Regulation of Sensory Neuron-specific Acid-sensing Ion Channel 3 by the Adaptor Protein Na+/H+ Exchanger Regulatory Factor-1*S

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Acid-sensing ion channels (ASICs) are cationic channels activated by extracellular protons. The ASIC3 subunit is largely expressed in the peripheral nervous system, where it contributes to pain perception and to some aspects of mechanosensation. We report here a PDZ-dependent and protein kinase C-modulated association between ASIC3 and the Na+/H+ exchanger regulatory factor-1 (NHERF-1) adaptor protein. We show that NHERF-1 and ASIC3 are co-expressed in dorsal root ganglion neurons. NHERF-1 enhances the ASIC3 peak current in heterologous cells, including F-11 dorsal root ganglion cells, by increasing the amount of channel at the plasma membrane. Perhaps more importantly, we show that the plateau current of ASIC3 can be dramatically increased (10–30-fold) by association with NHERF-1, leading to a significant sustained current at pH 6.6. In the presence of NHERF-1, the ASIC3 subcellular localization is modified, and the channel co-localizes with ezrin, a member of the ezrin-radixin-moesin family of actin-binding proteins, providing the first direct link between ASIC3 and the cortical cytoskeleton. Given the importance of the ASIC3 sustained current in nociceptor excitability, it is likely that NHERF-1 participates in channel functions associated with nociception and mechanosensation.

Physiopathological conditions such as ischemia, inflammation, tumors, or injury are associated with a decrease in extracellular pH, and tissue acidosis has been linked to pain in human volunteers (1–3). Nociceptive neurons display voltage-independent H+-gated cationic currents, which are in part mediated by acid-sensing ion channels (ASICs)² (4, 5). The ASIC family comprises six isoforms encoded by four different expression (21). This paper describes the adaptor protein NHERF-1 (Na+/H+ exchanger regulatory factor-1) as an accessory protein that teams up with ASIC3 to provide a new type of modulation of its function and of its subcellular localization in relation with the cytoskeleton.

**MATERIALS AND METHODS**

**Yeast Two-hybrid Screening**—An ASIC3 COOH-terminal bait corresponding to the last 68 amino acids of rat ASIC3 was used to screen a rat DRG cDNA library as previously described (18). For subsequent analysis, the full-length rat NHERF-1 and NHERF-2 cDNAs, encoding the 356- and 337-amino acid proteins, respectively, as well as the mutated forms of both ASIC3 and NHERF-1 were obtained by PCR (22).

**Immunoprecipitation and Western Blot**—COS-7 cells were transiently transfected with rat HA-NHERF-1 or HA-NHERF-2 (NH2-terminal HA tag), rat Myc-ASIC3 (NH2-terminal Myc tag), or both, by the DEAE-dextran method. F-11 cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Cells were scrapped in lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM
NaCl, 5 mM EDTA, 1% Triton X-100, and Roche Complete protease inhibitors (Roche Applied Science) 24–48 h post-transfection and then centrifuged for 30 min at 100,000 × g. For phosphorylation experiments, cells were treated for 20 min with a 100 nM concentration of the PKC activator phorbol 12-myristate 13-acetate (PMA) or with a 10 μM concentration of the PKC inhibitor chelerythrine (Sigma) before lysis. Supernatant was collected and immunoprecipitated with anti-HA 3F10 antibody (Roche Applied Science), anti-Myc A14 antibody (Santa Cruz Biotechnology), or anti-NHERF-1 antibody (Affinity Bioreagents). After incubation with protein A or protein G affinity gel (Sigma), beads were washed four times with lysis buffer and boiled in SDS-PAGE buffer for 5 min. Immunoprecipitated or cell lysate proteins were separated on 12% polyacrylamide gels by SDS-PAGE and electroblotted onto polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp.). Membranes were incubated with the 3F10 (1:1,000), A14 (1:500), anti-NHERF-1 (1:800), or anti-NHERF-2 (1:1,000) antibody for 1 h at room temperature or overnight at 4 °C. Peroxidase-conjugated (for chemiluminescence revelation; 1:10,000) secondary antibodies were used. Blots were revealed with the supersignal WestPico luminescent detection system (PerkinElmer Life Sciences).

Freshly isolated mouse dorsal root ganglia were homogenized on ice with a Dounce homogenizer in lysis buffer and then incubated for 30 min at 4 °C before centrifugation for 15 min at 10,000 × g. Supernatants were collected, and protein concentrations were determined by the Bradford method. Western blots were performed as previously described.

Surface Biotinylation in COS-7 Cells—Surface biotinylation experiments were carried out according to standard protocols. Briefly, COS-7 cells were transiently transfected by the DEAE-dextran method and used 48 h post-transfection. Cells were incubated on ice for 30 min with 1 mg/ml Sulfo-NHS-Biotin reagent (Pierce) dissolved in PBS (pH 8.0) and then washed twice with ice-cold PBS complemented with 100 mM glycine and incubated for 20 min in the same buffer on ice. Cell lysis was performed as previously described for the immunoprecipitation experiments. After the 100,000 × g centrifugation step, supernatant was mixed with 30–50 μl of streptavidin-agarose beads (Sigma) and incubated for 2 h at 4 °C. Beads were washed four times with lysis buffer and then heated in SDS-PAGE loading buffer for 20 min at 65 °C. Proteins were next resolved on a 12% polyacrylamide gel, and subsequent steps (transfer and Western blot analysis) were carried out as described previously.

Immunofluorescence and in Situ Hybridization—COS-7 cells were grown on 35-mm plates and transfected by the DEAE-dextran method. The next day, cells were dissociated with 1 mM EDTA in phosphate-buffered saline (PBS) and plated on glass coverslips in 24-well plates. One day later, cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, blocked with 10% horse serum, and incubated with primary antibodies anti-Myc A14 (1:800) or 9E10 (1:200; Santa Cruz Biotechnology, Inc.), anti-HA 3F10 (1:2,000), or anti-ezrin (1:700; gift of Dr. M. Arpin), followed by the secondary antibodies goat anti-rabbit Alexa 488 (1:1,500), donkey anti-rat Alexa 594 (1:1,500), or donkey anti-mouse Alexa 594 (1:1,500) (Molecular Probes, Inc., Eugene, OR). Staining was visualized using an Axioplan 2 microscope (Carl Zeiss). In situ hybridization was essentially performed as previously described (10, 18) with an antisense synthetic oligonucleotide corresponding to the rat ASIC3 sequence (CAACATGTCCTAAAGGGAGTGCCCG) and a sense primer for control experiments corresponding to the sequence GCCAGATATTGACCATGCTCTCTC. For fluorescent labeling, detection was performed with the ELF-97 mRNA in situ hybridization kit (Molecular Probes) based on the streptavidin-alkaline phosphatase interaction with the biotinylated probe and the formation of green fluorescent precipitates in the presence of the ELF-97 substrate. Detection of the NHERF-1 protein by immunohistochemistry was performed after the in situ hybridization with an anti-NHERF-1 rabbit polyclonal antibody (1:200; Affinity Bioreagents) followed by a goat anti-rabbit Texas Red secondary antibody (Jackson ImmunoResearch Laboratories). All experiments were done on at least two animals.

Reverse Transcription-PCR Analysis—Experiments were performed from adult rat total RNA as previously described (18). The following sense and antisense primers were used for PCR: ASIC3, CTG-GCAACGAGACTGGAGATTA and TGTAGTACGGACCGGTTTG (amplicon of 506 bp); NHERF-1, CCGTCTGCACCATGAAGAA and CTAGAGGGTGTGCTAAAGGTT (amplicon of 625 bp); and NHERF-2, ATACATCGGCTCTTGAGCCCC and CTGCTGAGGCTTTGGGAGGCT (amplicon of 576 bp). Twenty-five cycles of PCR (95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min) were performed,
except for β-actin, for which only 22 cycles were done. One-third of the PCR was resolved on a 2% agarose gel. The PCR conditions have been designed to avoid overamplification and allow a better comparison between tissues.

Transfection and Electrophysiology of COS-7 and F-11 Cells—COS cells were transfected with a mix of rat NHERF-1 and rat ASIC3 or ASIC3Δ5, cDNAs using the DEAE-dextran method. Cells were used for electrophysiological measurements 1–4 days after transfection. F-11 DRG cells cultured in Ham’s F-12 medium (Invitrogen) supplemented with 15% fetal bovine serum (ICN Biomedicals), 1 mM glutamine, and streptomycin (500 μg/ml). Currents were recorded within 3–5 days of DNA injections. In a 0.3-ml perfusion chamber, a single oocyte was gently impaled with two standard glass microelectrodes (1–2.5 megohms) filled with a 3 mM KCl solution and maintained under voltage clamp using a Dagan TEV 200 amplifier. Data acquisition and analysis were performed using pClamp software (Axon Instruments). All experiments were performed at 19 °C, and MES or acetic acid was used instead of HEPES to buffer ND96 solution pH ranging from 6 to 3.

Statistical Analysis—Data points represent the mean ± S.E. of n independent experiments. The one-way analysis of variance test followed by a Tukey post hoc test was used for statistical analysis using Prism software (version 4.03; GraphPad).

RESULTS
NHERF-1 and NHERF-2 Associate with the ASIC3 COOH Terminus in a Yeast Two-hybrid Assay—The ASIC3 COOH terminus shares homology with type I PDZ-binding motifs ((S/T)X(V/L)) (23). The yeast two-hybrid system was used to identify putative ASIC3-interacting proteins. A rat DRG cDNA library (18) was screened with the last 68 amino acids of rat ASIC3 as a bait. Among the isolated clones, one clone encoded a full-length NHERF-1 protein, also named EBP50 (ezrin-rac-104)dixin-moesin-binding phosphoprotein of 50 kDa), and another encoded a partial NHERF-2 protein, also identified as E3KARP (Na+/H+ exchanger 3 kinase-activator-1), or SIP-1 (SRY-interacting protein-1). NHERF-1 and NHERF-2 are PDZ-containing proteins that mediate specific protein-protein interactions and thereby can serve as adaptors. They have been involved in the regulation of the targeting and trafficking of specific integral

Oocyte Injection and Electrophysiology—Oocytes were kept at 19 °C in ND96 solution (containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 2 mM MgCl2, and 5 mM HEPES) supplemented with penicillin (6 μg/ml) and streptomycin (5 μg/ml). Currents were recorded within 3–5 days of DNA injections. In a 0.3-ml perfusion chamber, a single oocyte was gently impaled with two standard glass microelectrodes (1–2.5 megohms) filled with a 3 mM KCl solution and maintained under voltage clamp using a Dagan TEV 200 amplifier. Data acquisition and analysis were performed using pClamp software (Axon Instruments). All experiments were performed at 19 °C, and MES or acetic acid was used instead of HEPES to buffer ND96 solution pH ranging from 6 to 3.

Statistical Analysis—Data points represent the mean ± S.E. of n independent experiments. The one-way analysis of variance test followed by a Tukey post hoc test was used for statistical analysis using Prism software (version 4.03; GraphPad).
membrane proteins (for a review, see Ref. 24). They both contain two tandem PDZ domains followed by a carboxyl-terminal sequence that binds to members of the ezrin-radixin-moesin (ERM) family of membrane-cytoskeleton adaptors. The NHERF-1 and NHERF-2 clones isolated from the rat DRG library both contained functional PDZ domains, strongly suggesting an interaction between these PDZ domains and the COOH-terminal region of ASIC3.

**NHERF-1 and NHERF-2 Bind to ASIC3 through Their PDZ-1 Domain in Transfected COS Cells**

To confirm the two-hybrid results and test the interaction with the full-length proteins, binding between NHERFs and ASIC3 was assayed by coimmunoprecipitation in transfected COS-7 cells. Constructs encoding Myc-tagged ASIC3 and HA-tagged NHERF-1 and NHERF-2 or mutant variants were co-transfected in COS cells, and cell lysates were immunoprecipitated with anti-HA antibodies, followed by Western blot analysis. Wild-type ASIC3 interacted with NHERF-1 and NHERF-2 (Fig. 1, A (lane 1) and B (lane 7)), and association occurred regardless of which was initially precipitated (data not shown). This interaction was impaired by removing the last three amino acids of ASIC3, which form most of the PDZ binding motif (23) (ASIC3 Δ531–533 mutant; Fig. 1, A (lane 2) and B (lane 8)). As expected, NHERF-1 did not co-precipitate with ASIC1a and ASIC2a (Fig. 1A, lanes 3 and 4), two ASIC subunits with a different COOH-terminal PDZ-binding motif.

The two NHERF-1 PDZ domains have considerable structural homology but recognized distinct synthetic peptides matching the consensus sequence (S/T)(R/Y)L-COOH for PDZ-1 (similar to the COOH-terminal motif of ASIC3) and S(S/T)WL-COOH for PDZ-2 (25). In good agreement with this, versions of NHERF-1 and NHERF-2 made with a deletion of the first or the second PDZ domain demonstrated that the PDZ-1 domain of NHERF-1 and NHERF-2, but apparently not the PDZ-2 domain, was necessary for the interaction (Fig. 1, A (lanes 5 and 6) and B (lanes 9 and 10)). However, surface plasmon resonance measurements have shown that both PDZ domains of NHERF-1 can interact with an immobilized peptide corresponding to the COOH terminus of CFTR (comprising the TRL motif) but that the PDZ-1/CFTR complex is formed much faster than the PDZ-2/CFTR interaction (26). We cannot
Modulation of ASIC3 by NHERF-1

FIGURE 4. NHERF-1 is expressed in mouse and rat DRG neurons where it partially co-localizes with ASIC3. A, reverse transcription-PCR experiments; ASIC3, NHERF-1, and NHERF-2 mRNA levels have been assessed in different rat tissues (indicated at the top of each lane) by reverse transcription-PCR. Control amplification with β-actin was performed in parallel (bottom). Images show ethidium bromide staining of the product of 35 cycles (22 for β-actin) of amplification resolved on an agarose gel. B and C, Western blot experiments. Proteins from mouse and rat DRG lysates (∼20 μg) were blotted for NHERF-1 (B) or NHERF-2 (C). Lysate of COS cells (∼1 μg) transfected with recombinant rat NHERF-1 (B) or NHERF-2 (C) was used as a positive control (CTR). D, ASIC3 co-immunoprecipitates with endogenous NHERF-1 in rat F-11 DRG cells. Myc-tagged ASIC3 was transfected in F-11 cells, and cell lysates were immunoprecipitated (IP) with an anti-NHERF-1 antibody. Western blots were probed with anti-Myc antibody to detect ASIC3. Myc-ASIC3, was used as a control. E, expression of ASIC3 mRNA and NHERF-1 protein in rat DRG. Tissue sections were first treated for in situ hybridization (ASIC3, left) and then for immunohistochemistry (NHERF-1; middle). Co-localization of ASIC3 and NHERF-1 is shown in yellow in the merged images (right). Scale bar, 100 μm.

Therefore, the possibility of some weak interaction between ASIC3 and the PDZ-2 domain of NHERF-1 in certain conditions. NHERF-1 mutants with a COOH-terminal deletion of the last 30 or 60 amino acids were still able to associate with ASIC3 (supplementary Fig. 1), indicating that the ERM domain of NHERF-1 was not involved in the interaction. Levels of wild-type and mutant proteins were comparable in all of these experiments (Fig. 1, A (lanes 1 and 2 and lanes 5 and 6) and B (lanes 7–10)).

These results demonstrate that the ASIC3 COOH-terminal PDZ binding motif interacts with the PDZ-1 domain of NHERF-1 and NHERF-2. They also suggest a constitutive association of NHERFs and ASIC3 in transfected mammalian cells.

Phosphorylation of the ASIC3 COOH-terminal Domain Increases NHERF-1 Binding—Phosphorylation-dependent modulation of the interaction between PDZ proteins and PDZ binding motifs has been well documented. ASIC3 contains a conserved consensus site for phosphorylation by PKC in its intracellular COOH-terminal domain (Ser-523; Fig. 2A). Previous work has suggested that this residue is phosphorylated by PKC (20). We have investigated the impact of phosphorylation on this position on the interaction between NHERF-1 and ASIC3. PKC stimulation by PMA increased ASIC3 co-precipitation with NHERF-1 by ∼50% in COS-7 cells co-transfected with Myc-ASIC3 and HA-NHERF-1 compared with a condition where PKC was inhibited with chelerythrine (Fig. 2, B (lanes 1 and 2) and C). A further indication that phosphorylation of serine 523 was likely to be involved in this regulation included the observation that the nonphosphorylatable glycine mutant (523G) prevented the PMA effect (Fig. 2B, lane 3). In addition, a mutation mimicking phosphorylation in ASIC3 (523D) mimicked the PMA effect despite the presence of chelerythrine (Fig. 2B, lanes 4). All of these data strongly suggest that PKC phosphorylation of serine 523 in the COOH-terminal region of ASIC3 positively modulates the interaction between ASIC3 and NHERF-1.

NHERF-1 and NHERF-2 Increase ASIC3 Current in Xenopus Oocytes—We subsequently measured the effect of NHERF-1 and NHERF-2 on ASIC3 current in Xenopus oocytes. Both proteins were able to strongly potentiate the pH 5.0-evoked ASIC3 transient current amplitude when co-injected with the channel (∼6.9- and ∼7.7-fold increase, respectively; Fig. 3, A and B). The more dramatic effect was seen on the sustained current (∼30–40%–42–4-fold increase, respectively, Fig. 3C). The pH dependence of the peak and sustained currents was not altered (Fig. 3D); nor was the selectivity of the sustained current (Fig. 3E). The kinetics of desensitization were only slightly modified (supplementary Fig. 2). The NHERF-1 PDZ-1 domain, but not the PDZ-2 domain, and the last COOH-terminal region of ASIC3 were required for the NHERF-1 effect to occur on both the peak and sustained current (Fig. 3, B and C), consistent with the biochemical data described in Fig. 1. Deletion of the NHERF-1 ERM-binding domain (NHERF-1-DΔ390) decreased the effect of NHERF-1 on the ASIC3 transient current (Fig. 3B). The amplitude measured in the presence of NHERF-1-DΔ390 was still higher than the amplitude observed with ASIC3 expressed alone (5.87 ± 0.70 μA (n = 18) versus 1.57 ± 0.24 μA (n = 90), respectively, p < 0.01). However, the amplitude was significantly lower than the one of ASIC3 co-expressed with wild type NHERF-1 (5.87 ± 0.70 μA (n = 18) versus 9.96 ± 0.73 μA (n = 79), respectively, p < 0.05). This suggests that the effect on the peak current is partially dependent on the interaction with ERM proteins and thereby on the association of the channel with the cytoskeleton. The NHERF-1-DΔ390 mutant had no significant effect on the sustained current (Fig. 3C), which may reflect differences in the regulation by NHERF-1 of the peak and the sustained components of the ASIC3 current.

NHERF-1 Is Expressed in Native DRG Neurons, Where Its Distribution Partly Overlaps with That of ASIC3—We next examined the expression of NHERF-1 and NHERF-2 in native DRG neurons. NHERF-1 transcripts were detected in rat DRG by reverse transcription-PCR (Fig. 4A). The levels seemed lower than in epithelial tissues like lung and colon. However, the NHERF-1 protein was easily detected by Western blot in lysates from both rat and mouse DRG (Fig. 4B). These data confirmed the previous observation of high levels of the NHERF-1 protein in rat DRG neurons (27). On the other hand, NHERF-2 transcripts are barely detected in rat DRG (Fig. 4A),
and the protein was not detected in DRG neurons by Western blot (Fig. 4C) and immunohistochemistry (data not shown). The overlap in expression between ASIC3 and NHERF-1 was next analyzed in rat DRG by double in situ hybridization and immunohistochemistry. We used in situ hybridization to detect ASIC3 mRNA, and the NHERF-1 protein was subsequently identified on the same samples by immunohistochemistry (Fig. 4E). ASIC3, which has been previously shown to be expressed in nociceptive sensory neurons (9, 10), exhibited significant overlapping expression with NHERF-1 in rat DRG neurons (Fig. 4E, right).

We have not been able to confirm the specificity of the commercially available antibodies against ASIC3 on DRG tissues isolated from ASIC3-inactivated mice, including an antibody directed against the 21-amino acid sequence at the COOH terminus of rat ASIC3 (Chemicon). We have therefore transfected a Myc-tagged form of ASIC3 in the rat F-11 DRG cell line (see below) and have examined its association with endogenous NHERF-1. ASIC3 was detected by Western blot after immunoprecipitation with an anti-NHERF-1 antibody (Fig. 4D), indicating that Myc-ASIC3 and endogenous NHERF-1 interact in this neuronal DRG cell line. Sensory neurons thus express high levels of NHERF-1 together with ASIC3, and ASIC3 associates with endogenous NHERF-1 in F-11 DRG cells, strongly suggesting that both proteins interact in vivo. NHERF-1 rather than NHERF-2 appears as the genuine partner of ASIC3 in DRG neurons.

**NHERF-1 Co-localizes with ASIC3 and Alters Its Pattern of Expression in COS Cells**—NHERF-1 is known to participate in the regulation of the targeting or trafficking of several membrane proteins (24). We therefore examined the cellular distribution of ASIC3 and NHERF-1 by immunofluorescence to confirm and to explore the consequences of the association between these two proteins in mammalian cells. Fixed and permeabilized COS cells were stained for HA-tagged NHERF-1 and Myc-tagged ASIC3. When ASIC3 was expressed alone, ASIC3 immunoreactivity was predominantly observed in
intracellular compartments and around the nucleus, most probably representing the endoplasmic reticulum (ER) apparatus (Fig. 5A).

ASIC3 also displayed some labeling at the plasma membrane, consistent with electrophysiological recordings from transfected COS cells (9) (Fig. 8). We also observed a strong immunoreactivity overlapping the cell nucleus, possibly caused by nonspecific labeling or simply reflecting the fact that there is an overexpression of ASIC3 in the system. When NHERF-1 was expressed alone, NHERF-1 staining was observed both in the cytoplasm and at the plasma membrane (Fig. 5C). Co-expression of NHERF-1 and ASIC3 altered the distribution of ASIC3 and, to a lower extent, of NHERF-1 (Fig. 5, D–F).

The two staining patterns indicated a close colocalization (Fig. 5F). The effect is specific, since co-expression of NHERF-1 with ASIC1a instead of ASIC3 failed to produce a redistribution of ASIC1a (Fig. 5, P–R). Deletion of the PDZ domain interaction site by removing the last three amino acids in ASIC3 did not affect subcellular localization when the channel was expressed alone (Fig. 5B) but eliminated the NHERF-1-induced redistribution of ASIC3 and prevented co-localization when co-expressed with NHERF-1 (Fig. 5, G–I). In addition, deletion of the first PDZ domain in NHERF-1 also eliminated the redistribution of ASIC3 (Fig. 5, J–L), whereas deletion of the second PDZ domain had no noticeable effect (Fig. 5, M–O). NHERF-1 and

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**FIGURE 6.** ASIC3 co-localizes with endogenous ezrin in the presence of NHERF-1. COS cells were transfected with plasmids encoding either Myc-ASIC3 alone (A–C) or a construct designed for simultaneous expression of NHERF-1 and Myc-ASIC3 or Myc-ASIC3Δ2 (D–I). Cells were permeabilized and immunostained with anti-Myc (red) and anti-ezrin (green) antibody. A, D, and G and B, E, and H illustrate ASIC3 and endogenous ezrin staining, respectively, and C, F, and I are merged images. The arrowheads in F illustrate the co-localization of ASIC3 and ezrin at specific sites of the cell, probably at the plasma membrane. Scale bar, 10 μm.

**FIGURE 7.** Deletion of the ERM-binding domain in NHERF-1 prevents accumulation of ASIC3 at specific sites. COS cells were co-transfected with Myc-ASIC3 and HA-NHERF-1 (A–C) or a mutant form of HA-NHERF-1 lacking the ERM-binding domain (NHERF-1Δ60; D–F). A and D, NHERF-1 labeling; B and E, ASIC3 labeling, after cell permeabilization and immunostaining with anti-HA (red) and anti-Myc (green) antibody. The arrowheads in C show the accumulation of ASIC3 at specific sites probably in the membrane, which is dependent on the ERM-binding domain of NHERF-1. Scale bar, 10 μm.
ASIC3 thus co-localize in permeabilized COS cells in a PDZ-dependent way, and NHERF-1 regulates the ASIC3 cellular distribution, probably by increasing export of the channel from the ER.

Endogenous Ezrin Co-localizes with ASIC3 through Binding to NHERF-1, and ERM Proteins Participate in the Subcellular Localization of the Channel—The carboxyl-terminal region of NHERF-1 is known to interact with the FERM (four-point-one/ezrin/radixin/moesin) domain of activated ERM proteins. Therefore, we examined by immunocytochemistry in COS cells whether ezrin, a member of the ERM family expressed in these cells, was also present in the complex containing NHERF-1 and ASIC3. In nontransfected cells, endogenous ezrin showed in some cells a homogenous distribution throughout the cytoplasm, whereas in other cells it was accumulated in specific regions of the membrane, probably representing filopodia and microvilli (data not shown), presumably representing the activated state of the protein. As expected, cells transfected with a unique vector carrying ASIC3 and NHERF-1, driven by two different promoters, displayed a severe change in the distribution of ASIC3 (Fig. 3B) compared with ASIC3 alone (Fig. 6A). The channel did not co-localize with endogenous ezrin when expressed alone (Fig. 6, A–C), whereas good colocalization was observed in discrete regions upon NHERF-1 co-expression (Fig. 6F). In contrast, in cells co-expressing NHERF-1 and an ASIC3 mutant lacking the last three amino acids, no effect on the ASIC3 localization and no colocalization with endogenous ezrin were observed (Fig. 6, G–I).

ASIC3 co-expression with NHERF-1 did not seem to modify the endogenous ezrin distribution, i.e. to participate in the activation and/or the translocation of ezrin to the membrane (data not shown). Deletion of the last 60 amino acids in NHERF-1 removes the ERM-binding domain and precludes the accumulation of NHERF-1 in specific regions observed in a subset of transfected COS cells (Fig. 7A), resulting in a diffuse distribution of the protein throughout the cell (Fig. 7D). ASIC3 relocalization was not prevented by the NHERF-1 deletion mutant, which still co-localized with the channel (Fig. 7, D–F), in good agreement with the data obtained in Xenopus oocytes showing a significant increase in the ASIC3 current upon co-expression with the NHERF-1Δ60 mutant (Fig. 3B). However, ASIC3 did not accumulate at discrete sites anymore (Fig. 7, compare F with C), which may be relevant to the significant decrease in the amplitude of the ASIC3 current observed in oocytes co-expressing the NHERF-1Δ60 mutant as compared with wild-type NHERF-1 (Fig. 3B).

These results indicate that ASIC3 interacts specifically with a protein complex including NHERF-1 and ERM proteins like ezrin. Furthermore, ERM proteins seem to be involved, through their interaction with NHERF-1, in the recruitment of ASIC3 to specific regions, probably in the plasma membrane.

NHERF-1 Increases ASIC3 Surface Expression in Transfected COS Cells—The functional data obtained in Xenopus oocytes, the redistribution of ASIC3 in COS cells upon NHERF-1 expression, and the effect of the ERM-binding domain mutant indicated that NHERF-1 probably influences the cell surface expression of ASIC3. This possibility was directly examined by surface biotinylation on COS cells. Cells expressing Myc-tagged ASIC3 together with NHERF-1 were treated with a membrane-impermeable biotinylation reagent. In the presence of NHERF-1, a greater fraction of ASIC3 was biotinylated (i.e. associated with the plasma membrane) compared with the control condition without NHERF-1 or with an ASIC3 protein lacking the PDZ-binding motif.

FIGURE 8. NHERF-1 increases the surface expression of ASIC3 in COS-7 cells. A, cell surface biotinylation analysis of ASIC3 in transiently transfected COS-7 cells expressing Myc-tagged ASIC3 alone or together with NHERF-1 or CIPP. After surface biotinylation, labeled proteins were immunoprecipitated with streptavidin-agarose beads and immunoblotted with anti-Myc antibody to detect ASIC3. B, densitometric quantification of the immunoprecipitated ASIC3 as shown in A (n = 4; **, p < 0.01 compared with ASIC3 alone). C, typical pH 5.0-evoked currents recorded from COS-7 cells transfected with ASIC3 or ASIC3 + NHERF-1. Whole-cell currents were recorded at −50 mV, and the dotted lines represent the zero current level. The bars above each current trace indicate the time at which external acidifications (from pH 7.4 to pH 5.0) were applied to the cells. The inset represents the endogenous NHERF-1 protein in COS-7 detected by Western blot from whole cell lysate of nontransfected COS-7 cells (−20 µg of proteins; left) or COS cells transfected with HA-NHERF-1 (−2 µg of proteins; right) as a control. D, statistical analysis of the ASIC3 transient (left) and sustained (right) current densities measured from COS-7 cells transfected with ASIC3, ASIC3 + NHERF-1, or ASIC3Δ3 + NHERF-1 (n = 12–51; **, p < 0.01 compared with ASIC3 alone).
The PDZ protein CIPP was used as a positive control and also enhanced the cell surface expression of ASIC3 (Fig. 8, A and B), which was consistent with our previous data (18). These results were further confirmed by patch clamp analysis of COS cells transfected with ASIC3, ASIC3 + NHERF-1, or ASIC3 + NHERF-1ΔPDZ1. The pH 5.0-evoked ASIC3 peak current density was increased ~2.4-fold in the presence of NHERF-1 (Fig. 8D, left). The effect was absent when the COOH-terminal deletion mutant of ASIC3 (ASIC3Δ3) was co-transfected with NHERF-1. The potentiating effect of NHERF-1 was also observed on the pH 5.0-evoked sustained current. This effect was again more important than the one observed on the transient current (~11.8- versus ~2.4-fold increase, respectively), and it was not observed when ASIC3Δ3 was co-transfected with NHERF-1 (Fig. 8D, right). However, the amplitude of the effect was probably underestimated due to the presence of endogenous NHERF-1 in COS cells (Fig. 8C, inset). Taken together, these data indicate that NHERF-1 is able to increase the cell surface expression of ASIC3 in COS cells.

The Potentiating Effect of NHERF-1 Is Observed in the F-11 DRG Cell Line—In order to confirm the effect of NHERF-1 in neuronal cells, we next tried to measure this effect in the F-11 DRG cell line. This sensory neuronal cell line is a hybrid of mouse neuroblastoma and embryonic rat DRG neurons (28). As mentioned before, the NHERF-1 protein was detected in F-11 cells (Fig. 4D, bottom). These cells also displayed endogenous ASIC1 currents but no ASIC3 currents.3 We have therefore transfected ASIC3 in F-11 cells, together or not with NHERF-1. NHERF-1 was, once more, able to increase both the transient and the sustained phase of the current at pH 5.0, in a way very similar to COS cells (~2- and ~9.4-fold increase, respectively; Fig. 9, A–C). Perhaps most importantly, the effect was still present at pH 6.6 (~2.2- and ~6.1-fold increase; Fig. 9, D and E), where it generated a large sustained current compared with ASIC3 alone (Fig. 9, D and E). These data confirm the capacity of NHERF-1 to positively regulate ASIC3 at moderately acidic pH in a neuronal DRG-derived cell environment.

3 A. Baron, personal communication.
FIGURE 10. Model of the interaction of NHERF-1 with ASIC3. NHERF-1 associates with ASIC3, probably in the ER, and favors the trafficking of the channel to the plasma membrane. At the membrane, NHERF-1 can directly modulate ASIC3 activity but also links the channel to the cortical actin cytoskeleton through the ERM proteins. This participation in the accumulation of ASIC3 in discrete regions. NHERF-1 has the ability to homo- or heterodimerize and probably serves as an adapter via its two PDZ domains between ASIC3 and membrane receptors such as G-protein-coupled receptors and/or intracellular effectors (M) like phospholipase C. Only the association of NHERF-1 with COOH-terminal phosphorylated ASIC3 is shown. NHERF-1 can also associate with ASIC3 later in the plasma membrane, and PKC phosphorylation could be a way to control the cellular localization of the channel.

DISCUSSION

The protein network associated with ASIC3 remains insufficiently understood despite the importance of this channel in modulating high intensity pain stimuli (14, 15), in acid-induced hyperalgesia (11), in cardiac ischemia (12), in inflammation (10, 13), and in mechanosensation (14–16). The present work identifies the adaptor protein NHERF-1 as an abundant protein in DRG neurons, where it is frequently co-expressed with ASIC3. NHERF-1 associates with the channel COOH-terminal region through its first PDZ domain. This association modulates ASIC3 subcellular localization, both through the trafficking of the channel to the plasma membrane and through its association with the cytoskeleton via ERM proteins. NHERF-1 also regulates ASIC3 activity and has a very large effect on the sustained component of the current.

NHERF-1 Drastically Increases the ASIC3 Sustained Current—NHERF-1 increases both the peak and the sustained current of ASIC3. However, the impact on the plateau current is much larger, with an up to 30-fold increase in Xenopus oocytes. NHERF-1 is therefore the most potent modulator of ASIC3 described so far. The effect on the peak current in COS cells and F-11 cells (~2.4- and ~2-fold increase, respectively) is well correlated with the increased amount of channel in the plasma membrane (~2-fold). On the other hand, NHERF-1 enhances the plateau current more than predicted by the increase in surface expression, suggesting that, besides its role in trafficking to the cell surface, it can directly modulate the ASIC3 channel activity as well. One possibility could be the existence of two channel states for ASIC3, one associated with the transient current and one associated with the sustained current. NHERF-1 would preferentially stabilize the conformation corresponding to the sustained component and would increase, for example, the open probability of the channel, as seen for CFTR (26). Although the NHERF-1/ASIC3 interaction does not change the pH dependence of the channel, it generates a large sustained current at pH values as high as 6.6, in a pH range relevant to pathophysiological conditions, such as ischemia, inflammation, or tissue injury (30). The fast transient component of the ASIC3 current is probably rapidly inactivated when pH decreases slowly, which is likely to happen during the onset of a tissue acidosis, because of its strong desensitization and the pH dependence of inactivation (9). This emphasizes the importance of the slowly activating sustained component of the ASIC3 current in sensory neurons (31). It has been shown that action potentials are only spontaneously triggered during the peak phase and not during the sustained depolarization due to the plateau phase of ASIC3-like currents in DRG neurons (32, 33). However, this sustained depolarization modulates the neuronal excitability in response to another depolarizing stimulus (32). NHERF-1, by increasing the plateau phase of the ASIC3 current, may therefore potentely increase nociceptor responsiveness in acidic pH conditions reached for instance during tissue injury, ischemia, tumors, or inflammation.

NHERF-1 May Assemble Interactive Sets of Proteins for Regulating ASIC3 Activity—One important function of the NHERF family of proteins is to form molecular scaffolds. NHERF-1 contains two PDZ domains followed by the carboxyl-terminal ERM binding domain. It can self-associate to form homodimers (34, 35), and it can also form heterodimers with NHERF-2 (36). Self-association occurs preferentially between homologous PDZ domains (e.g. PDZ-2 to PDZ-2) (35), which facilitates simultaneous association of different proteins with similar PDZ binding specificity toward NHERF-1. The PDZ domains of NHERF-1 have been shown to interact with a number of signaling proteins, G-protein-coupled receptors, and ion-transporting proteins, such as phospholipase C, Na+/H+ exchanger 3, cystic fibrosis transmembrane conductance regulator, mammalian TRP channels TRPC4 and TRPC5, κ-opioid receptor, and P2Y1 purinergic receptor (24). Some of these proteins are expressed in DRG neurons, like TRPC4 and TRPC5, κ-opioid receptor (37), κ-opioid receptor (38), and P2Y1 (39). There may be, for example, a functional coupling through NHERF-1 between ASIC3 and metabotropic ATP receptors, in a way similar to the recently described potentiation of TRPV1 by P2Y2 (40). This would be particularly interesting given the importance of ATP in peripheral pain sensitization and inflammatory hyperalgesia.

NHERF-1 Modulates the ASIC3 Subcellular Localization and Trafficking to the Plasma Membrane—NHERF-1 modulates the trafficking of several target proteins (24) and also regulates the number of active...
ASIC3 channels in the plasma membrane. In addition, the presence of NHERF-1 allows co-localization of ASIC3 with endogenous ezrin in COS-7 cells. This protein as well as other members of the Merlin and ERM family of membrane-cytoskeletal linker proteins bind to the COOH-terminal tail of NHERF-1 (41–43). ERM proteins provide a regulated link between the membrane and the cortical filamentous (F)-actin cytoskeleton and play a crucial role in the integration of cortical functions (for a review, see Refs. 44 and 45). NHERF-1 therefore provides the first direct link between ASIC3 and the cortical actin cytoskeleton. Moesin and radixin are expressed in DRG neurons (27), where they probably associate with ASIC3 via NHERF-1. Our data suggest that this interaction participates in the surface expression of ASIC3, probably by anchoring the channel to the cytoskeleton and preventing or delaying its internalization. ERM proteins are also expected to participate in the targeting of ASIC3 to specialized subcellular regions of the neuron, as suggested by the immunocytochemical experiments in COS cells. All PKC activators and particularly factors that are known to enhance pain perception, such as bradykinin (46), may change the cellular effectors and/or membrane receptors (Fig. 10). Unveiling the precise roles of NHERF-1 in sensory neurons in relation to ASIC3, but also to other ion channels and receptors, will definitely help to better clarify their functions and regulations of these channels in nociception and mechanismanesthesia. Moreover, these data highlight the fact that besides their well documented roles in polarized epithelia (29), NHERF proteins emerge as important players in neuronal function.

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