The acid-sensing ion channel (ASIC) subunits ASIC1, ASIC2, and ASIC3 are members of the amiloride-sensitive Na\(^+\) channel/degenerin family of ion channels. They form proton-gated channels that are expressed in the central nervous system and in sensory neurons, where they are thought to play an important role in pain accompanying tissue acidosis. A splice variant of ASIC2, ASIC2b, is not active on its own but modifies the properties of ASIC3. In particular, whereas most members of the amiloride-sensitive Na\(^+\) channel/degenerin family are highly selective for Na\(^+\) over K\(^+\), ASIC3/ASIC2b heteromultimers show a nonselective component. Chimeras of the two splice variants allowed identification of a 9-amino acid region preceding the first transmembrane (TM) domain (pre-TM1) of ASIC2 that is involved in ion permeation and is critical for Na\(^+\) selectivity. Three amino acids in this region (Ile-19, Phe-20, and Thr-25) appear to be particularly important, because channels mutated at these residues discriminate poorly between Na\(^+\) and K\(^+\). In addition, the pH dependences of the activity of the F20S and T25K mutants are changed as compared with that of wild-type ASIC2. A corresponding ASIC3 mutant (T26K) also has modified Na\(^+\) selectivity. Our results suggest that the pre-TM1 region of ASICs participates in the ion pore.

ASICS\(^*\) belong to the NaC/DEG family of sodium channels whose members are involved in physiological functions as diverse as Na\(^+\) reabsorption in epithelia (epithelial Na\(^+\) channel), mechanotransduction (degenerins of Caenorhabditis elegans), and neurotransmission (Phe-Met-Arg-Phe-amide peptide-gated Na\(^+\) channel (FaNaC)). ASIC1, ASIC2, and ASIC3 (previously named ASIC (1), MDEG (2), and DRAASIC (3), respectively) are located in the central nervous system (ASIC1 and ASIC2) or in sensory neurons (ASIC1 and ASIC3). They are activated by a drop in extracellular pH. It has been proposed that ASICS in sensory neurons mediate the pain accompanying tissue acidosis (3). ASIC2b (previously named MDEG2) is a splice variant of ASIC2b that is not form a pH-activated channel by itself but acts as a modulatory subunit when associated with other ASIC subunits such as ASIC3. The ASIC3 response to a drop in pH is biphasic, with both current components being carried mainly by Na\(^+\) (3). Co-expressing ASIC3 with ASIC2b does not change the characteristics of the transient current, but the sustained current becomes nonselective (4). The native proton-gated current in dorsal root ganglia also shows a transient Na\(^+\)-selective component and a sustained nonselective component (5). Therefore, the ASIC2b/ASIC3 association appears to be a good candidate for the H\(^+\)-gated channel involved in pain sensation (4).

The ASIC2 and ASIC2b splice variants differ only in the first 185 N-terminal amino acids of ASIC2. Chimeric constructs of these splice variants led us to identify a region before the first transmembrane (TM1) domain of ASIC2 that controls the ion selectivity and the pH dependence of the channel. Point mutations in this particular region of the ASIC2 protein have led to identification of amino acids that play a key role in defining the biophysical properties of the channel. Corresponding mutations in ASIC3 induce similar changes in ion selectivity. These results indicate the important role of the region preceding the first transmembrane domain (pre-TM1) in the pore formation of ASICS.

**Experimental Procedures**

Molecular Biology—We used the previously described constructions of FaNaC in pRC/CMV vector (Invitrogen) (6) and of ASIC2, ASIC2b, and ASIC3 in pCI vector (Promega) (1–4). For the generation of chimeras, two complementary primers containing the sequence of the desired junction between ASIC2 and ASIC2b were chosen. Two different PCRs were performed, one on the ASIC2 plasmid with a junction primer and a specific ASIC2 primer, and the other on the ASIC2b plasmid with the other junction primer and a specific ASIC2b primer. Fragments were purified with the Wizard PCR Prep DNA purification system (Promega). Both fragments were mixed with the specific primers, and another PCR was performed. The PCR product was digested and subcloned in ASIC2 or ASIC2b expression plasmids. The PCR was carried out using the Expand High Fidelity PCR system (Boehringer Mannheim), and the subcloned fragments were sequenced. Five percent Me\(_2\)SO or GC Melt (CLONTECH) was added for PCR on the GC-rich ASIC2b 5′-translated region.

Site-directed mutagenesis was performed by PCR according to the QuickChange Site-Directed Mutagenesis Kit protocol (Stratagene) or with a variation of the method used for chimeras. Expression in COS Cells—COS cells, at a density of 20,000 cells/35-mm diameter Petri dish, were co-transfected with expression vectors containing CDS receptor cDNA and the PCI-ASIC vectors (a 1:5 ratio) using the DEAE-dextran method. Cells were used for electrophysiologically measurements 1–3 days after transfection. Successfully transfected cells were recognized by their ability to bind to CD8 antibody-coated beads (7).

Electrophysiology—Ion currents were recorded using either the whole cell or outside-out patch-clamp technique and stored on a hard disk for later off-line analysis. The pipette solution contained 120 mM KCl, 30 mM NaCl, 2 mM MgCl\(_2\), 5 mM EGTA, and 10 mM HEPES/KOH (pH 7.2). The bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM MgCl\(_2\), 2 mM CaCl\(_2\), and 10 mM HEPES/NaOH, pH 7.5 (Na\(^+\)-rich solution) or 145 mM KCl, 2 mM MgCl\(_2\), 2 mM CaCl\(_2\), and 10 mM HEPES/NaOH, pH 7.2 (K\(^+\)-rich solution).
The Pre-TM1 Domain of ASICs in the Ion Pore

Fig. 1. Reversal potentials of chimeric ASIC2/ASIC2b channels. A, topological organization of ASIC2 and its splice variant, ASIC2b. TM1 and TM2 indicate the transmembrane domains. The region common to ASIC2 and ASIC2b is indicated in light gray. B, horizontal bars in the schematic representation of chimeras indicate the junction positions between ASIC2 (gray) and ASIC2b (white). Numbers beneath the bars indicate amino acid position in ASIC2. This corresponds to the following positions in ASIC2b: 86 (C1), 66 (C2), and 57 (C3). I/V (current/voltage) curves shown on the right were obtained from whole cell recordings. Currents were normalized with respect to the peak current recorded at −60 mV. C, the change in current reversal potential depends on the junction position. Peak currents reversed at 32 ± 2 mV for wild-type ASIC2, at 7 ± 1 mV for C1, at 11 ± 3 mV for C2, and at 39 ± 2 mV for C3.

Table 1
Na⁺/K⁺ selectivity and inactivation time constants for ASIC2 mutants

An alignment between the pre-TM1 regions of ASIC2 and ASIC2b is shown above the table. Motifs conserved among all NaC/DEG family members are framed. Each ASIC2 residue was mutated in its ASIC2b counterpart. Mutations causing the largest change in Na⁺ selectivity (I19P, F20S, and T25K) are indicated with a dot. Reversal potentials and time constants for inactivation were determined for each mutant. Standard errors of the mean are shown. Asterisks indicate a significant difference from wild-type ASIC2, with p < 0.05, using an unpaired t test.

| Mutant     | E_{rev} (mV) | P_{Na⁺}/P_{K⁺} | τ (s) |
|------------|--------------|----------------|-------|
| ASIC2 WT   | 32 ± 2       | 9.8            | 3.2 ± 0.5 |
| I17G       | 33 ± 1       | 11.2           | 2.8 ± 0.8 |
| Q18R       | 31 ± 2       | 9.2            | 6.8 ± 0.1 |
| I19P       | * 20 ± 2     | 3.3            | 4.1 ± 0.8 |
| F20S       | * 10 ± 3     | 1.7            | 4.4 ± 0.5 |
| A21L       | * 23 ± 4     | 4.1            | 3.2 ± 0.1 |
| N22S       | * 25 ± 2     | 4.8            | 6.3 ± 1.5 |
| T23R       | * 27 ± 2     | 5.6            | 4.0 ± 1.0 |
| S24T       | * 30 ± 2     | 7.8            | 4.1 ± 1.1 |
| T25K       | * 6 ± 2      | 1.4            | 6.6 ± 1.2 |
| T25D       | * 17 ± 2     | 2.7            | 2.6 ± 0.2 |
| T25V       | * 7 ± 3      | 1.5            | 3.0 ± 0.1 |

Fig. 2. Outside-out patch recordings of ASIC2 F20S and T25K mutants. Outside-out patches were excised from transfected COS cells and exposed to a solution buffered at pH 5. Currents were normalized with respect to the peak current recorded at −60 mV. Na⁺ equilibrium potential = 40.1 mV. Voltage dependence of the peak current for ASIC2-F20S (A) and ASIC2-T25K (B) in a Na⁺-rich solution is shown. C and D, currents elicited at −60 mV by a drop in pH from pH 7.3 to pH 5 in a Na⁺-rich extracellular medium (left panel) or in a medium that had its Na⁺ replaced by K⁺ (right panel) for ASIC2-F20S (C) and ASIC2-T25K (D). The corresponding I/V (current/voltage) relationships obtained by a voltage ramp from −80 mV to 60 mV in Na⁺- or K⁺-containing medium are shown underneath (bottom panel). The currents recorded in the K⁺-rich extracellular solution reversed at −2 mV for ASIC2-F20S and 4 mV for ASIC2-T25K.

KOH, pH 7.3 (K⁺-rich solution). Changes in extracellular pH were induced by shifting one of six outlets of a microperfusion system in front of the cell patch. Test solutions with a pH < 5 were buffered with 10 mM MES rather than HEPES but were identical to the control solution in all other respects. Experiments were carried out at room temperature (20 °C to 24 °C).

RESULTS

The ASIC2 and ASIC2b splice variants are different in their first half, which includes the N-terminal region, the first transmembrane domain, and one-third of the extracellular loop. They are identical from amino acid 186 of ASIC2 onward (Fig. 1A). Replacement of the N-terminal intracellular region of ASIC2 (amino acids 1–42) with that of ASIC2b led to functional chimeras that displayed different properties compared with wild-type ASIC2 (Fig. 1, B and C, chimera C1). ASIC2 was selective for sodium over potassium (4, 8), as confirmed by the current reversal potential of 32 ± 2 mV (n = 19; pNa⁺/pK⁺ = 9.8), which is close to the sodium equilibrium potential of 41 mV. Chimeric C1 did not discriminate well between Na⁺ and K⁺ because the reversal potential (E_{rev}) was 7 ± 1 mV (n = 6), corresponding to a pNa⁺/pK⁺ ratio of 1.5. Chimeric C2, with a junction at amino acid 26 of the ASIC2 sequence, also had a lower selectivity for Na⁺ over K⁺ as compared with ASIC2 (E_{rev} = 11 ± 3 mV; n = 4; pNa⁺/pK⁺ = 1.9). Chimeric C3, with a junction at amino acid 17, was at least as selective for Na⁺ as the wild-type ASIC2 channel (E_{rev} = 32 ± 2 mV; n = 5).

The modification in ion selectivity observed with chimeras can thus be associated with a 9-amino acid region of ASIC2, from Ile-17 to Thr-25, located before the TM1. These nine amino acids were then systematically replaced in ASIC2 by those that are present in the ASIC2b sequence, and the reversal potential was determined for each mutant (Table 1). Shift-
ing the pH from pH 7.4 to pH 5 activated all mutant channels, with current amplitudes similar to that of wild-type ASIC2 (data not shown). Six mutants had reversal potentials that were significantly lower than that of ASIC2 (Table I). The largest changes in selectivity were observed for the I19P, F20S, and T25K mutants, with pNa−:pK+ values of 3.3 (n = 7), 1.7 (n = 5), and 1.4 (n = 8), respectively, whereas the pNa−:pK+ value calculated for wild-type ASIC2 was 9.8, as described previously (8). A similar change in selectivity occurred when Thr-25 was mutated for an apolar Val or a negatively charged Asp residue instead of a Lys residue. The pNa−:pK+ ratio was 2.7 for T25D (n = 4) and 1.5 for T25V (n = 4) (Table I).

The ASIC2α current inactivates exponentially and completely after a change in pH. The time constant for inactivation, which is 3.2 s for the wild-type, was also modified for some mutants. Mutants Q18R, N22S, and T25K had a time constant of inactivation that was twice as large as that for the wild-type (Table I).

The two mutants that are particularly altered in their ion selectivity, F20S and T25K, were analyzed in more detail. Unlike ASIC2, unitary currents recorded from the T25K and F20S mutants were very noisy, so that single-channel properties could not be determined. Reversal potentials for outside-out patches (ASIC2-F20S, 8 ± 2 mV, n = 4; ASIC2-T25K, 12 ± 2 mV, n = 4) were similar to these for the whole cell (Table I; Fig. 2, A and B). When extracellular Na+ was replaced by K+, proton-gated currents elicited by a pH step from pH 7.3 to pH 5 were almost as large as in the presence of Na+ (Fig. 2, C and D). The currents recorded in the K+-rich extracellular solution reversed at −2 and 4 mV for the ASIC2-F20S and ASIC2-T25K mutants, respectively.

The pH of half-maximal activation (pH0.5) of the F20S and T25K mutants was shifted to a more alkaline pH as compared with that of wild-type ASIC2. Whereas the pH0.5 value was 4.4 for wild-type ASIC2 and 4.7 for ASIC2-I19P, it was 5.7 and 5.4 for ASIC2-F20S and ASIC2-T25K, respectively (Fig. 3).

Mutations corresponding to ASIC2-I19P, F20S, and T25K were then introduced in other channels of the NaC/DEG family (Fig. 4A). This led to a loss of function for some of the mutants such as FaNaC-N51K, ASIC1-F21S, ASIC1-T26K, and ASIC3-F21S. However, two ASIC3 mutants, ASIC3-V20P and ASIC3-T26K, could still be activated by a drop in extracellular pH. ASIC3 normally displays biphasic kinetics with a rapidly inactivating current and a sustained current (3), and both currents are carried essentially by Na+ (Ref. 3; Fig. 4C; reversal potential, 32 ± 3 mV (n = 5), pNa−:pK+ = 9.8). Both components of ASIC3-V20P were also selective for Na+. Their reversal potentials of 26 ± 2 mV (n = 6) correspond to pNa−:pK+ ratio of 5.2 (Fig. 4C). On the contrary, ASIC3-T26K, like ASIC2-T25K, displayed a reduced Na+/K+ selectivity. The reversal potential for both the transient current and the sustained current was 9 ± 3 mV (n = 7), corresponding to a pNa−:pK+ ratio of 1.7 (Fig. 4C).

Neither component of the ASIC3-T26K mutant showed a change in pH0.5 with respect to the wild-type (Fig. 4B).

**DISCUSSION**

ASICs are a new class of cation channels activated by extracellular acidification. They belong to the NaC/DEG superfAMILY of ion channels (9). Subunits of this family have a topological organization comprising two transmembrane segments and a large extracellular loop (10–13) and assemble into tetramers, as demonstrated for both FaNaC (6) and ENaC (14, 15). Selective functional domains in control of the pore selectivity, the sensitivity to amiloride, and the gating properties have been identified for some members of the family (16–19), but there are few structural data on the ASICs (8).

The participation of the transmembrane domain TM2 and perhaps TM1 in the ion pore has not been directly demonstrated for ASICs but is well documented for ENaC (20) and another ligand-gated channel, FaNaC (21). The region located just before TM2 has been shown to be important for gating and...
for the pharmacological properties of ASIC2. The ASIC2 mutations G430T, G430V, or G430F, which correspond to mutations leading to gain of function or neurodegeneration in C. elegans degenerins (22–25), cause large changes in pH dependence and the inactivation process as well as changes in amiloride sensitivity (8). Participation of the pre-TM2 region in ion selectivity and conductance has also been established for ENaC (17, 18).

The present study focuses on the pre-TM1 domain of ASICs. This region is highly conserved among NaC/DEG family members and is involved in the gating properties of the epithelial sodium channel. Mutations of a conserved glycine in the pre-TM1 domain of β-ENaC is associated with type I pseudohypoaldosteronism (26) and leads to a reduction of ENaC activity. ENaC channels containing α-ENaC or β-ENaC subunits mutated at this glycine residue display a reduction in open probability, with no change in ion conductance and selectivity (27). However, a participation of this region in the ion permeation or the selectivity filter was never reported.

Our results demonstrate an involvement of the pre-TM1 domain of ASIC2 in both the pH dependence and the ion pore properties. F20S and T25K mutations in ASIC2 change the pH dependence from a pH0.5 of 4.4 to 5.7 (F20S) and 5.4 (T25K). The pH dependence of ASICs is associated with several regions of the protein because mutations in the pre-TM2 position can also modify the pH0.5 value (8).

Of particular interest is the modification of ASIC2 ion selectivity after mutations in the pre-TM1 domain. The I19P, F20S, and T25K mutations cause a 3- to 7-fold reduction in the Na⁺:K⁺ permeability ratio. The large changes in selectivity are not restricted to ASIC2 mutants because both the peak and sustained components of the ASIC3-T26K mutant show a 6-fold reduction in the Na⁺:K⁺ permeability ratio.

This loss of ion selectivity is associated with the replacement of ASIC2 and ASIC3 pre-TM1 residues by amino acids present in ASIC2b. In sensory cells, ASIC2b associates with ASIC3 and then confers a nonselective character to its sustained component (4). The pre-TM1 domain of ASIC2b may thus be one of the structural elements responsible for the loss of Na⁺ selectivity of this sustained component. The T26K mutation in ASIC3 changes the reversal potential of the peak and sustained currents in parallel, whereas co-expression of ASIC3 and ASIC2b influences the sustained current but not the peak current. This discrepancy between the properties of the heteromeric ASIC3/ASIC2b and the mutated ASIC3 T26K channel could be explained by the existence of two channel modes for ASIC3, one associated with the transient current, and one associated with the sustained current. The ASIC2b subunit would associate only with the conformation corresponding to the channel mode responsible for the sustained component and would modify its properties by changing its ionic selectivity. The T26K mutation introduced in ASIC3 would change the ion selectivity of both modes (transient and sustained currents) of the ASIC3 channel.

This study suggests that the pre-TM1 domain contributes to the ion pore of ASICs and plays a crucial role in the selectivity filter, possibly as a re-entrant loop from the cytoplasmic side. The present data suggest a model for the ion pore of ASICs that involves the transmembrane domains (TM2 and perhaps TM1) and the pre-TM2 and pre-TM1 regions. This model could possibly be suitable for other members of the NaC/DEG family.

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