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Mammalian ASIC2a and ASIC3 Subunits Co-assemble into Heteromeric Proton-gated Channels Sensitive to Gd^{3+}*

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Proton receptors of the acid-sensing ion channel (ASIC) family are expressed in sensory neurons and thus could play a critical role in the detection of noxious acidosis. To investigate the subunit composition of native ASICs in peripheral and central neurons, we co-injected human as well as rodent ASIC2a and ASIC3 subunits in *Xenopus* oocytes. The amplitudes of acid-induced biphasic responses mediated by co-expressed ASIC2a and ASIC3 subunits were much larger (as much as 20-fold) than the currents mediated by the respective homomers, clearly indicating functional association. The reversal potential of the ASIC2a+3 current (≥+20 mV) reflected a cationic current mainly selective for sodium. The sensitivity to pH or amiloride of single *versus* co-expressed ASIC subunits was not significantly different; however, gadolinium ions inhibited ASIC3 and ASIC2a+3 responses with much higher potency (IC_{50} ~40 μM) than the ASIC2a response (IC_{50} ≥1 mM). Biochemical interaction between ASIC2a and ASIC3 subunits was demonstrated by co-purification from transfected human embryonic kidney (HEK293) cells and *Xenopus* oocytes. Our *in situ* hybridization data showed that rat ASIC2a and ASIC3 transcripts are co-localized centrally, whereas reverse transcription-polymerase chain reaction data led us to detect co-expression of human ASIC2a and ASIC3 subunits in trigeminal sensory ganglia, brain, and testis where they might co-assemble into a novel subtype of proton-gated channels sensitive to gadolinium.

Sensory as well as central mammalian neurons respond to application of acidic solutions on their plasma membrane by the opening of cation channels sensitive to extracellular protons (1). Detection of acidosis in the periphery by primary sensory neurons plays a physiological role of protection by informing the central nervous system of noxious conditions of inflammation, hypoxia, or tissue damage. Recently, the molecular basis of acid sensing has progressed with the discovery of two types of cation channels directly gated by high concentrations of protons and highly expressed in primary sensory neurons: the vanilloid receptor (VR1) (2, 3) and the acid-sensing ion channels or ASICs[1] (4). ASICs belong to a supergene family of channel subunits with two transmembrane domains and a cysteine-rich extracellular loop including also the *Caenorhabditis elegans* degenerins (5–9), the amiloride-sensitive epithelial sodium channels (10), the FMRFamide-gated channel of *Helix aspersa* (11), as well as the orphan rat BLINaC (12), human INaC (13), and *Drosophila* RPK and PPK subunits (14–16).

Currently three genes and five major transcripts coding for ASIC subunits have been cloned and characterized from mammals. ASIC1a (also named ASIC or BNaC2) subunits are highly enriched in primary sensory neurons of dorsal root and trigeminal ganglia but are also expressed in most brain regions (17) and respond to low external pH (pH_{50} = 6.4) by mediating an amiloride-sensitive and sodium-selective current with fast desensitization kinetics (18). Rodent ASIC1b (also named ASICβ) corresponds to a splicing variant of ASIC1a with a restricted expression in sensory neurons (19). When expressed in heterologous systems, rat ASIC1b subunits assemble in homomeric channels similar to ASIC1a with regard to the functional properties (19). In an analogous situation to the *ASIC1* gene, the rodent *ASIC2* gene is alternatively spliced to code for two major variants, ASIC2a and 2b, differing in their first 185 and 236 amino-terminal residues, respectively. Neuronal ASIC2a (also named BNaC1, MDEG or BNC1) subunits have a widespread distribution in the nervous system (17, 20, 21) and assemble into proton-gated channels with a fast desensitization and a low sensitivity to pH (22). Widespread rat ASIC2b (also named MDEG2) subunits show high levels of expression in sensory neurons (22). They do not assemble in pH-sensitive channels by themselves, but they associate with other ASIC partners and change the ion selectivity (22). Finally, homomeric ASIC3 receptors (also named DRASIC) respond to low pH applications by a biphasic current with a fast desensitizing phase followed by a late sustained current that might mediate a component of the prolonged pain induced by acidosis (23–25). Interestingly, despite high levels of expression in primary sensory neurons, major differences in the central and peripheral distribution of ASIC3 subunits have been reported between rats and humans (23, 24).

Like most ligand-gated ion channels, native ASICs are believed to be heteromeric complexes composed of different subunits assembled around the central conducting pore. Rat ASIC1a and ASIC2a have been shown to interact with ASIC2b *in vitro,* and the resulting heteromeric channels have slower inactivation kinetics and a lower selectivity for sodium ions than the respective homomeric counterparts (22, 26). Recom-

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[1] The abbreviations used are: ASIC, acid-sensing ion channel; RT-PCR, reverse transcriptase-polymerase chain reaction; GFP, green fluorescent protein; EGFP, enhance green fluorescent protein.

binant rat ASIC2b is also able to associate with ASIC3 to mediate a biphasic acid-induced response with a slow and sustained phase that is less selective for sodium ions than that of ASIC3 alone (22). The specific amino-terminal sequence of the ASIC2b subunit preceding the first predicted transmembrane domain seems to play a critical role in this modulation of ion selectivity (27). The set of native heteromeric ASICs could vary between species because of differences in their specific repertoire of ASIC subunit genes and corresponding variants as well as in their regulation of developmental and anatomical patterns of expression. Indeed the ortholog of rodent ASIC2b mRNA has not yet been identified in humans, strongly suggesting that it represents a minor transcript, and ASIC3 shows a widespread distribution in human tissues contrasting with its restricted localization in rat, mainly limited to sensory ganglia, brainstem, and cerebellum. The present work describes electrophysiological, biochemical, and anatomical evidence for a novel type of heteromeric association between ASIC2a and ASIC3 subunits generating gadolinium-sensitive proton-gated channels in humans and rodents.

EXPERIMENTAL PROCEDURES

Molecular Biology—The cDNA coding for human ASIC3 subunit was cloned as previously reported (23). Rat ASIC3 cDNA was amplified by RT-PCR from adult trigeminal ganglia reverse-transcribed cDNA using the Expand polymerase mix (Roche Molecular Biochemicals) and gene-specific primers (forward, AGGATGAAACCTCGCTCCGGACT; reverse, GCCTTGTGACGAGGTAACA) based on the published sequence (25). Human ASIC2a in pcDNA3 was kindly provided by Dr François Besnard (Sanofi-Synthelabo, Rueil Malmaison, France). Rat ASIC2a subunit cDNA was isolated by RT-PCR from whole brain random-primed reverse-transcribed cDNA with Expand and the specific primers (forward, CAGTTGCAGAACTGCACAG; reverse, GTGTCAGCAGGCAATCTC) based on the published sequence (17). All full-length subunits were then subcloned in pcDNA3 or pcDNA3.1 vector (Invitrogen), sequenced, and validated by functional tests in *Xenopus* oocytes. Amino-terminal tagging of ASIC subunits with hexahistidine or with EGFP was performed by subcloning corresponding ASIC cDNAs in pcDNA3-His-N (Invitrogen) or pEGFP-C2 (CLONTECH) vectors, respectively. Carboxyl-terminal tagging of hASIC3 with the FLAG epitope (Sigma) was obtained first by PCR mutagenesis of the native stop codon of hASIC3 into an *Xho*I site and then by in-frame ligation of the mutated hASIC3 subunit to a *Xho*I-*Xba*I FLAG-containing cDNA cassette.

In situ hybridization of adult rat brain sections with ^{35}S-UTP-labeled riboprobes corresponding to specific protein domains from Ser^{61} to Met^{153} and from Ser^{382} to Pro^{502} of rat ASIC2a and ASIC3 subunits, respectively, was performed as described previously (28).

To document the co-expression of ASIC2a and ASIC3 mRNA in various human tissues, specific oligonucleotide primers were designed and used in a duplex RT-PCR protocol to amplify fragments of 470 and 340 base pairs, respectively, enabling the co-amplification of both messages from a single sample. The reaction was carried out with a *Taq* and *Pwo* polymerase mix (Expand, Roche Molecular Biochemicals), according to the manufacturer's instructions. Briefly, the reaction mix included 0.5 mM deoxy NTPs, 1 μM forward primer (ASIC2a, TCTTTGCCAACACCTCCACC; ASIC3, AGTGGCCACCTTCCTCTA) and reverse primer (ASIC2a, CTCCTGCCCTTTGAACTTGC; ASIC3, CAGTCCAGCAGCATGTCATC) and 1 ng RT-cDNA template. Cycling conditions were as follows: initial denaturation step of 2 min at 94 °C, then 35 cycles of 45 s at 94 °C, 45 s at 58 °C, and 1 min at 72 °C, followed by a final extension step of 10 min at 72 °C. RT-cDNAs were either purchased from a commercial source (Multiple Tissue cDNA Panels, CLONTECH) or prepared from poly(A)$^+$ RNA (0.5 μg) with the Thermoscript enzyme mix (Life Technologies Inc., Life Sciences). Negative controls contained only water as template.

Electrophysiology—Oocytes surgically removed from adult *Xenopus laevis* were treated for 2 h at room temperature with 1 mg/ml type I collagenase (Sigma) in Barth solution (83 mM NaCl, 5 mM NaOH, 1 mM KC1, 0.82 mM $MgSO_4$·7 H_2O, 2.4 mM $NaHCO_3$, pyruvic acid 550 mg/l, 10 mM HEPES pH 7.4) under constant agitation. Selected oocytes at stage IV-V were defolliculated manually before nuclear microinjection of 2.5–5 ng of ASIC2a and/or ASIC3 subunits in pcDNA3 vector. Subunits were co-injected at a 1:1 cDNA ratio. After 2–4 days of expression at 19 °C in Barth solution containing 50 μg/ml gentamicin and 1.8 mM $CaCl_2$, currents were recorded in a two-electrode voltage-clamp configuration, using an OC-725B amplifier (Warner Instruments). Currents were acquired and digitized at 500 Hz on a MacIntosh IIci computer with an A/D NB-MIO-16XL interface (National Instruments), and then recorded traces were postfiltered at 100 Hz in Axograph (Axon Instruments). Drugs were prepared in a modified Ringer's solution containing 97 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 10 mM acetic acid, 10 mM HEPES (Sigma) brought to pH 7.3 with NaOH. Test solutions were prepared by lowering the pH with HCl and applied to oocytes by constant perfusion (10–12 ml/min) at room temperature. Osmolarity of all solutions was adjusted to 235 mosM with choline chloride.

All currents were measured at their peak. When fast and slow phases were partially superimposed, the amplitude of the fast phases was measured at the inflection point of the shoulder. Dose-response curves were fitted to the Hill sigmoidal equation to calculate pH_{50} and IC_{50} values using the Prism 2.0 software package (Graphpad Software). Results are expressed as mean ± S.E., and statistical comparisons were performed using one-way or two-way analysis of variance with Bonferroni post-hoc test where applicable.

Western Blotting—Injected oocytes were collected in phosphate-buffered saline, pelleted at low speed centrifugation, and homogenized in 10 volumes of 10 mM HEPES buffer and 0.3 M sucrose, pH 7.4, containing a protease inhibitor mixture (Sigma). Membranes from lysates were solubilized with 1% Triton X-100 for 2 h at 4 °C and pelleted at 14000 × *g* for 5 min. Membrane proteins within supernatants were used for Western blots. Solubilized proteins were incubated with 100 μl of equilibrated anti-FLAG M2 affinity gel (Sigma) or Ni^{2+}-NTA resin (Qiagen) for 2 h at 4 °C under agitation. Then resin beads were washed six times in Tris-buffered saline containing 1% Triton X-100. Proteins specifically bound to the Ni^{2+}-NTA resin were eluted with 500 mM imidazole, and samples were mixed with 2× SDS-containing loading buffer. Proteins bound to the M2 resin were eluted directly in the loading buffer. Proteins were then separated on a 10% SDS-polyacrylamide gel and transferred overnight onto nitrocellulose. Immunostaining was performed either with mouse anti-Xpress peptide monoclonal antibody (1:1000) (Invitrogen) for detection of hexahistidine-Xpress-tagged ASIC subunits or with rabbit anti-GFP polyclonal antibodies (1:5000) (Molecular Probes) for detection of EGFP-tagged ASIC subunits. Washout of unbound primary antibodies was followed by incubation with peroxidase-labeled anti-mouse (Amersham Pharmacia Biotech) or anti-rabbit (Jackson Immunologicals) secondary antibodies (1:2000) for visualization by enhanced chemiluminescence (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

A robust response to low pH was recorded between 24 and 72 h after nuclear co-injection of rat ASIC2a and ASIC3 subunit cDNAs in *Xenopus* oocytes. Both the maximal amplitude (peak) and the total current induced by application of extracellular pH 4 were significantly larger in oocytes co-injected with ASIC2a and ASIC3 than in oocytes injected with single subunits, when recorded under the same conditions (Fig. 1*A*). Moreover, this potentiation could not be explained in amplitude or shape by the algebraic sum of the two types of currents mediated by homomeric ASIC2a and ASIC3 channels. Therefore the observed phenotype was by itself an evidence of functional interaction between these two subunits of proton-gated channels. The association of rat ASIC2a and ASIC3 subunits in heteromeric receptors gave rise to a biphasic response with a fast phase followed by a slow and sustained phase typically of larger amplitude. Although there was a significant difference between the fast phases of rat ASIC3 (0.3 ± 0.1 μA) and ASIC2a+3 currents (6.3 ± 1.3 μA, $p < 0.05$), no potentiation was found when we compared the sum of the fast phases mediated by homomeric rat ASIC2a (3.9 ± 0.9 μA) and ASIC3 to the fast phase of heteromeric rat ASIC2a+3 ($p = 0.28$) (Fig. 1*B*). However, the amplitude of the sustained current mediated by homomeric rat ASIC3 was dramatically increased in the heteromeric response (0.4 ± 0.1 μA *versus* 8.0 ± 0.9 μA, $p < 0.0001$) (Fig. 1*B*).

The phenotype of the human homomeric ASIC2a-mediated response to low pH was similar to that of the rat with two kinetics of desensitization (Fig. 2*A*). Human ASIC3 receptors

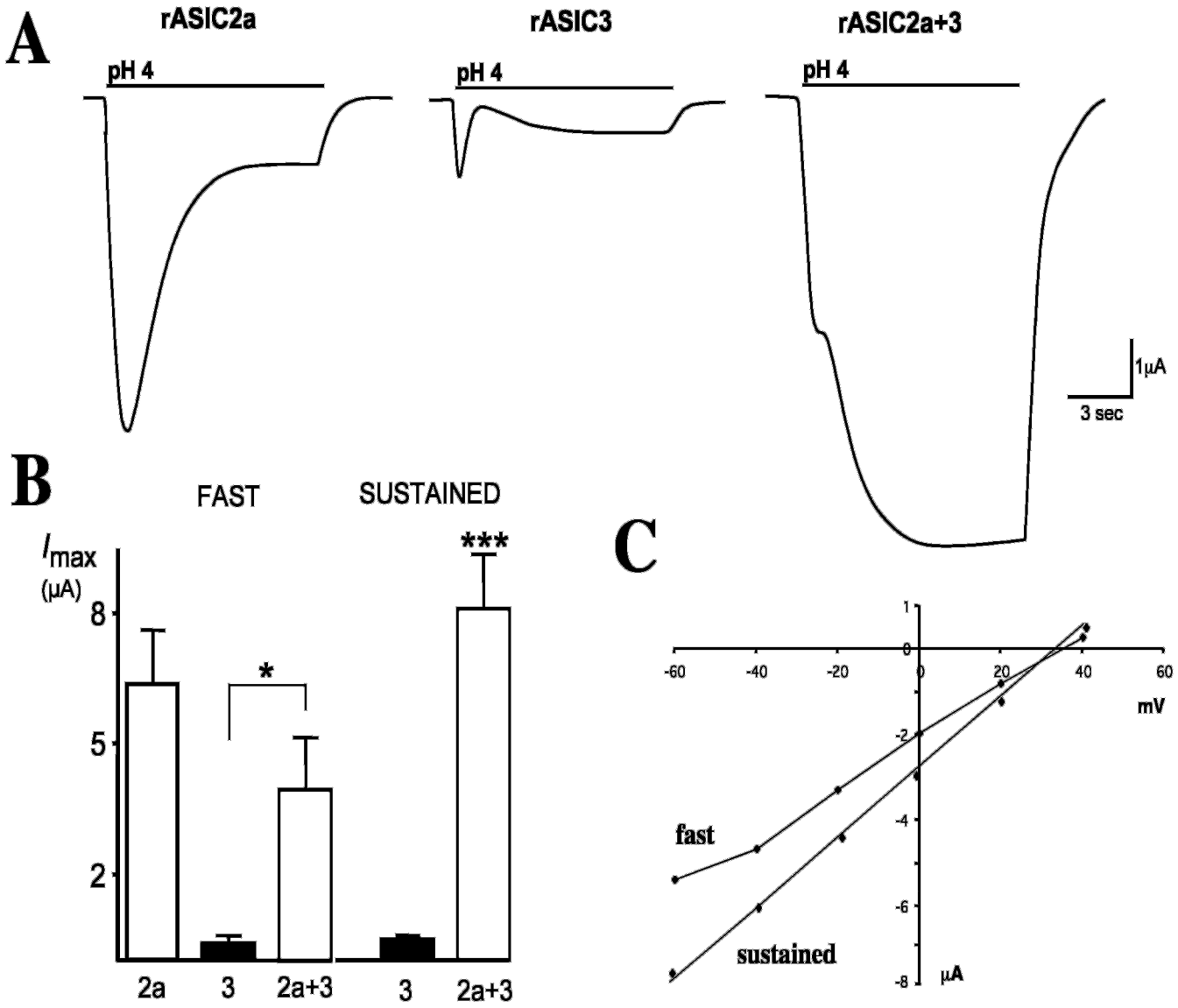

FIG. 1. **Potentiation of electrophysiological responses to low extracellular pH by co-expression of rat ASIC2a and ASIC3 subunits in *Xenopus* oocytes.** *A*, representative phenotypes of homomeric rat ASIC2a, homomeric rat ASIC3, and heteromeric rat ASIC2a+3 responses. *B,* amplitudes of both phases of ASIC2a, ASIC3, and ASIC2a+3 currents (n is 9–16). *, $p < 0.05$; ***, $p < 0.001$. *Bars* indicate mean ± S.E. *C*, current-voltage relationship of the fast desensitizing and the sustained phase of a typical rat ASIC2a+3 response.

responded slowly to low pH and did not desensitize during the 10 s of stimulation. Interestingly, in these series of experiments, we did not record a fast desensitizing phase with the human ASIC3 response as previously reported by de Weille *et al.* (24) and Babinski *et al.* (23). Technical variables related to the speed of application of acid solutions could be eliminated because biphasic responses with a fast phase were reproducibly recorded in oocytes injected with rat ASIC3 subunits (Fig. 1*A*). The biphasic response might be because of the expression of two populations of proton-gated channels. Indeed the differential sensitivity of the two phases to the antagonist amiloride suggests that ASIC3 subunits are able to assemble into fast and slow subtypes of homomeric proton-gated channels with different pharmacological properties. Specific levels of subunit phosphorylation or the translation of endogenous additional channel subunits, for example, could underlie such variability in the expression of biphasic human ASIC3 phenotypes. Nevertheless, a clear potentiation of the response to pH 4 was also recorded in oocytes co-injected with human ASIC2a and ASIC3 subunits. In contrast to rat ASIC2a+3, the increased response with the co-expressed human subunits was observed with both the fast (2.4 ± 0.6 μA *versus* 5.9 ± 0.8 μA, $p < 0.01$) and the sustained current (0.3 ± 0.03 μA *versus* 4.1 ± 0.6 μA, $p < 0.0001$), giving a phenotype that was more distinctly biphasic (Fig. 2*A*). Rat and human heteromeric currents reversed at +39.7 ± 0.8 mV and +34.9 ± 1.6 mV for the fast phases respectively, and at +33.1 ± 1.4 mV and +23.2 ± 1.5 mV for three sustained phases, as illustrated in Figs. 1*C* and 2*C*. Thus, in both species the reversal potential of the two phases was ≥+20 mV, indicating that sodium ions were the main charge carriers of the ASIC2a+3 inward current. This is consistent with the role of the amino-terminal pre-transmembrane region of ASIC2a in the selectivity for sodium ions (27). Thus the association of ASIC2a with ASIC3 subunits generates a novel phenotype of non-desensitizing proton-gated channels that are mainly sodium-selective. The small currents recorded in cells injected with ASIC3 alone (see Figs. 1*A* and 2*A*) suggest that ASIC3 subunits do not assemble favorably into homomeric channels *in vitro*. The differences in kinetic properties between rat and human homomeric ASIC3 might be because of specific functional determinants not yet identified, as suggested by the

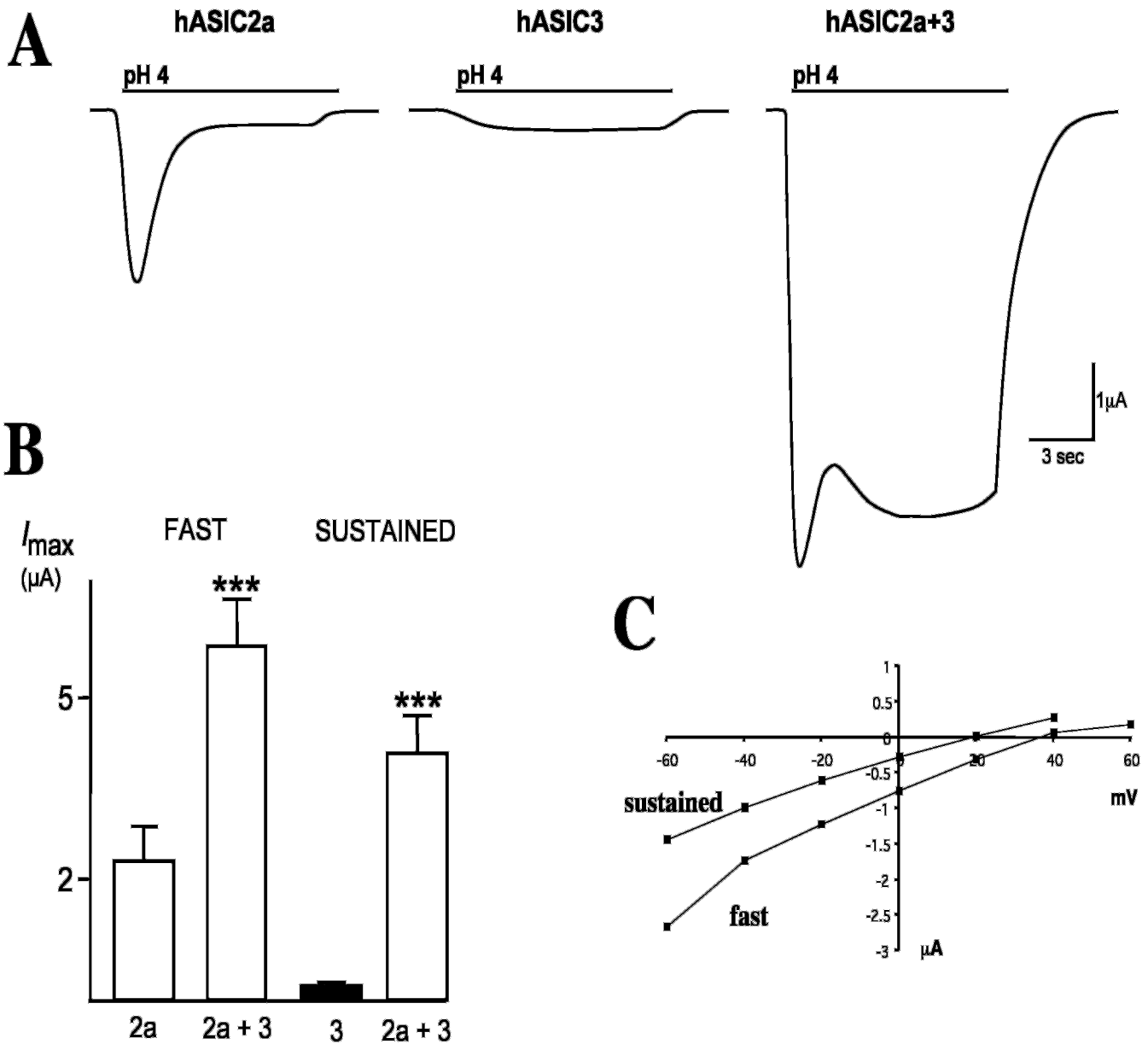

FIG. 2. **Functional interaction of human ASIC2a and ASIC3 subunits in *Xenopus* oocytes.** *A*, representative phenotype of currents mediated by homomeric human ASIC2a, homomeric human ASIC3, and human ASIC2a+3 receptors. *B*, potentiation of current amplitudes of both the fast and sustained phases of ASIC2a+3 (n is 19–47). ***, $p < 0.001$. *Bars* indicate mean ± S.E. *C,* current-voltage relationships of both phases of a typical heteromeric hASIC2a+3 response show reversal potentials of >+20 mV that are characteristic of mainly sodium-selective channels.

relatively low homology (83% identity) between their primary structures.

From this data in *Xenopus* oocytes, we can also infer that ASIC2a and ASIC3 subunits are candidate partners when co-expressed *in vivo*. Indeed, our results strongly suggest that when ASIC2a and ASIC3 subunits are co-expressed, the resulting biphasic currents are mainly mediated by heteromeric channels. However, the actual stoichiometry still remains to be elucidated, and we cannot exclude the possibility that the fast and sustained currents represent two distinct channels with varying composition of ASIC2a and ASIC3 subunits.

We tested the sensitivity of the response to low extracellular pH mediated by heteromeric ASIC2a+3 receptors. From the dose-response curves from both species shown in Fig. 3*A*, we could conclude that the fast phase of human ASIC2a+3 (pH_{50} = 4.6 ± 0.1) is slightly more sensitive to extracellular protons than the fast phase of rat ASIC2a+3 (pH_{50} = 4.3 ± 0.03) and the sustained phases of both rat and human (rat pH_{50} = 4.1 ± 0.02, human pH_{50} = 4.2 ± 0.02). All Hill slopes were found to be >1, indicating that at least two protonations have to occur before activation. Amiloride, a known antagonist for this family of sodium channels, has been shown to inhibit ASIC2a (26) as well as the fast phase of homomeric ASIC3 currents (23–25) so we challenged the heteromeric ASIC2a+3 receptors with low pH in the presence of 100 μM amiloride (Fig. 3*B*). The fast phase of the heteromeric response was significantly inhibited in rat and human ASIC2a+3 (60.3 ± 4.4% *versus* 45.6 ± 9.1% remaining current). However, the sustained phase was more reduced by amiloride in the human (47.4 ± 8.3%) than in the rat (75.9 ± 3.5%). These pharmacological results are in agreement with the previously reported differential sensitivity of the two phases of homomeric ASIC3 to pH and amiloride (23, 24), supporting our hypothesis that the slow kinetics and the potentiation of the sustained phase of ASIC2a+3 are mainly conferred by ASIC3.

Lanthanides including gadolinium (Gd^{3+}) have been re-

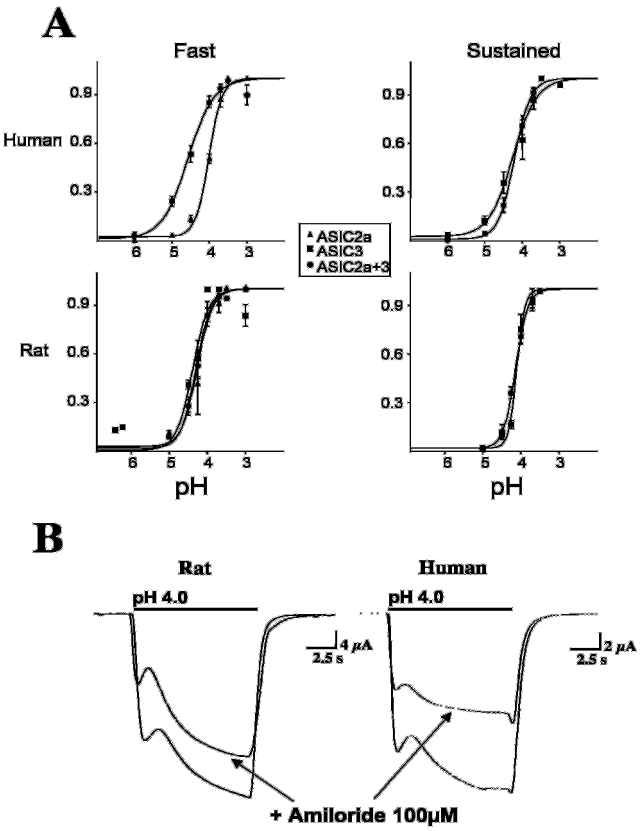

FIG. 3. **Pharmacological properties of the ASIC2a+3 subtype of proton-gated channels.** *A,* sensitivity of early/fast and late/sustained phases of rat and human ASIC2a+3-mediated responses to extracellular protons (n = 6 for each group of experiments on human, n is 17–50 for rat). *Bars* indicate mean ± S.E. *B,* differential sensitivity of the low pH-induced responses of rat and human ASIC2a+3 heteromers to 100 μM amiloride.

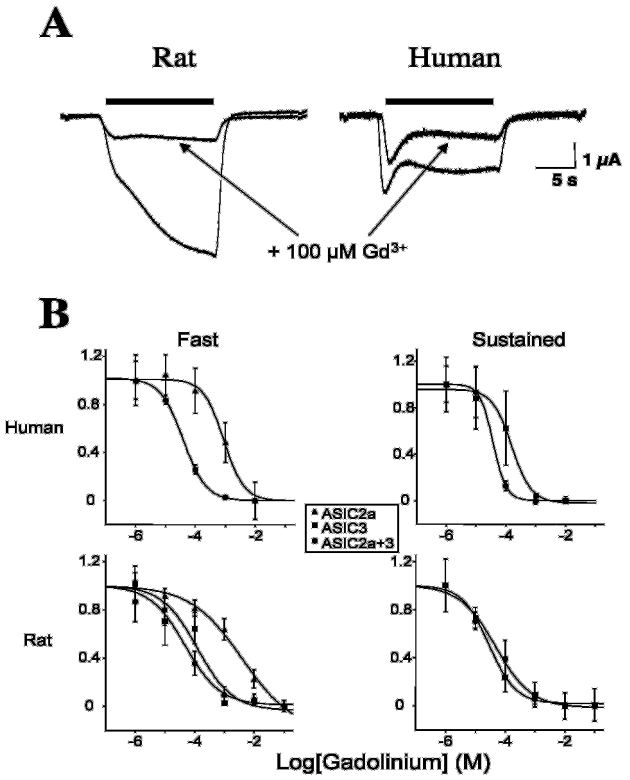

FIG. 4. **Heteromeric ASIC2a+3 and homomeric ASIC3 proton-gated channels are blocked by Gd^{3+} ions.** *A,* inhibitory effects of 100 μM Gd^{3+} on low pH-induced responses on human and rat ASIC2a+3 channels. *B,* sensitivity of both phases of human and rat ASIC2a+3 subtypes to inhibition by Gd^{3+} (n = 10 for each group of experiments). *Bars* indicate mean ± S.E.

ported to block some types of stretch-activated responses in neurons (29, 30). Because ASIC subunits belong to the same gene family as *C.elegans* degenerins, which are believed to be mechanoreceptor subunits (5–8), we decided to investigate the impact of trivalent ions on the activity of homomeric ASIC2a and ASIC3 and heteromeric ASIC2a+3 proton-gated channels. Application (with or without preincubation) of 100 μM Gd^{3+} with low pH decreased significantly the total response by inhibiting both phases of the current mediated by rat or human ASIC2a+3 (Fig. 4*A*). To our knowledge, this is the first example of an inhibitory effect of Gd^{3+} ions on ASIC proton-gated channels. The fast phase of ASIC2a+3 was found to be as sensitive as the slow phase (IC_{50}s = 49.5 ± 13.9 μM *versus* 50.3 ± 13.1 μM in rat, and 38.8 ± 0.5 μM *versus* 37.8 ± 1.2 μM in human) (Fig. 4*B*). Dose-response curves also demonstrated that the Gd^{3+} inhibition is much more potent on the fast phase of ASIC2a+3 than on the homomeric ASIC2a (IC_{50} = 49.5 μM *versus* >1 mM, $p < 0.001$ in human) (Fig. 4*B*). Furthermore, for both fast and sustained phases, ASIC3 and ASIC2a+3 receptors seemed to display the same sensitivity to Gd^{3+}, indicating that ASIC3 subunits might be the main determinants of the Gd^{3+} effect on the heteromeric response. Gd^{3+} inhibition of ASIC3 and ASIC2a+3 might be therefore related to an involvement of ASIC3 subunit-containing channels in the transduction of mechanosensory stimuli in rodents and humans.

We tested the biochemical interactions between ASIC2a and ASIC3 subunits using immunodetection in Western blot. To co-purify and visualize these two ASIC subunits, we tagged them with hexahistidine-Xpress or EGFP for ASIC2a and with hexahistidine-Xpress or FLAG epitope for ASIC3. The addition of the artificial epitopes created fusion subunits with modified intracellular domains that had the potential to disturb their native functional properties, so we checked their ability to assemble into functional homomeric and heteromeric channels when expressed in *Xenopus* oocytes. As illustrated in Fig. 5*A*, epitope-tagged ASIC2a and ASIC3 subunits correctly assemble into heteromeric channels and respond to extracellular protons as their wild-type counterparts do, with similar kinetics and sensitivity. Thus the addition of the 8-amino acid FLAG peptide, of the 40-amino acid hexahistidine-Xpress polypeptide, or even of the bulky 251-amino acid EGFP tag did not prevent the ASIC subunits from assembling, translocating to the surface of the oocytes, and being activated by low pH. We then purified human ASIC3-containing complexes with either the hexahistidine-binding Ni^{2+}-NTA resin or the FLAG peptide-binding M2 monoclonal antibody resin to check for co-purification of associated ASIC2a partners (Fig. 5*B*). A major band of M_r = 61.4 ± 2.7 (n = 2) corresponding to the human ASIC2a-His_6 subunit co-purified with human ASIC3-FLAG. Similarly, a major band of M_r = 91.9 ± 3.1 (n = 4) corresponding to human ASIC2a-EGFP co-purified with ASIC2a-His_6 as well as with the ASIC3-FLAG subunits. The validity of the labeling was controlled by the absence of specific signal in oocytes injected with epitope-tagged ASIC2a subunits only (Fig. 5*B*). We obtained identical results on association from mammalian human embryonic kidney (HEK293) cells transiently co-transfected with these subunits (data not shown). The apparent sizes of ASIC2a-His_6 and ASIC2a-EGFP subunits, as well as the difference of M_r 30.5 between these two epitope-tagged forms of ASIC2a, were in good correlation with the predicted molecular weights of the corresponding protein constructs. Therefore these experiments of co-purification demonstrate for the first time that ASIC2a

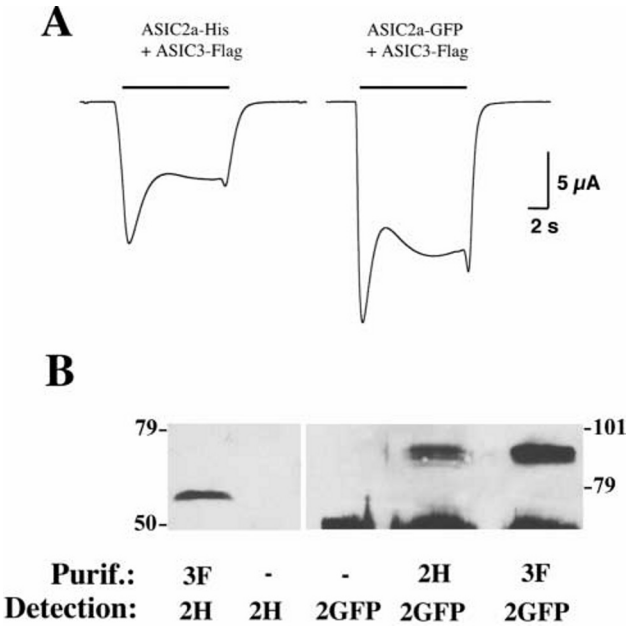

FIG. 5. **Biochemical association of ASIC2a and ASIC3 subunits.** *A,* electrophysiological characterization of epitope-tagged human subunits co-injected in *Xenopus* oocytes and assembly in heteromeric ASIC2a+3 proton-gated channels. *B,* Western blot of the co-purification of hexahistidine-tagged human ASIC2a (*2H*) and EGFP-tagged human ASIC2a (*2GFP*) with FLAG-tagged human ASIC3 (*3F*) (see "Experimental Procedures" for details). Sizes of markers are indicated in kDa.

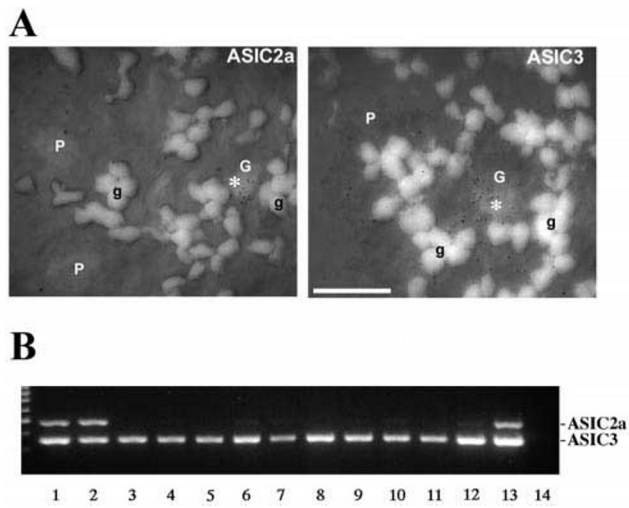

FIG. 6. **Cellular co-localization and regional co-expression of ASIC2a and ASIC3 subunits in rodents and humans.** *A, in situ* hybridization showing cellular co-localization of ASIC2a and ASIC3 subunit mRNA in Golgi neurons (*G*, *) in the granular layer of adult rat cerebellum. *P*, Purkinje cells; *g*, granule cells, *scale bar* = 20 μm. *B,* detection of co-expression of ASIC2a and ASIC3 transcripts in several central and peripheral human tissues using RT-PCR amplification. *Lane 1*, whole brain; *lane 2,* cerebellum; *lane 3*, kidney; *lane 4,* liver; *lane 5,* lung; *lane 6,* pancreas; *lane 7*, colon; *lane 8*, lymphocytes; *lane 9*, ovary; *lane 10*, prostate; *lane 11*, small intestine; *lane 12*, testis; *lane 13*, trigeminal ganglia; and *lane 14*, negative control.

and ASIC3 subunits can physically associate with high affinity to produce a heteromeric proton-gated channel complex.

Because the levels of rat ASIC2a mRNA are very low to undetectable in trigeminal or dorsal root sensory ganglia (22), we looked for native co-expression of rat ASIC2a and ASIC3 subunits in the rat brain using *in situ* hybridization. At the regional level, we detected moderate but significant amounts of both central ASIC2a and ASIC3 mRNA in the cerebellar cortex. Autoradiography at higher cellular resolution on emulsion-dipped sections allowed us to specifically localize these two subunits in the population of Golgi interneurons in the granular layer (Fig. 6*A*). Golgi cells are inhibitory interneurons that make γ-aminobutyric acid (GABA)-ergic synapses on the glomeruli of the mossy fibers (31). Thus the expression of ASIC2a and ASIC3 subunits make them susceptible to respond to local acidosis by a sustained inhibition of the main input to the cerebellum. Subcellular localization of these channels would help to understand their exact function during normal and pathological activity of the cerebellar network.

In human tissues, we relied on RT-PCR analysis to detect the co-expression of ASIC2a and ASIC3 subunits. As illustrated in Fig. 6*B*, expected RT-PCR products of 470 and 340 base pairs, corresponding to specific regions of ASIC2a and ASIC3 mRNA, respectively, were amplified from several central and peripheral structures where the *ASIC3* gene has been previously shown to be transcribed (23). Both subunits were found to be expressed in whole brain, cerebellum, testis, and trigeminal sensory ganglia (Fig. 6*B*). Low levels of ASIC2a signal were also visible in liver, colon, and ovary. These anatomical data suggest that the ASIC2a+3 subtype of proton receptor might be more widespread in the central nervous system, in sensory ganglia, and in peripheral tissues of human than in rat. Furthermore, the expression of ASIC2a subunits in human sensory ganglia also suggests the presence of homomeric ASIC2a proton-gated channels in sensory neurons. As ASIC2b is the only major variant of the *ASIC2* gene in the primary sensory neurons of rat, our data support the idea that the regulation of transcription of *ASIC2* is species-specific. Although the exact contribution of ASIC2a+3 receptors to the response of human sensory neurons to low extracellular pH is not yet documented, these differences of distribution could also mean that in humans the heteromeric ASIC2a+3 receptors could correspond to a major sensory subtype, in marked contrast to what has been reported for the rat. Completion of the analysis of the human *ASIC2* gene will definitely answer the question about the existence of exon(s) coding for an ASIC2b variant. In any case, we provided evidence that ASIC2a+3 heteromers, likely a major neuronal subtype in humans, will have to be taken into consideration when designing potential therapeutic agents acting on proton-gated channels. The existence of this novel ASIC phenotype illustrates the functional diversity of ion channels involved in the nociceptive response of primary sensory neurons to acidosis.

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