The exact subunit combinations of functional native acid-sensing ion channels (ASICs) have not been established yet, but both homeric and heteromeric channels are likely to exist. To determine the ability of different subunits to assemble into heteromeric channels, a number of ASIC1a, ASIC1b, ASIC2a, ASIC2b, and ASIC3-containing homo- and heteromeric channels were studied by whole-cell patch clamp recordings with respect to pH sensitivity, desensitization kinetics, and level of sustained current normalized to peak current. Analyzing and comparing data for these three features demonstrated unique heteromeric channels in a number of co-expression experiments. Formation of heteromeric ASIC1a+2a and ASIC1b+2a channels was foremost supported by the desensitization characteristics that were independent of proton concentration, a feature none of the respective homomeric channels has. Several lines of evidence supported formation of ASIC1a+3, ASIC1b+3, and ASIC2a+3 heteromeric channels. The most compelling was the desensitization characteristics, which, besides being proton-independent, were faster than those of any of the respective homomeric channels. ASIC2b, which homomerically expressed is not activated by protons per se, did not appear to form unique heteromeric combinations with other subunits and in fact appeared to suppress the function of ASIC1b. Co-expression of three subunits such as ASIC1a+2a+3 and ASIC1b+2a+3 resulted in data that could best be explained by coexistence of multiple channel populations within the same cell. This observation seems to be in good agreement with the fact that ASIC-expressing sensory neurons display a variety of acid-evoked currents.

It is well established that tissue acidification, which may be present in inflammatory and ischemic conditions, causes pain (1–3). In line with this, peripheral sensory neurons exhibit sensitivity toward acid by activating several types of depolarizing currents (4–6). Although the repertoire of ion channels responsible for these currents is not fully known, the family of acid-sensing ion channels (ASICs) is believed to be an important constituent. The ASIC family is a member of the ENaC/DEG superfamily, which also includes the amiloride-sensitive epithelial sodium channels (ENaCs), the mechanically gated degenerins of Caenorhabditis elegans (DEGs), and a neuropeptide-gated channel of Helix aspersa (FaNaC). The membrane topology of all channels within this superfamly comprises two transmembrane domains, intracellular N and C termini and a large extracellular loop with a number of conserved cysteine residues (7). Currently, four genes encoding six ASIC transcripts have been cloned and characterized from mammalian organisms. ASIC1a (BNaC2) and ASIC1b (ASIC1b) are the products of alternatively spliced transcripts of the ASIC1 gene that differ in the N-terminal region, including the first transmembrane domain and the proximal part of the large extracellular domain (8–10). ASIC2a (BNaC1, MDEG) and ASIC2b (MDEG2) are alternatively spliced forms of the ASIC2 gene product. These, too, have unique N termini and share the C-terminal amino acids (11). Splice variants have not yet been identified for ASIC3 (DRASIC) (12, 13) and ASIC4 (SPASIC) (14, 15). Except for ASIC2b and ASIC4, all subunits have the ability to form functional homomeric channels when expressed in Xenopus laevis oocytes or mammalian cells. The functional and pharmacological properties of homomeric ASIC1a match one type of acid-evoked current described in peripheral sensory neurons (16, 17), and the properties of ASIC3-mediated currents mimic acid-evoked currents in cardiac sensory neurons, believed to mediate the pain of angina (18). Different ASIC subunits often co-localize within the same neurons (19), and heteromeric channels are likely to exist, as is the case for most ligand-gated ion channels. However, the exact composition of endogenous acid-sensitive channels is at present largely unknown.

To study heteromerization of ASICs, we examined co-expression of different ASIC subunits in Chinese hamster ovary (CHO) cells. By analyzing currents obtained in whole-cell voltage clamp experiments using ultrafast solution exchange for providing acidification, data for all possible combinations of ASICs (except ASIC4) were obtained. These data reveal unique properties for several of the homomerically expressed ASIC subunits and clearly demonstrate the formation of heteromeric channels in a number of co-expression experiments. Interestingly, not all the tested co-expreressions led to channels that could be identified as unique; in the case of experiments involving co-expression of three different subunits, the data suggested existence of multiple channel populations. To summarize, this is the first comprehensive study of ASICs co-expressed in a mammalian expression system.

**EXPERIMENTAL PROCEDURES**

Cloning of ASICs—Poly(A) mRNA was purified from fetal rat dorsal root ganglion (DRG) neurons using the Oligotex® direct mRNA mini kit (Qiagen) according to the manufacturer’s protocol. ASIC1b, ASIC2b, and ASIC4 were cloned from fetal rat DRG poly(A) mRNA (Clontech) using reverse transcription PCR. First and second strand cDNA was obtained in a one-tube reverse transcriptase–PCR reaction using Avian myoblastosis virus reverse transcriptase,
Heteromeric Expression of ASICs

DYNAzyme™ DNA polymerase (Finzymes), and the following primer sets (MWG Biotech): ASIC1a –53s TGGAGGGCTGCGCTTCCGAG and ASIC1a 1678as ATGCAGTTAAGTTCCAGGGTGAC; ASIC1b –50s ATGCAAGTTAAGTTCCAGGGTGAC and ASIC1b 1678as ATGCAGTTAAGTTCCAGGGTGAC; ASIC2a 1614as CTCGCCCTGAGTTCTCTGAG and ASIC2a 1614as CTCGCCCTGAGTTCTCTGAG; ASIC2b 1614as CTCGCCCTGAGTTCTCTGAG and ASIC2b 1614as CTCGCCCTGAGTTCTCTGAG; ASIC3 –6s GCCGCCATGAGCCGGAGCGG- and ASIC3 719as ACTCGGATCCCCACCTCAAAC; and ASIC3 543s AATCTCCTCCAGGGT; ASIC2b 6s GCCGCCATGAGCCGGAGCGG- and ASIC2b 6s GCCGCCATGAGCCGGAGCGG-; ASIC2a 1538as TGTGACGAGGTAACAGGT. and ASIC1a 1538as TGTGACGAGGTAACAGGT; ASIC1b 1538as TGTGACGAGGTAACAGGT; and ASIC3 515s GTTGCGCTGAACTCTACAGT and ASIC3 1602as CTAGACGTGTCAGGAGTTAACAGT.

The cDNAs were cloned into pSwaS, which is a custom-designed vector derived from pZero™ (Invitrogen), and positive clones were sequenced bidirectionally. ASIC1a, ASIC2a, and ASIC4 were subcloned directly into the pNS1z vector. The ASIC1b fragment, which encoded the N-terminal part of the channel, was subcloned into pNS1z-ASIC1a, substituting the corresponding part of ASIC1a. ASIC3 was cloned in two overlapping fragments and subcloned in pNS1z in a unique restriction site of ASIC3 (BarG1) within the overlapping region. The pNS1z vector is a customized vector derived from pCDNA3 (Invitrogen) with expression of the insert under control of the cytomegalovirus promoter.

Expression of ASICs—All constructs were expressed in CHO-K1 cells (ATCC No. CCL61). CHO-K1 cells were cultured at 37 °C in a humidified atmosphere of 5% CO2 and 95% air and passed twice/week. Cells were maintained in Dulbecco’s modified Eagle’s medium (10 mM HEPES, 2 mM GlutaMAX) supplemented with 10% fetal bovine serum and 2 mM l-proline (Invitrogen). CHO-K1 cells were co-transfected with the plasmids expressing ASICs and a plasmid encoding enhanced green fluorescent protein using the LipofectAMINE PLUS kit (Invitrogen) according to the manufacturer’s protocol. When more than one ASIC subunit was expressed, this was done in a 1:1 ratio. For each transfection, cells were in an amount of that would yield whole-cell currents within a reasonable range (0.5–10 nA) to avoid saturation of the patch clamp amplifier: ASIC1a and ASIC1b, 800 ng; ASIC2a and ASIC3, 400 ng; ASIC1a+1b, 2 × 250 ng; ASIC1a+2a, 2 × 100 ng; ASIC1a+2b and ASIC2a+2b, 2 × 400 ng; ASIC1a+3 and ASIC2a+3, 2 × 25 ng; ASIC1b+3, 2 × 75 ng; ASIC1b+2a and ASIC2a+2b, 2 × 200 ng; ASIC1a+2a+3, ASIC1a+2b+3, and ASIC1a+2a+2b+3, 3 × 75 ng. Electrophysiological measurements were performed 16–48 h after transfection.

Electrophysiology—All experiments were performed under voltage clamp using conventional whole-cell patch clamp methods (20) at 20–22 °C. A Macintosh G4 computer was used to control an EPC-9 amplifier (List Elektronische Geräte) via an ITC-HDG interface. The experimental conditions were defined by the Pulse software accompanying the amplifier, and data were sampled at 2 kHz and low pass-filtered at 667 Hz. Pipettes were pulled from borosilicate glass (Modulohm) using a horizontal electrode puller (Zeitiz-Instrumente). The pipette was filled with an intracellular solution containing 120 mM KCl, 31 mM KOH, 2 mM MgCl2, 10 mM EGTA, and 10 mM HEPES adjusted to pH 7.2. The tip of the electrode was a silver chloride pellet electrode (In Vivo Metric) fixed to the experimental chamber. The electrodes were zeroed with the open pipette in the bath just prior to sealing, and the pipette resistances were 1.5–3.0 MΩ. Series resistance compensation was set at 80%.

Coverslips with cells were transferred to the recording chamber (Buchi, Warsaw Instruments) mounted on the stage of an inverted microscope (Olympus). Transfected cells were identified by the emission of green fluorescence when exposed to UV light. After gigaseal formation, the whole-cell configuration was attained by suction.

Cells were continuously superfused at a rate of 2.5 ml/min with an extracellular solution (Na-R) containing 140 mM NaCl, 4 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, and 5 mM MES adjusted to pH 7.4. Test solutions were made from standard Na-B, adjusting pH with HCl. Rapid pH changes were achieved by placing a piezo-driven double-barreled application pipette (theta tube) in front of the cell. Using this system, complete solution exchange can be achieved in <1 ms, as measured by the liquid junction potential shift (data not shown). Currents were measured at the peak of the response and normalized to the current evoked by a pH 4 stimulus.

Data Analysis—Using the software GraphPad Prism™ 3.0, pH dose-response curves were fitted to the equation $I_{\text{max}} = \frac{1}{1+K_a \cdot (\text{EC}_{50})^{-B}}$, where $K_a$ denotes the proton concentration [H+] yielding 50% activation and $n$ is the Hill coefficient. In a few instances, the current was not fully saturated at pH 4 when fitting to this equation. In these cases, data were subsequently normalized to the value of $I_{\text{max}}$ at saturation as estimated from the fit to obtain a maximal $I_{\text{max}}$ of 1.0. Time constants of desensitization were calculated using IgorPro software (WaveMetrics) by fitting the falling phase of the evoked current to a single exponential function $I(t) = I_0 \cdot (1 - e^{-t/T})$, where $I_0$ is the time constant of desensitization, $\tau_{\text{desens}}$, equals $1/k_2$, $K_a$ denotes the amplitude of the non-desensitizing current, and $K_a + K_p$ is the peak current, i.e. all ASIC subunit combinations yielded currents that desensitized incompletely at pH 4.0. Results are presented as mean ± S.E., and comparisons were made using a two-tailed t test or one-way analysis of variance. A p value of less than 0.05 was considered to be significant.

RESULTS

ASIC subunits were transiently expressed, either singly or as combinations, in CHO cells. All functional channels yielded transient acid-evoked whole-cell currents that displayed varying levels of sustained current. These whole-cell currents were characterized with respect to three different properties: 1) the pH sensitivity (pH50 and Hill slope); 2) the time constant of desensitization ($\tau_{\text{desens}}$, measured at various pH values; and 3) the amplitude of the residual non-desensitizing current at pH 7.4 (pH50 of Channel 1). Some ASIC channels exhibited desensitization kinetics that were dependent on the applied proton concentration, whereas others had kinetics that were independent of proton concentration. When comparing channels with different kinetic properties, values of $\tau_{\text{desens}}$ determined at a proton concentration close to the pH50 values or in some cases pH50 values for activation were used.

Homomeric Expression of ASIC Subunits—The expression of ASIC1a, ASIC1b, ASIC2a, or ASIC3 in CHO cells resulted in functional acid-sensitive channels (Fig. 1, A–E), but ASIC2b and ASIC4 could not be activated even at pH 4. Whereas ASIC1a, ASIC1b, and ASIC2a displayed transient activation characteristics, ASIC3 was found to have two different activation profiles. In some cells ASIC3 had a transient activation profile (Fig. 1D), but in other cells a slower activating current followed the transient current (Fig. 1E). The proton affinities for the various channels varied by two orders of magnitude (Fig. 2A and Table I), with ASIC3 being the most sensitive subunit (pH50 = 6.4) and ASIC2a the least sensitive (pH50 = 4.5). Although pH50 was similar for the two ASIC1 splice variants, activation of ASIC1a occurred across a larger pH range, whereas ASIC1b was activated and saturated within a narrow pH range (Fig. 2A). This is clearly reflected in the Hill coefficients, with ASIC1a and ASIC1b having Hill slopes of 0.75 and 4.8, respectively. The Hill coefficients for ASIC2a and ASIC3 were intermediate to those of the two ASIC1 splice variants (Table I).

The values of $\tau_{\text{desens}}$ were strongly dependent upon the proton concentration used for activation of ASIC1a, ASIC1b, and ASIC2a, as the rate of desensitization increased with increasing proton concentrations (Figs. 2, B and C). This is a feature commonly observed for ligand-gated ion channels. In contrast, the desensitization rate of the transient current of ASIC3 was independent of the applied pH (pH 6.5–4.0; $p = 0.25$) (Fig. 2B). Despite their different pH50 and Hill slopes, no significant
of the test solution is decreased. ND, not determined. Data are represented as mean ± S.E.

| Half-maximum pH activation (pH$_{50}$) | Hill slope | Mean time constant of desensitization at pH$_{50}$ (τ$_{desens}$) | Sustained current normalized to peak current (I/Ip, pH 4.0) |
|--------------------------------------|------------|---------------------------------------------------------------|----------------------------------------------------------|
| ASIC1a                               | 5.8        | 0.64 ± 0.09, n = 4                                            | 2.5 ± 1.5, n = 7                                          |
| ASIC1b                               | 6.1        | 0.89 ± 0.11, n = 4                                            | 4.4 ± 1.9, n = 6                                          |
| ASIC2a                               | 4.5        | 1.4 ± 0.2, n = 7                                              | 7.5 ± 0.8, n = 20                                         |
| ASIC3                                | 6.4        | 0.29 ± 0.02, n = 9                                            | 26 ± 2.2, n = 25                                         |
| ASIC1a/ASIC1b                        | 6.0        | 1.3 ± 0.06, n = 7                                            | 5 ± 1.3, n = 18                                          |
| ASIC1a/ASIC2a                        | 5.5        | 0.41 ± 0.07, n = 5                                            | 5.7 ± 1.7, n = 15                                         |
| ASIC1a/ASIC2b                        | 6.2        | 0.97 ± 0.19, n = 5                                            | 3.5 ± 1.2, n = 17                                         |
| ASIC1a/ASIC3                         | 6.3        | 0.16 ± 0.03, n = 5                                            | 3.7 ± 0.6, n = 17                                         |
| ASIC1b/ASIC2a                        | 4.9        | 1.0 ± 0.08, n = 7                                            | 14 ± 1.5, n = 14                                         |
| ASIC1b/ASIC2b                        | ND         | ND                                                            | ND                                                       |
| ASIC1b/ASIC3                         | 6.0        | 0.23 ± 0.01, n = 4                                            | 15 ± 2.3, n = 25                                         |
| ASIC2a/ASIC2b                        | 4.8        | 3.5 ± 0.6, n = 5                                              | 17 ± 1.6, n = 13                                         |
| ASIC2a/ASIC3                         | 5.7        | 0.19 ± 0.02, n = 6                                            | 105 ± 45, n = 21                                        |
| ASIC2b/ASIC3                         | 6.5        | 0.23 ± 0.02, n = 5                                            | 19 ± 4.0, n = 26                                         |
| ASIC1a/ASIC2a/ASIC3                 | 5.1        | 0.37 ± 0.02, n = 14                                            | 14 ± 2.5, n = 37                                         |
| ASIC1b/ASIC2a/ASIC3                 | 6.3        | 0.21 ± 0.04, n = 6                                            | 2.7 ± 0.9, n = 18                                         |
| ASIC1b/ASIC2b/ASIC3                 | 4.9        | 0.55 ± 0.09, n = 10                                            | 18 ± 2.9, n = 21                                         |

The sustained current remaining at pH 4.0 was in the range of 3–8%, relative to the peak current, for ASIC1a, -1b, and -2a (Table I). ASIC3 displayed a significantly larger sustained current of 26% (p < 0.001) (Table I).

ASIC1a Co-expressed with ASIC1b, ASIC2a, ASIC2b, or ASIC3—Co-expression of ASIC1a and one other ASIC subunit gave rise to functional acid-sensitive channels in all cases (Fig. 1, F–I). Proton sensitivities of the different subunit combinations fell into two categories. The ASIC1a + 1b, ASIC1a + 2a, and ASIC1a + 2b combinations all showed proton sensitivities similar to ASIC1a (Fig. 3, A and B, respectively), whereas the sensitivity of the ASIC1a + 3 combination was indistinguishable from that of ASIC3 homomeric channels (Fig. 3C).

For three of four of these combinations desensitization kinetics were affected in one way or another relative to the respective homomeric receptors; only ASIC1a + 2b appeared unchanged compared with the ASIC1a homomer receptor. The desensitization rate of the ASIC1a + 1b combination was dependent on the proton concentration as was the case for the respective homomeric channels (Fig. 4A). Contrary to this, the rates for the ASIC1a + 2a and ASIC1a + 3 combinations were independent of the proton concentration (p > 0.08 and p > 0.48, respectively) (Fig. 4, B and C). Comparing desensitization rates at pH$_{50}$, ASIC1a + 1b was significantly slower than ASIC1a and ASIC1b (p < 0.001 and p < 0.05, respectively), whereas ASIC1a + 3 was significantly faster than ASIC1a and ASIC3 (p < 0.001 and p < 0.05, respectively). Due to the pH dependence of ASIC1a desensitization, a comparison of the rates of ASIC1a + 2a and ASIC1a was complex with significant differences at some proton occupancy levels but not at others. However, measured at pH = 4.0 (pH$_{100}$), ASIC1a + 2a desensitization was significantly slower than that of ASIC1a (p < 0.05) but faster than that of ASIC2a (p < 0.001).

The sustained current at pH 4.0 of all the combinations was in the range of 3–6% of peak currents, which is not significantly different from homomeric ASIC1a (Table I). Interestingly, when compared with homomeric ASIC3, the ASIC1a + 3 combination displayed a major reduction of the level of sustained current (p < 0.001).

ASIC1b Co-expressed with ASIC2a, ASIC2b, or ASIC3—ASIC1b co-expressed with ASIC2a or ASIC3 in CHO cells yielded functional channels (Fig. 1, J–K); however, contrary to all other combinations of subunits, ASIC1b + 2b did not exhibit proton-activated currents in 30 of 36 cells. In the transfection procedure for ASIC1b + 2b, an increase of DNA levels to the highest level possible (before cellular death occurred) was tried, but still, in the few cells that did show a proton-evoked current, the amplitude was very low (<100 pA). For this reason it was concluded that ASIC1b + 2b is a non-functional combination, and no data are presented. The proton sensitivity of the ASIC1b + 2a combination (Fig. 3D) resembled that of ASIC2a, whereas the sensitivity of ASIC1b + 3 (Fig. 3E) was almost identical to that of homomeric ASIC1b.

The values of τ$_{desens}$ of both ASIC1b + 2a and ASIC1b + 3 were independent of the proton concentration used for stimulation (p > 0.23 and p > 0.15, respectively) (Fig. 4, D and E). Again, comparing proton-independent with proton-dependent desensitization is problematic. In general, desensitization rates of ASIC1b + 2a could be characterized as intermediate to the respective homomorphic channels; ASIC1b + 2a was significantly faster than ASIC2a at pH$_{50}$ (p < 0.05) but significantly slower than ASIC1b at pH$_{100}$ (p < 0.05). The general picture of ASIC1b + 3 was one of a channel desensitizing faster than the respective homomorphic channels (Fig. 4E). Indeed, it desensitized significantly faster than ASIC1b at pH$_{50}$ (p < 0.001); however, despite the pH independence of both ASIC3 and ASIC1b + 3 significant differences were not observed for all proton occupancy levels. Still, at pH$_{100}$ (pH = 4.0) ASIC1b + 3 clearly desensitized significantly faster than ASIC3 (p = 0.01).

For the ASIC1b + 2a combination the level of sustained current at pH 4.0 relative to peak current was 14%, which represents a significant increase compared with either homomorphic channel (p < 0.001). The level of sustained current for the ASIC1b + 3 combination (15%) was intermediate to the levels determined for the respective homomorphic channels (Table I).

Co-expression of ASIC2a + 2b, ASIC2a + 3, and ASIC2b + 3—Acid-sensitive channels were formed from co-expressing dual combinations of ASIC2a, ASIC2b, and ASIC3 (Fig. 1, L–O). The activation characteristics of ASIC2a + 3 resembled ASIC3 with two different profiles. In some cells ASIC2a + 3 had an apparent simple transient activation profile (Fig. 1M), whereas in other cells a slower activating current followed the transient current (Fig. 1N). Despite functionality of all these subunit combinations, the acid sensitivity of ASIC2a and ASIC3 was largely different...
unaffected by the presence of ASIC2b (Fig. 3, D and F, respectively), whereas the sensitivity of ASIC2a+3 was intermediate to either homomeric channel (Fig. 3F).

Overall, desensitization kinetics of ASIC2a+2b and ASIC2b+3 were identical to the kinetics of homomeric ASIC2a and ASIC3, respectively (p > 0.05) (Fig. 4, F and G). The \( \tau_{\text{desens}} \) values of ASIC2a+3 were independent on the proton concentration (p > 0.54) (Fig. 4G). At pH\textsubscript{50} ASIC2a+3 desensitized significantly faster than ASIC2a (p < 0.01); however, although desensitization of ASIC2a+3 always appeared faster than ASIC3 (Fig. 4G), this difference did not reach significance. Interestingly, the desensitization phase of ASIC2a+3 was masked at pH ≤ 4.5 due to activation of a prominent sustained current (see below).

Whole-cell currents measured from cells co-expressing ASIC2a+2b maintained a level of sustained current of 17% at
pH 4.0; this represents a significant increase relative to the 7.5% determined for homomeric ASIC2a (p < 0.0001) (Table I). Interestingly the ASIC2a/H110013 combination displayed a pronounced mean sustained current of 105% at pH 4. The reason for a measured sustained current larger than the peak current is the second slower activating current observed in some cells, which frequently exceeds the amplitude of the transient peak. The sustained current of 105% was significantly greater than the values corresponding to both homomeric ASIC2a and ASIC3 (p < 0.05). The level of sustained current determined for ASIC2b/H110013 was of a similar magnitude to that of ASIC3 homomers (p > 0.16) (Table I).

Co-expression of ASIC1a+2a+3—Co-expressing these three subunits gave rise to channels with peculiar characteristics (Fig. 1P). The proton sensitivity of acid-evoked currents in these cells was highly unusual (Fig. 5A), with activation starting at pH 6.5 and not fully saturating at pH 4, which is also clearly reflected in a low Hill slope of 0.65 (Table I).

The τdesens values of ASIC1a+2a+3-mediated whole-cell currents displayed unusual pH dependence, in the range of pH 6–4.5, in that τdesens actually increased with increasing proton concentration (p < 0.01) (Fig. 6A). The desensitization rate at pH50 was similar to that of ASIC1a+2a (p > 0.05) but significantly slower when compared with the rates of ASIC1a+3 and ASIC2a+3 (p < 0.001).

The level of sustained current at pH 4.0 did not differ significantly from that of ASIC1a+2a and ASIC1a+3 (p > 0.05) but was significantly lower than that of ASIC2a+3 (p < 0.01) (Table I).

Co-expression of ASIC1a+2b+3—Although this co-expression experiment yielded functional channels (Fig. 1Q) the channel characteristics were similar to those of ASIC1a+3. First, the proton sensitivity of acid-evoked currents was virtually identical to that of ASIC1a+3-mediated currents (Fig. 5B). Next, desensitization kinetics were not significantly different from those of ASIC1a+3 at any pH value (p > 0.05) (Fig. 6B). Finally, the level of sustained current normalized to peak current at pH 4.0 was not significantly different from that of ASIC1a+3 (p > 0.35) (Table I).

Co-expression of ASIC1b+2a+3—The last triple co-expression tested also yielded functional acid-sensitive channels (Fig. 1R) but again with peculiar characteristics. With respect to the proton sensitivity, the ASIC1b+2a+3 combination was reminiscent of ASIC1a+2a+3 (Fig. 5A) and was thus less acid-sensitive than any of the corresponding pairwise subunit combinations (Table I).

Time constants of desensitization for ASIC1b+2a+3-mediated currents were also unusual compared with other subunit combinations, as τdesens increased at increasing proton concentration (Fig. 6A). The desensitization rate at pH50 was signifi-
cantly faster than that of ASIC1b (p < 0.01) but signifi-
cantly slower than those of ASIC1b and ASIC2a (p < 0.05).

The mean level of sustained current at pH 4.0 was not
significantly different from that of ASIC1b (p > 0.05) and
ASIC2a+3 (p < 0.05), whereas it was significantly reduced
compared with that of ASIC2a+3 (p < 0.05) (Table I).

**DISCUSSION**

Results from co-expressing subunits, such as the ASIC sub-units, can potentially be quite complex. In the simplest system either homomeric or heteromeric channels are formed; however, besides the possibility of coexistence of homomeric and heteromeric channels, the heteromeric channels themselves could be a mixed population with different stoichiometries. Assuming a tetrameric assembly of the ASIC channel (21), co-expressing two subunits gives rise to the possibility of hav-
ing two homomeric channels along with three different hetero-
meric channel assemblies. When co-expressing three subunits these numbers rise to a possibility of having three homomeric channels along with 12 heteromeric channels. The currents measured from co-expression experiments may therefore reflect the presence of homomeric and/or heteromeric channels, which may or may not have distinct characteristics. Thus, presence of heteromeric ASIC channels could only be identified to the extent that they displayed biophysical properties distinct from the homomeric channels. Generally, the features studied here (pH sensitivity, desensitization rate, and sustained current at pH 4) proved to be very informative in identifying the existence of heteromeric channels, and the present data demonstrate that a number of distinct heteromeric channels are formed.

**Homomeric ASICs Show Variable Properties**—Initially, the
FIG. 4. Desensitization kinetics of double ASIC combinations. The rate of desensitization is indicated by mean time constants of desensitization (τ_{desens}). A, ASIC1a+1b desensitizes more slowly than either homomeric channel, reflected in higher τ_{desens}, and as observed for both homomers, desensitization is increased at decreasing pH. B, desensitization of ASIC1a+2a is independent of proton concentration and shows no obvious resemblance to either homomeric channel. There are no significant differences between τ_{desens} of ASIC1a and ASIC1a+2b. C, ASIC1a+3
different homomeric channels were characterized to enable identification of channels formed from co-expression experiments. As described previously, ASIC1a, ASIC1b, ASIC2a, and ASIC3 all expressed well as homomeric channels (8, 10–12), whereas ASIC2b and ASIC4 did not yield functional acid-sensitive channels (11).

Interestingly, the pH dose-response curves of ASIC1a and ASIC1b differed markedly despite similar pH50 values; activation and saturation of ASIC1a occurred over a range of three pH units, whereas ASIC1b activated and saturated within a single pH unit. Waldmann et al. (8) reported a much steeper pH dose-response curve for ASIC1a expressed in Xenopus oocytes, and Chen et al. (9) showed more gradual activation of ASIC1b expressed in COS-7 cells. Hence, this feature may be critically dependent upon cell type and/or the solution application system used for whole-cell recording. In agreement with previous findings, ASIC2a homomeric channels were the least acid-sensitive (i.e. displaying a low pH50) (11), whereas ASIC3 homomers were the most acid-sensitive (i.e. high pH50) (12).

The desensitization rate of ASIC1a, ASIC1b, and ASIC2a was clearly increased when the stimulation pH was lowered (i.e. proton concentration increased). Conversely, the time constant of desensitization for ASIC3 remained constant despite changes in pH.

**Heteromeric ASIC Channels with Distinct Properties**—A heteromeric ASIC1a+1b channel with functionally unique properties has not been reported previously; however, the present data support its existence. Currents from cells co-expressing ASIC1a+1b desensitized significantly slower at pH50 than currents corresponding to either homomeric channel. Moreover, the proton sensitivity and Hill slope of ASIC1a+1b were indistinguishable from those of homomeric ASIC1a, which would appear inconsistent with a mixture of homomeric channels. If only homomers were present, the very steep proton dose-response curve of ASIC1b would be expected to affect the shallow dose-response curve of homomeric ASIC1a.

Co-expression of either ASIC1 splice variant with ASIC2a resulted in heteromeric channels clearly identifiable from the respective homomeric channels. Contrary to the homomeric channels, the ASIC1a+2a and ASIC1b+2a combinations both displayed pH-independent rates of desensitization. Moreover, proton sensitivities of activation for both combinations were

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**Fig. 5. Proton sensitivity of triple ASIC combinations.** Shown is the sensitivity of indicated channels to lowering of pH from a holding of pH 7.4. Currents are not fully saturated at pH 4, and therefore these have subsequently been normalized to ILmax at saturation as estimated from the initial pH dose-response fit. A, ASIC1a+2a+3 and ASIC1b+2a+3 both exhibit shallow activation curves with low pH50 values and low Hill slopes. B, the pH sensitivity of ASIC1a+2b+3 resembles that of ASIC1a+3 with similar pH50 and hill slope. n = at least four cells for each data point. Bars indicate mean ± S.E.

**Fig. 6. Desensitization kinetics of triple ASIC combinations.** The rate of desensitization is indicated by mean time constants of desensitization (τdesens). A, desensitization of both ASIC1a+2a+3 and ASIC1b+2a+3 is slowed with increasing proton concentration indicated by an increase in τdesens. B, desensitization kinetics of ASIC1a+2b+3 is constant across the applied pH range and does not differ from ASIC1a+3 at any pH value. n = at least four cells for each data point.

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inconsistent with a predominance of homomeric channels. Considering the large separation of the pH dose-response curves and Hill slopes between the ASIC1 variants and ASIC2a, a mixture of independent homomeric channels would be anticipated to result in biphasic pH dose-response curves or curves with extremely low Hill slopes. However, this was not observed; ASIC1a–2a had a pH50 value similar to homomeric ASIC1a but an increased Hill slope, whereas ASIC1b–2a had a pH50 value similar to homomeric ASIC2a with an unaltered Hill slope.

As noted above, ASIC3 was characterized by displaying pH-independent desensitization kinetics. Interestingly, this phenotype was conferred to the channels resulting from the co-expressions of ASIC3 with ASIC1a, ASIC1b, or ASIC2a. Furthermore, these co-expressions all gave rise to channels with accelerated desensitization kinetics (–0.2 s) relative to any of the respective homomeric channels. However, this observation might in each case argue in favor of assembly of heteromeric channels. In agreement with this, it was reported previously that desensitization of acid-evoked currents from DRG neurons of ASIC3-null mice was slowed compared with wild type animals, suggesting that presence of ASIC3 might shorten the response to acid in vivo (22, 23). A striking observation in the co-expression experiments with ASIC3 was the large amplitude of sustained current evoked by stimulation of ASIC2a–3 at pH 4.0. The summed contributions of homomeric ASIC2a and ASIC3 channels cannot explain the magnitude of this current, strongly suggesting the assembly of functional ASIC2a–3 heteromeric channels. This was also noted by Babinski et al. (24), who co-expressed ASIC2a and ASIC3 in Xenopus oocytes.

Heteromerization of ASIC1a–3, ASIC1b–3, and ASIC2a–3 was supported by the fact that the DNA amounts used for these transient transfections were reduced considerably compared with expression of the same subunits singly. This could suggest very efficient assembly and/or transport of such heteromeric channel complexes to the cell membrane.

Interestingly, whenever co-expressing two subunit types, the pH dose-response curves usually ended up being very similar to one of the curves for the respective homomeric channels. Only in the case of ASIC2a–3 an easily identifiable “new” dose-response curve appeared, which was located between the curves for the respective homomers.

Does ASIC2b Constitute a Functional Subunit?—The present data do not provide convincing evidence that ASIC2b participates in any functional channel complex, although it previously has been suggested to constitute a modulatory subunit (11). When ASIC2b was co-expressed with ASIC1a or ASIC3 or both in combination, minor changes to features such as pH dose-response curves could be observed, but nothing significant. In accordance with this, Lingueglia et al. (11) reported that ASIC2b was devoid of effect on the identical set of properties of ASIC1a and ASIC3. However, these authors found that ASIC2b altered the permeation properties of ASIC3 from being Na+-selective to being non-selective for cations, suggesting that heteromultimerization of these subunits had occurred. With regards to ASIC2a–2b co-expression, the relative level of sustained current at pH 4.0 appeared increased by the presence of ASIC2b, an observation also reported by Lingueglia et al. (11). However, there were no other clear indications suggesting that ASIC2b influenced the properties of ASIC2a. Strangely, the most obvious “effect” of ASIC2b appeared to be suppression of ASIC1b function. Compared with homomeric expression of ASIC1b, the fraction of cells displaying acid-evoked currents was highly reduced by the presence of ASIC2b, and acid-evoked currents of the few responsive cells were of low amplitude. This might suggest a dominant negative effect of ASIC2b on ASIC1b.

Three Different Subunits Appear Not to Form a Distinct Channel—As described in the preceding sections, ASIC1a, ASIC2a, and ASIC3 are capable of assembling into heteromeric complexes when co-expressed pairwise. Therefore, we surmised that assembly of all three of these subunits into single ASIC channels could be a realistic possibility. However, experiments involving co-expression of the three subunits yielded no conclusive evidence in favor of such an interaction. Two lines of evidence rather point toward existence of multiple channel populations. The pH dose-response relationship was unusual with activation taking place over a broad pH range, thereby yielding a very low Hill coefficient; the desensitization rate for ASIC1a–2a–3 decreased with increasing proton concentration in a large part of the pH range examined, which is contrary to the properties of all other investigated combinations of subunits. These observations could be explained by assuming that multiple channel populations were present. Considering the very efficient assembly of in particular heteromeric ASIC1a–3 and ASIC2a–3 channels but also ASIC1a–2a, it might seem unlikely that homomeric channels contribute significantly. On the other hand, the most obvious way to create a shallow dose-response relation is by having a mixture of channels with dose-response relations widely separated over the pH range, which suggests a presence of ASIC2a homomeric channels. Thus, a mixture of ASIC1a–3 and ASIC2a channels could theoretically account for a large part of the unexpected observations. The difference in pH50 of these two channel types would result in a low Hill coefficient of the activation profile. Furthermore, at high pH values (pH 5.5–6.5), the rapid desensitization of ASIC1a–3 would be observed, whereas the slower desensitization of ASIC2a would dominate at lower pH values (pH 4.0–5.0). It is of course entirely possible that other combinations, such as ASIC1a homomers as well as ASIC1a–2a and ASIC2a–3 heteromers, also contribute to the data observed. It should be noted, however, that the relatively modest amplitude of sustained current at pH 4.0 determined for ASIC1a–2a–3 (14%; Table I) seems to argue against the presence of large amounts of ASIC2a–3 heteromers.

Co-expression of ASIC1b with ASIC2a and ASIC3 gave very similar results, suggesting that expression of either ASIC1a or ASIC1b with ASIC2a and ASIC3 resulted in channel populations that were alike. Again assuming a tetrameric assembly of the ASIC channel (21), the apparent inability of channel formation involving three different subunits combined with the highly efficient channel formation with two different subunits could perhaps suggest an assembly consisting of two dimers.

ASIC Channels in DRG Neurons—At least three different types of acid-evoked currents have been recorded from DRG neurons (4–6). Benson et al. (23) showed that co-expression of ASIC1a–2a–3 could reproduce the biophysical properties of a rapidly desensitizing acid-evoked current of mouse DRG neurons. Indeed, the fast desensitization at pH 6 observed by these authors was reproduced in the present study; however, as noted above, the rate of desensitization was reduced at lower pH values. In this context, it should be noted that the pH value giving half-maximal activation of current in the present study (pH50 = 5.1) was substantially less than the value (pH50 = 6.4) reported by Benson et al. (23), a fact which may relate to the use of different expression systems and/or other differences in experimental conditions.

Understanding the molecular composition of native ASIC channels is an important step in understanding their physiological role in sensory transduction and also has clinical implications, as these channels provide potential targets for the
pharmacological modulation of sensory stimuli, including pain. However, identification of native ASICs is complicated by the lack of pharmacological tools, as only a single specific ASIC1a antagonist has been reported (16). By using a combination of non-steroidal anti-inflammatory drugs, which show different patterns of antagonism toward ASICs, Voilley et al. (17) showed that acid-evoked currents of DRG neurons are likely to reflect the presence of at least four different populations of ASIC channels. It may therefore very well be that the ASIC currents recorded from native DRG neurons reflect the concerted activity of multiple types of ASIC channels with distinct molecular compositions. This hypothesis seems to be compatible with the present ambiguous data obtained for cells co-expressing ASIC1a+2a+3, which also indicate that multiple channels form when these subunits are allowed to interact.

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pH Dependency and Desensitization Kinetics of Heterologously Expressed Combinations of Acid-sensing Ion Channel Subunits
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