essentially as a homo-trimer stabilized by intersubunit disulfide bonds. Surprisingly, the apparent proton affinities for activation and desensitization of Y424C-G428C were similar to those of controls. It is possible that an undetectable pool of channels composed of non-linked subunits is responsible for the observed change in current in response to extracellular acidification in oocytes expressing Y424C-G428C. However, our data indicate that Y424C-G428A and functional G428C that also have a mutation at residues Lys-422, Tyr-424, or Glu-425 were modified by MTSET, the only exception was Y424C-G428C. We consider it unlikely that the lack of response of Y424C-G428C to MTSET is consequence of a structural change that limit MTSET access to the target Cys residues.

The pore of ASIC1 in the presumed desensitized-like state has an hourglass shape, where the narrowest point of the hourglass is defined by the intersection of the TM2 segments (Fig. 5b). It is clear that conformational restrictions on the outer vestibule of the pore arising from intersubunit 424–428 disulfide bonds do not prevent the channel from cycling through the resting, open and desensitized states (Figs. 5 and 7 and Table 1). Our data suggest that the distance between residues 424 and 428 (when not linked) varies between resting and desensitized states. With respect to the pore, these disulfide bonds would...
restrict any substantial radial TM2 tilt leading to pore widening (Fig. 10). However, a simultaneous twist of the TM2 helices to maintain good bond geometry could widen the pore in an aperture-like fashion (see Fig. 10). The transmembrane architecture of ASIC1 suggests that a modest circumferential TM2 tilt may be allowed during pore opening. We propose that such coordinated motions in the clockwise direction (from the extracellular perspective) mediate ion pore opening. Indeed, normal mode analysis, a computational approach to predict collective dynamics and inherent flexibilities in biological molecules, suggested that proton binding to the extracellular region of ASIC1 drives an intrinsic rotation of the TM segments (29). In summary, our results indicate that opening of the ion pore could be accomplished by a concerted global rotation of the TM2 helices.

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