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The Cytoskeletal Protein α-Actinin Regulates Acid-sensing Ion Channel 1a through a C-terminal Interaction*

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The acid-sensing ion channel 1a (ASIC1a) is widely expressed in central and peripheral neurons where it generates transient cation currents when extracellular pH falls. ASIC1a confers pH-dependent modulation on postsynaptic dendritic spines and has critical effects in neurological diseases associated with a reduced pH. However, knowledge of the proteins that interact with ASIC1a and influence its function is limited. Here, we show that α-actinin, which links membrane proteins to the actin cytoskeleton, associates with ASIC1a in brain and in cultured cells. The interaction depended on an α-actinin-binding site in the ASIC1a C terminus that was specific for ASIC1a *versus* other ASICs and for α-actinin-1 and -4. Co-expressing α-actinin-4 altered ASIC1a current density, pH sensitivity, desensitization rate, and recovery from desensitization. Moreover, reducing α-actinin expression altered acid-activated currents in hippocampal neurons. These findings suggest that α-actinins may link ASIC1a to a macromolecular complex in the postsynaptic membrane where it regulates ASIC1a activity.

Acid-sensing ion channels (ASICs)[2] are H^+-gated members of the DEG/ENaC family (1–3). Members of this family contain cytosolic N and C termini, two transmembrane domains, and a large cysteine-rich extracellular domain. ASIC subunits combine as homo- or heterotrimers to form cation channels that are widely expressed in the central and peripheral nervous systems (1–4). In mammals, four genes encode ASICs, and two subunits, ASIC1 and ASIC2, have two splice forms, a and b. Central nervous system neurons express ASIC1a, ASIC2a, and ASIC2b (5–7). Homomeric ASIC1a channels are activated when extracellular pH drops below 7.2, and half-maximal activation occurs at pH 6.5–6.8 (8–10). These channels desensitize in the continued presence of a low extracellular pH, and they can conduct Ca^{2+} (9, 11–13). ASIC1a is required for acid-evoked currents in central nervous system neurons; disrupting the gene encoding ASIC1a eliminates H^+-gated currents unless extracellular pH is reduced below pH 5.0 (5, 7).

Previous studies found ASIC1a enriched in synaptosomal membrane fractions and present in dendritic spines, the site of excitatory synapses (5, 14, 15). Consistent with this localization, *ASIC1a* null mice manifested deficits in hippocampal long term potentiation, learning, and memory, which suggested that ASIC1a is required for normal synaptic plasticity (5, 16). ASICs might be activated during neurotransmission when synaptic vesicles empty their acidic contents into the synaptic cleft or when neuronal activity lowers extracellular pH (17–19). Ion channels, including those at the synapse often interact with multiple proteins in a macromolecular complex that incorporates regulators of their function (20, 21). For ASIC1a, only a few interacting proteins have been identified. Earlier work indicated that ASIC1a interacts with another postsynaptic scaffolding protein, PICK1 (15, 22, 23). ASIC1a also has been reported to interact with annexin II light chain p11 through its cytosolic N terminus to increase cell surface expression (24) and with Ca^{2+}/calmodulin-dependent protein kinase II to phosphorylate the channel (25). However, whether ASIC1a interacts with additional proteins and with the cytoskeleton remain unknown. Moreover, it is not known whether such interactions alter ASIC1a function.

In analyzing the ASIC1a amino acid sequence, we identified cytosolic residues that might bind α-actinins. α-Actinins cluster membrane proteins and signaling molecules into macromolecular complexes and link membrane proteins to the actin-cytoskeleton (for review, Ref. 26). Four genes encode α-actinin-1, -2, -3, and -4 isoforms. α-Actinins contain an N-terminal head domain that binds F-actin, a C-terminal region containing two EF-hand motifs, and a central rod domain containing four spectrin-like motifs (26–28). The C-terminal portion of the rod segment appears to be crucial for binding to membrane proteins. The α-actinins assemble into antiparallel homodimers through interactions in their rod domain. α-Actinins-1, -2, and -4 are enriched in dendritic spines, concentrating at the postsynaptic membrane (29–35). In the postsynaptic membrane of excitatory synapses, α-actinin

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[2] The abbreviations used are: ASIC, acid-sensing ion channel; NMDA, *N*-methyl-D-aspartate; CHO, Chinese hamster ovary; EGFP, enhanced green fluorescent protein (GFP); HA, hemagglutinin; PBS, phosphate-buffered saline; siRNA, small interfering RNA; MES, 4-morpholineethanesulfonic acid; RT, reverse transcription; TMAOH, tetramethylammonium hydroxide.

connects the NMDA receptor to the actin cytoskeleton, and this interaction is key for Ca^{2+}-dependent inhibition of NMDA receptors (36–38). α-Actinins can also regulate the membrane trafficking and function of several cation channels, including L-type Ca^{2+} channels, K^+ channels, and TRP channels (39–41).

To better understand the function of ASIC1a channels in macromolecular complexes, we asked if ASIC1a associates with α-actinins. We were interested in the α-actinins because they and ASIC1a, both, are present in dendritic spines, ASIC1a contains a potential α-actinin binding sequence, and the related epithelial Na^+ channel (ENaC) interacts with the cytoskeleton (42, 43). Therefore, we hypothesized that α-actinin interacts structurally and functionally with ASIC1a.

EXPERIMENTAL PROCEDURES

Expression Constructs—Mouse ASIC1a was cloned into pMT3 (44) for heterologous expression. Human α-actinin-1 was a gift of C. Otey, University of North Carolina, and α-actinin-2, -3, and -4 were gifts of M. Sheng, Massachusetts Institute of Technology. Full-length human α-actinin-1–4 were cloned into pEGFP vectors (Clontech Laboratories) to generate enhanced green fluorescent protein (EGFP)-α-actinin fusion proteins; previous studies showed that the localization and function of α-actinins were unaffected by GFP tags (45–47). A dominant-negative N-terminal-truncated (missing amino acid 3–249) human α-actinin-1 (rod-actinin) (48) was a gift of A. Huttenlocher, University of Wisconsin. Rod-actinin contains the central α-actinin spectrin domain including the C terminus but lacks the N-terminal head domain. Dimers containing rod-actinin can bind other proteins, but the absence of the head domain interrupts cross-linking to the filamentous actin cytoskeleton (48, 49). Rod-actinin would be expected to interfere with all α-actinins, and consistent with that conclusion, it disrupted the effect of α-actinins in human embryonic kidney cells, which express both α-actinin-1 and -4.[3] Chinese hamster ovary (CHO) cells also express α-actinin-1 and α-actinin-4 (50). HA-tagged ASIC1a was made by adding the influenza hemagglutinin epitope (YPYDVPDYAGV) to the N terminus of ASIC1a.

Cell Culture and Transfection—For patch clamp studies CHO cells were transfected with 1–6 μg of DNA using the TransFast Lipid™ reagent (Promega Madison, WI) and cultured on glass coverslips in 35-mm Petri dishes. To identify transfected cells using epifluorescence, we used EGFP at an ASIC1a:EGFP ratio of 6:1. Channel and α-actinin constructs were transfected at 1:1 ratio. Empty vector DNA was used to maintain a constant final DNA concentration for all transfections. Experiments were performed at room temperature 2–3 days after transfection.

Hippocampal Neuronal Culture and Transfection—Rat E18 hippocampal neurons (Brain Bits, Springfield, IL) were transfected after 7–8 days in culture using calcium phosphate transfection. Conditioned medium was collected and replaced by freshly prepared neurobasal medium with B-27 supplement (Invitrogen) 30 min before transfection. A total of 5 μg of vector DNA was added in a transfection mix consisting of 50 ml of water, 5 ml of $CaCl_2$ (2.5 M), and 60 ml of 2× PBS per dish. After 3 h of incubation at 37 °C and after an 8-min Hanks' balanced salt solution washing step, conditioned media was reapplied to cultures. At day 10, neurons were used for whole-cell, patch clamp experiments.

Immunoprecipitation and Immunoblotting—Whole mouse brains from adult animals were homogenized in ice-cold buffer (5 ml/brain) containing 300 mM sucrose, 10 mM Tris (pH 7.5), 20 mM NaCl plus protease inhibitors (Complete Mini, EDTA-free, Roche Applied Science) using a glass Teflon homogenizer. Samples were spun for 2 min in a tabletop centrifuge at 2000 × *g* to remove large debris. Supernatants were centrifuged for 30 min at 135,000 × *g* in a Beckman TLA100.3 rotor at 4 °C. The membrane pellet was washed once on ice and then solubilized in 6 ml of 50 mM Tris (pH 7.5), 150 mM NaCl, protease inhibitors (Complete Mini) with 1% Triton X-100. For immunoprecipitation, samples were centrifuged (10 min, 700 × *g*) to remove particulate debris and precleared by the addition of protein G-agarose (Roche Applied Science, 50 μl, 15 mg/ml). Either undiluted affinity-purified ASIC1 antibody (raised in rabbits against the 22-amino acid peptide from the C terminus of ASIC1 and purified as described previously (16)) or a mouse monoclonal α-actinin antibody BM-75.2 that binds to all four actinin isoforms (1 μl, Sigma-Aldrich) was added to 750 μl of protein extract and incubated for 3 h while rotating at 4 °C. For precipitation, protein G-agarose beads (Roche Applied Science, 50 μl 15 mg/ml) were added, and samples were rotated overnight at 4 °C. After centrifugation, 3 wash steps were performed to clear samples (buffer 1: 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycolate, protease inhibitors (Complete Mini); buffer 2: 50 mM Tris (pH 7.5), 500 mM NaCl, 0.1% Nonidet P-40, 0.05% sodium deoxycolate; buffer 3: 10 mM Tris (pH 7.5), 0.1% Nonidet P-40, 0.05% sodium deoxycolate). Bound proteins were extracted at 95 °C in SDS sample buffer (0.125 mM Tris (pH 7.5), 3.4% SDS, 17% glycerol, 67 mM dithiothreitol, 0.008% bromphenol blue) for 10 min and were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

Co-immunoprecipitation from COS-7 cells was performed 48 h after electroporation (10^6 cells, 10 or 15 μg of DNA at 1:1 ASIC1a:α-actinin ratio per sample). Electroporation was performed with 400 μl of prechilled cell suspension (120 mM KCl, 25 mM HEPES, 0.15 mM NaCl, 10 mM KPO_4, 2 mM EGTA, 5 mM $MgCl_2$ with 2 mM ATP and 5 mM glutathione using a single 25-s pulse of 0.320 V and 975 microfarads. Cells in 100-mm dishes were washed twice with ice-cold PBS and harvested in lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, and protease inhibitors (Complete Mini). Supernatants from cell extracts were pre-cleared with protein A-Sepharose (Pierce) gently agitated for 1 h at 4 °C. Either ASIC1 antibody (16) or rabbit polyclonal anti-GFP antibody (Clontech, Living Colors Full-length A.v. polyclonal antibody) were added to 250–500 μl of protein extract after 1 h of rotation at 4 °C. Protein A-Sepharose was added followed by incubation overnight with rotation at 4 °C. Immunoprecipitates absorbed to protein A-Sepharose were washed three times as described above. The immunoprecipitated proteins were resuspended in SDS sample buffer and electrophoresed. For immunoblotting, primary anti-

[3] J. W. Hell, unpublished observation.

bodies were detected with horseradish peroxidase-conjugated goat-anti rabbit or anti-mouse IgG from ECL (GE Healthcare). SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used for detection.

CHO cells were transfected with HA-ASIC1a:α-actinin:GFP at 1:1:1 ratio. For control, α-actinin was replaced with GFP so the ratio was 1:2 for HA-ASIC1a:GFP. Biotinylation of CHO cells was performed 2 days after Lipofectamine-mediated transfection. Cells were washed 3× with ice-cold PBS+/+, and 3 ml of 0.5 mg/ml NHS-biotin in PBS+/+ was added to each 10-cm dish followed by incubation at 4 °C for 30 min with gentle rocking. Cells were washed once with PBS+/+, and 0.1 M glycine in PBS+/+ was added to quench the reaction followed by 2 washes with PBS+/+. Cells were lysed in PBS with 1% Nonidet P-40, 0.5% deoxycholate, 0.5% SDS, and freshly added proteinase inhibitors (Roche Applied Science). Cell lysate was sonicated briefly and cleared by centrifugation. Protein concentration was quantified using a modified Bradford assay kit (Bio-Rad). For Neutravidin pulldown, 60 μl of a 50% slurry of Neutravidin beads were added to 200 μl of cell lysate and incubated at 4 °C overnight with gentle rotation. Beads were washed 3× with wash buffer (Tris 50 mM (pH 7.4), 1% Triton X-100). Beads were then boiled in 80 μl of SDS sample buffer with or without reducing agent.

RT-PCR—Rat hippocampal neurons and rat muscle tissue were lysed in cell lysis buffer, and first-strand cDNA was synthesized using the Cells-to-cDNA II Kit (Ambion). For PCR, the primer pairs for detection of α-actinin-1 were 5′-gatgcagacaaggagcgcct-3′ (bp 1887–1906) and 5′-gggacccaacgtgccggag-3′ (bp 2495–2513); for α-actinin-2 they were 5′-agaatgaggtggagaaggtga-3′ (bp 1787–1807) and 5′-tggaaggtgaccgtgccttg-3′ (bp 2467–2486); for α-actinin-3 they were 5′-ctgcagctggagtttgctcg-3′ (bp 1607–1625) and 5′-tgtgctccatgctgtagacc-3′ (bp 2196–2215), and for α-actinin-4 they were 5′-agcaatcacatcaagctgtcg-3′ (bp 1906–1927) and 5′-ccacactcatgatccggttg-3′ (bp 2542–2561). RT-PCR products were sequenced to confirm identity.

RNA Interference—We generated small interfering RNAs (siRNAs) against each of the four rat α-actinin isoforms using OligoEngine™ to locate 19 nucleotides within exons of the target and immediately downstream of AA dinucleotides and to exclude identity to other sequences in the NCBI data base. Sequences with potential polymerase III termination sites and mRNA splice sites were avoided.

The 19-nucleotide sense sequence and the inverted antisense sequence were connected by a 9-nucleotide spacer to allow stem-loop formation. At the 3′ end, a penta-thymidine motif provided a polymerase III termination site. siRNAs were cloned into pSilencer™ vector (Ambion) for expression of the respective sequences under the U6 promotor. DsRed and its cytomegalovirus promoter from pDsRed2-N1 were cloned into the Kpn site upstream of the U6 promoter within the pSilencer™ vector; this vector was used for identification of transfected neurons. To test for efficacy and specificity of siRNA, CHO cells were co-transfected with either one of the siRNAs and EGFP-tagged α-actinin at a 10:1 cDNA ratio (total 11 μg DNA) using Lipofectamine 2000 in Opti-MEM I media according to the manufacturer's recommendation (Invitrogen). RNAi1 targeted at 476–494 from the start sequence of rat α-actinin-1 (the sense chain was 5′-gatctccaacgtcaacaagttcaagagacttgttgacgttggagatctttttt-3′. RNAi2 targeted at 224–242 from the start sequence of rat α-actinin-2; the sense chain was 5′-tggcctgatggatcatgagttcaagagactcatgatccatcaggccatttttt-3′. RNAi3 targeted at 455–473 from the start sequence of rat α-actinin-3; the sense chain was 5′-aacgagaagctgatggaggttcaagagacctccatcagcttctcgtttttttt-3′. RNAi4 targeted at 493–511 from the start sequence of rat α-actinin-4; the sense chain was 5′-gactatccaggagatgcagttcaagagactgcatctcctggatagtctttttt-3′.

Electrophysiology—Whole-cell, patch clamp recordings were performed on CHO cells on 10-mm glass coverslips continuously superfused with a bath solution containing: 128 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM MES, 5.55 mM glucose, adjusted to pH 7.4 with TMAOH. The standard pipette solution contained 10 mM NaCl, 121 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 2 mM Na_2ATP, 10 mM HEPES adjusted to pH 7.25 with TMAOH. For whole-cell, patch clamp experiments with hippocampal neurons the bath solution contained 100 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM MES, 10 mM glucose adjusted to pH 7.4 with TMAOH. The pipette solution contained 10 mM NaCl, 70 mM potassium gluconate, 10 mM KCl, 10 mM EGTA, 1 mM $MgCl_2$, 3 mM Na_2ATP, 25 mM HEPES adjusted to pH 7.25 with TMAOH. Bath solutions with different pH values were adjusted with TMAOH and applied with a rapid solution exchanger (RSC-200 & EVH-9, Biologic Science Instruments). Recording pipettes were pulled from capillary glass with a micropipette puller (Sutter instruments) and polished (MF830, Narishige, Japan). Pipette resistances ranged from 2 to 5.5 megaohms.

Recordings were made at room temperature using an AXOPATCH 200B amplifier with pCLAMPex 8.1 software (Axon Instruments). Data were analyzed using Clampfit (Axon Instruments). Amplitude was determined by subtracting the base-line current at pH 7.4 from the peak current amplitude. The τ of desensitization (τ_{des}) was calculated by fitting the data to a single exponential equation with Clampfit (Axon Instruments). Membrane potential was held at −70 mV. Data are shown as the mean ± S.E. Statistical differences were determined by two-tailed Student's *t* test.

RESULTS

ASIC1a Contains a Putative Binding Site for α-Actinin—In the C terminus of ASIC1a, we identified an amino acid motif that resembled sequences in the cytosolic portions of the NMDA receptor subunit 1 and the P2X7 purinoceptor, two ion channels known to physically interact with α-actinin (Fig. 1) (38, 51, 52). The potential α-actinin-binding site in ASIC1a lies in the cytosolic portion of the subunit and would, therefore, be accessible for interaction with other cytoskeletal proteins. The sequence was not present in ASIC2 or ASIC3, suggesting that α-actinin might specifically interact with ASIC1. Because ASIC1a and -1b splice variants share the same C terminus, interactions would be predicted to occur with both subunits.

ASIC1a Associates with α-Actinin-1 and α-Actinin-4—To test the hypothesis that ASIC1a and α-actinin interact, we expressed ASIC1a with each of the four α-actinin isoforms in COS-7 cells. Immunoprecipitating ASIC1a co-precipitated

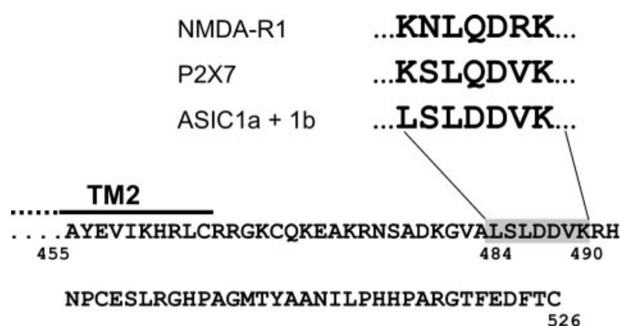

FIGURE 1. **The cytosolic C terminus of ASIC1a contains a putative α-actinin-binding site.** Analysis of the ASIC1 C terminus revealed an amino acid motif that is present in α-actinin binding cation channels. The *gray box* highlights the conserved motif in the primary sequence of the mouse ASIC1a cytosolic C terminus.

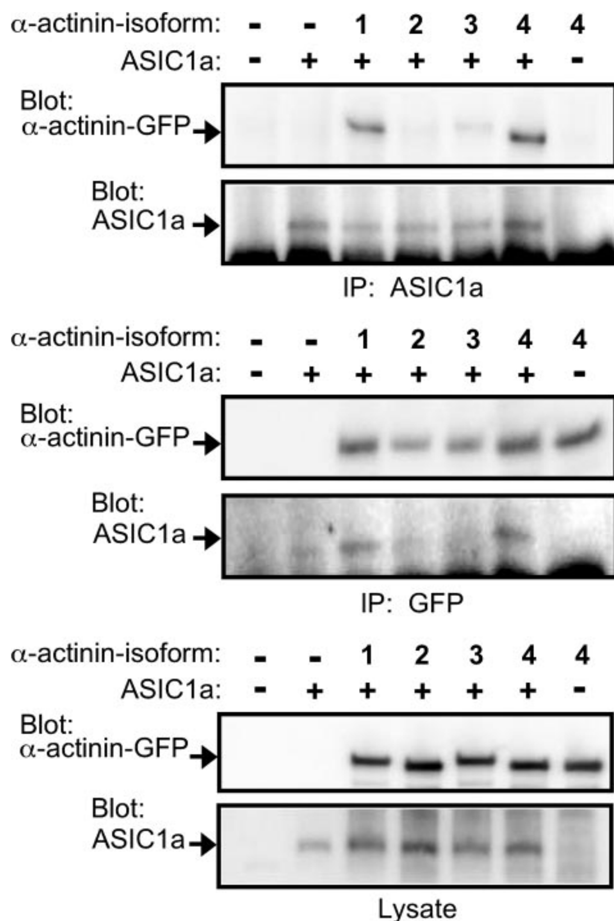

FIGURE 2. **α-Actinin and ASIC1a interacted in a heterologous expression system.** Shown are Western blots showing isoform-specific interactions between α-actinins and ASIC1a. Because the localization and function of α-actinin is not affected by GFP tags (45–47), we co-expressed EGFP-tagged isoforms of α-actinin-1, -2, -3, or -4 with ASIC1a in COS-7 cells. For immunoprecipitation (*IP*), either anti-ASIC1a (*top panels*) or anti-GFP (*middle panels*) antibodies were used. Western blots were probed with anti-GFP or anti-ASIC1a antibodies as indicated. Note that anti-ASIC1a antibody co-precipitated EGFP-tagged α-actinin-1 and α-actinin-4, and conversely, anti-GFP antibody co-immunoprecipitated ASIC1a with α-actinin-1 and α-actinin-4. The *lower panel* shows Western blots of total cell lysates (2% of total lysate) used for co-immunoprecipitation experiments.

α-actinin-1 and α-actinin-4 (Fig. 2). Conversely, precipitating α-actinin-1 or α-actinin-4 co-precipitated ASIC1a. ASIC1a weakly co-precipitated α-actinin-3, but not the converse, and

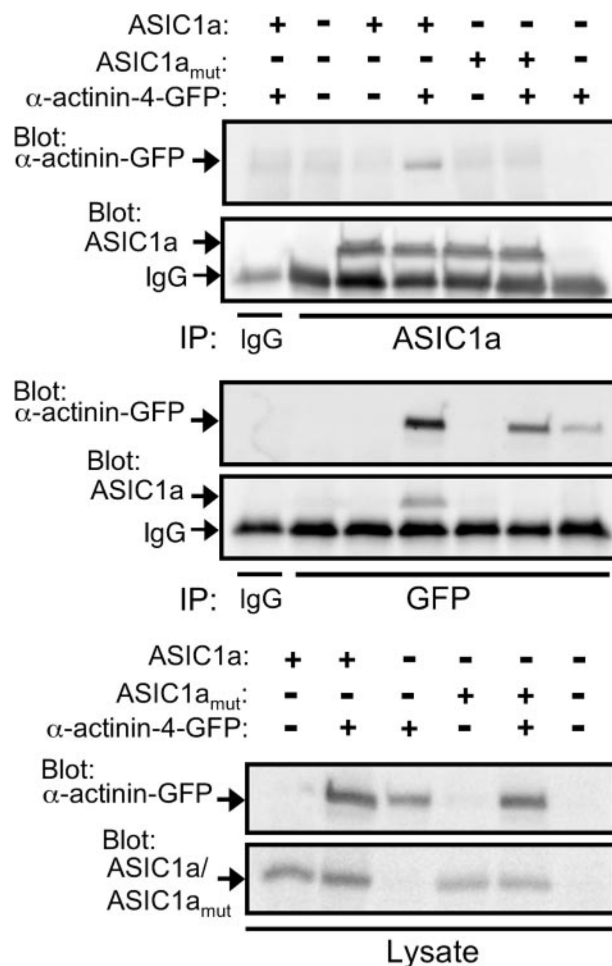

FIGURE 3. **α-Actinin and ASIC1a$_{mut}$ did not interact.** COS-7 cells were transfected with ASIC1a or ASIC1a$_{mut}$ alone or together with EGFP-labeled α-actinin-4. For immunoprecipitation (*IP*), either anti-ASIC1a (*upper panels*) or anti-GFP (*middle panels*) antibodies were used (IgG indicates nonspecific mouse or rabbit IgG used as a control). Western blots were probed with either anti-ASIC1a or anti-GFP antibody. Note that wild-type ASIC1a, but not ASIC1a$_{mut}$, co-immunoprecipitated with α-actinin-GFP. Conversely, α-actinin-GFP co-immunoprecipitated with wild-type ASIC1a but not ASIC1a$_{mut}$. The *lower panels* show total cell lysates (2% of total lysate) used for co-immunoprecipitation experiments.

we did not detect an interaction with α-actinin-2. These data suggest that ASIC1a resides in close proximity with at least two of the actinins, α-actinin-1 and α-actinin-4.

To learn whether the potential α-actinin binding motif in the C terminus of ASIC1a was involved in the interaction between ASIC1a and α-actinin, we expressed α-actinin-4 with an ASIC1a variant that contained mutations in the potential α-actinin binding motif (ASIC1a$_{mut}$ residues ^{484}LS$\underline{L}$D$\underline{D}$V$\underline{K}^{490}$ mutated to ^{484}LS$\underline{A}$D$\underline{A}$V$\underline{A}^{490}$). Mutating the C-terminal motif prevented ASIC1a from co-precipitating α-actinin-4 (Fig. 3). Likewise, α-actinin-4 failed to co-precipitate ASIC1a$_{mut}$. These results suggest a direct association between α-actinin-4 and ASIC1a that depends on the α-actinin binding motif. For most of the remainder of the studies, we focused on α-actinin-4.

α-Actinin Did Not Affect Cell Surface Expression of ASIC1a—To determine whether α-actinin might regulate surface expression of ASIC1a, we biotinylated cell surface proteins in CHO cells transfected with ASIC1a. Co-expressing ASIC1a with

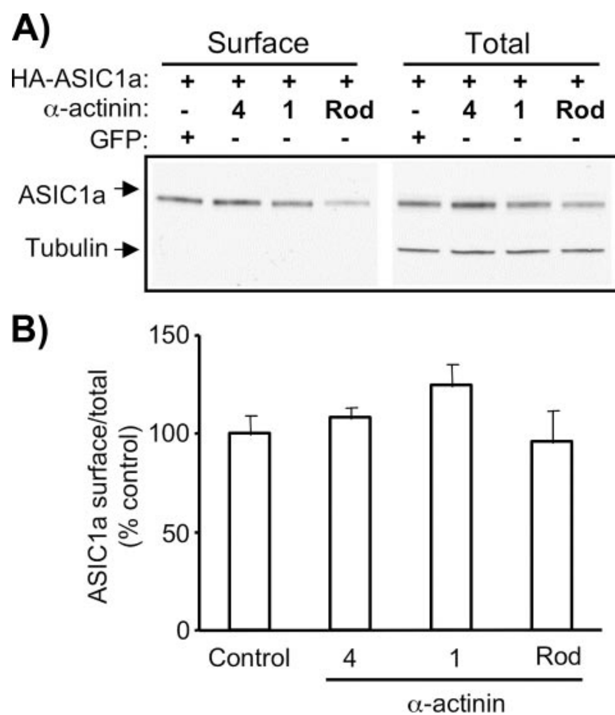

FIGURE 4. **α-Actinin did not alter cell surface expression of ASIC1a.** *A*, CHO cells were transfected with HA-ASIC1a and either GFP (as a control), α-actinin-4, α-actinin-1, or rod-actinin. After 48 h, surface proteins were biotinylated and pulled down with Neutravidin beads. The blot was with anti-HA and anti-tubulin antibodies. Lanes with total protein were loaded with 20% of the cell lysate used for Neutravidin binding. *B*, quantification of ASIC1a protein surface:total ratio ($n = 6$ from 3 separate experiments). There were no significant differences between experimental groups.

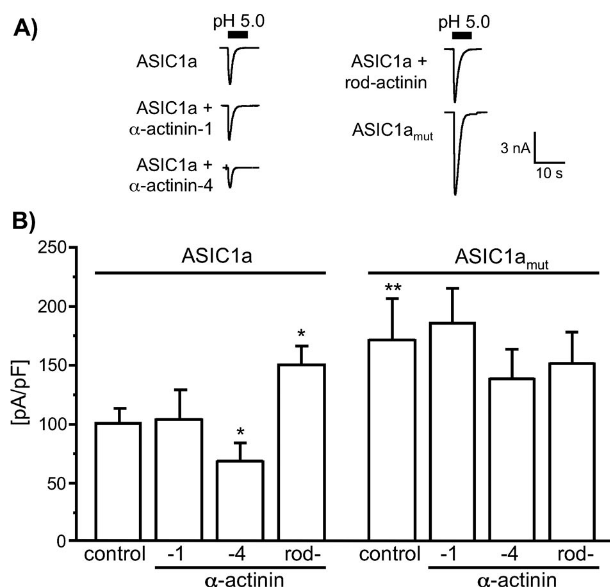

FIGURE 5. **α-Actinin regulated ASIC1a acid-evoked current in CHO cells.** *A*, representative currents evoked by application of pH 5.0 to CHO cells expressing ASIC1a or ASIC1a$_{mut}$ alone or co-expressing ASIC1a with α-actinin-1, α-actinin-4, or rod-actinin. *B*, mean current densities evoked by pH 5.0 superfusion of CHO cells expressing the indicated constructs. Data are normalized to currents obtained in CHO cells expressing wild-type ASIC1a alone. $n = 17-48$. Mean H^+-gated current densities in CHO cells expressing ASIC1a$_{mut}$ were not affected by co-expression with α-actinin-1, α-actinin-4, or rod-actinin ($n = 7-17$). *, $p < 0.02$ compared with ASIC1a (*left*) or ASIC1a$_{mut}$ (*right*) alone. **, $p < 0.05$ compared with ASIC1a.

α-actinin-4, α-actinin-1, or rod-actinin did not alter the fraction of ASIC1a on the cell surface (Fig. 4, *A* and *B*).

α-Actinin Influences ASIC1a Current Density—To learn whether α-actinin also regulates the function of ASIC1a, we co-expressed them and measured currents. α-Actinin-4 reduced ASIC1a current density, whereas α-actinin-1 had no effect (Fig. 5, *A* and *B*). These results suggest that even though both α-actinins interact with ASIC1a, they have selective functional effects. As an additional test of the effect of α-actinin, we co-expressed ASIC1a with rod-actinin, a dominant-negative construct (48, 49). We found that the dominant-negative rod-actinin fragment had the opposite effect of α-actinin-4 and increased current density (Fig. 5, *A* and *B*).

Finding that rod-actinin increased current amplitude suggested that it disrupted an interaction between ASIC1a and an endogenous α-actinin. Such an interaction predicts that ASIC1a$_{mut}$, which did not interact with α-actinin, would have a greater current density than ASIC1a. Consistent with this idea, ASIC1a$_{mut}$ generated more current than ASIC1a (Fig. 5, *A* and *B*). In addition, α-actinin-1 and α-actinin-4 failed to alter current produced by ASIC1a$_{mut}$. These data also suggest that the effect of α-actinin-4 on current results from a direct interaction with ASIC1a.

α-Actinin Alters the Properties of ASIC1a Currents—The findings that α-actinin-4 did not change the amount of ASIC1a on the cell surface whereas it decreased current density suggested that α-actinin-4 must have also altered the properties of the current generated by ASIC1a or increased the proportion of silent channels. To test this possibility, we examined several characteristics of ASIC1a current, including the pH sensitivity, the rate of desensitization, and the time course of recovery from desensitization.

Co-expressing α-actinin-4 increased the pH sensitivity of ASIC1a (Fig. 6*A*, Table 1). Conversely, the dominant-negative rod-actinin had the opposite effect, reducing pH sensitivity. Consistent with these data, when we disrupted the ASIC1a α-actinin-binding site (ASIC1a$_{mut}$), pH sensitivity fell, and α-actinin-4 and rod-actinin failed to alter the pH sensitivity of this variant (Fig. 6*B*, Table 1). These results indicate that the interaction with α-actinin influenced the sensitivity of ASIC1a to extracellular protons.

After acid-evoked activation, ASIC1a currents desensitize in the continued presence of acid (Fig. 5*A*). We found that neither α-actinin-1 nor -4 coexpression altered the time constant of ASIC1a current desensitization (τ_{des}) (Fig. 7). However, disrupting the α-actinin-binding site in ASIC1a$_{mut}$ or co-expressing rod-actinin with ASIC1a increased τ_{des}. Moreover, disrupting the α-actinin binding motif prevented the effect of rod-actinin. These data suggest that α-actinin can alter the desensitization rate of ASIC1a channels, an effect that is mediated in part by interactions between ASIC1a and ubiquitously expressed α-actinins.

ASIC1a currents also show a characteristic time-dependent recovery from desensitization in acidic solution (8, 10). We activated and desensitized ASIC1a currents with a 60-s exposure to pH 6.0 solution (Fig. 8*A*). Then, after varying time intervals at pH 7.4, we re-exposed the channels to pH 6.0. Co-expressing α-actinin-4 accelerated recovery from desensitization (Fig. 8*B*). Conversely, co-expressing a domi-

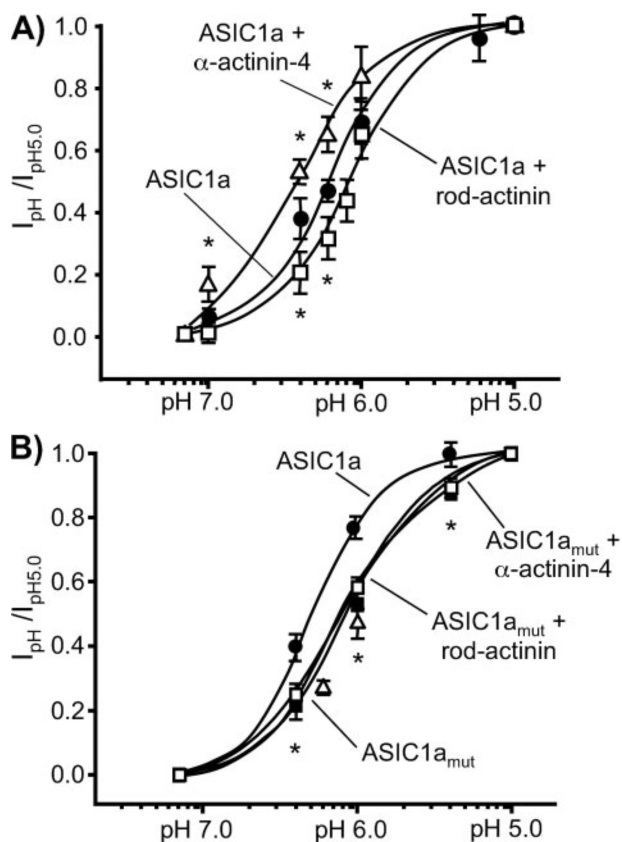

FIGURE 6. **α-Actinin modulated pH sensitivity of ASIC1a current in CHO cells.** *A*, pH sensitivity of ASIC1a expressed alone in CHO cells (●) or ASIC1a co-expressed with either α-actinin-4 (△) or rod-actinin (□). α-Actinin-4 increased pH sensitivity of ASIC1a, whereas rod-actinin decreased pH sensitivity (*. $p < 0.05$, n = 3–16 for each data point). *I* indicates peak current amplitude at given pH, and $I_{pH5.0}$ indicates peak current amplitude at pH 5.0. *B*, ASIC1a$_{mut}$ (■, n = 6–13) had a reduced pH sensitivity compared with wild-type ASIC1a (●, n = 6) (*, $p < 0.05$). Note that neither co-expressing rod-actinin with ASIC1a$_{mut}$ (□, n = 6–13) nor coexpressing α-actinin-4 with ASIC1a$_{mut}$ (Δ, n = 4–13) altered the pH sensitivity of ASIC1a$_{mut}$.

TABLE 1

α-Actinin and pH sensitivity of ASIC1a in CHO cells

Data are pH values evoking 50% of current compared to current induced by pH 5.0.

Transfected DNA	Half-maximal activation, pH_{50}
ASIC1a	6.46 ± 0.09
ASIC1a + α-actinin-4	6.67 ± 0.14
ASIC1a + rod-actinin	6.30 ± 0.07
ASIC1a$_{mut}$	6.28 ± 0.05
ASIC1a$_{mut}$ + α-actinin-4	6.22 ± 0.06
ASIC1a$_{mut}$ + rod-actinin	6.29 ± 0.06

nant-negative rod-actinin with ASIC1a or disrupting the α-actinin binding motif with ASIC1a$_{mut}$ slowed the rate of recovery.

These results indicate that an association with α-actinin-4 altered several characteristics of ASIC1a channel function. They also implicate the α-actinin binding motif in the ASIC1a C terminus. The effects of the dominant-negative rod domain and the ASIC1a$_{mut}$ variant suggest that ASIC1a interacts with α-actinins that are endogenous in the heterologous cells we used to express ASIC1a. Therefore, in the studies described below we tested for an effect of endogenous α-actinins.

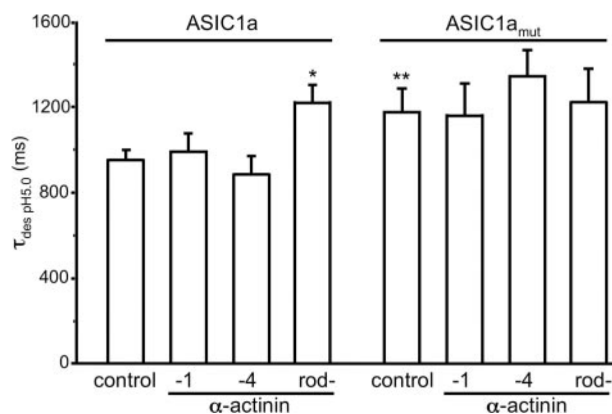

FIGURE 7. **Effect of α-actinin on desensitization of ASIC1a and ASIC1a$_{mut}$ currents.** Data are time constants of desensitization (τ_{des}) of currents evoked by pH 5.0. τ_{des} of acid-activated current in CHO cells expressing ASIC1a$_{mut}$ were not affected by co-expression of α-actinin-1, α-actinin-4, or rod-actinin. n = 16–43 for currents with ASIC1a and the α-actinin variants, and n = 6–15 for currents with ASIC1a$_{mut}$ and the α-actinin variants. *, $p < 0.01$ compared with ASIC1a alone (*left*) or ASIC1a$_{mut}$ (*right*). **, $p < 0.05$ compared with ASIC1a.

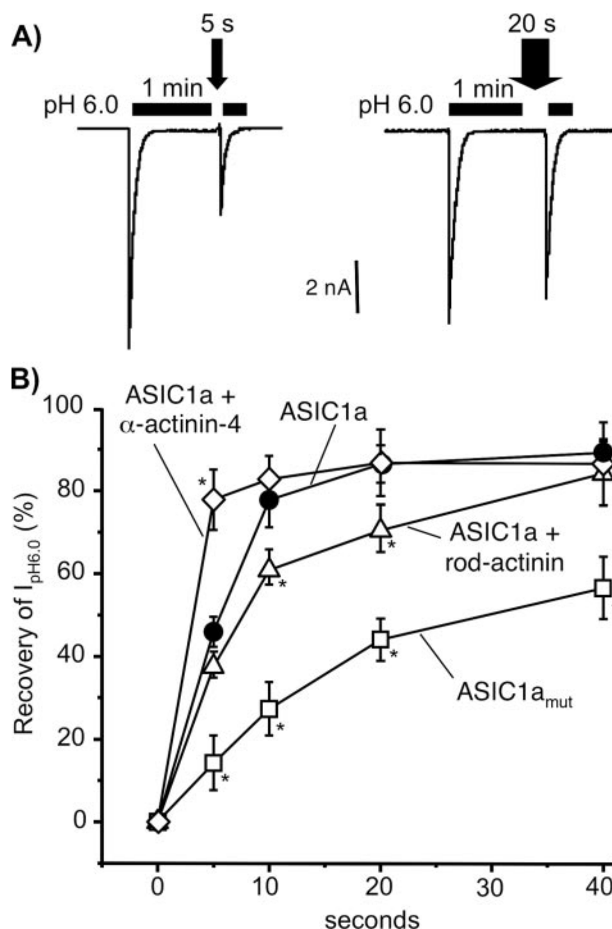

FIGURE 8. **α-Actinin accelerates recovery from desensitization for ASIC1a currents.** Acid-activated currents were desensitized by pH 6.0 application for 60 s followed by application of pH 7.4 for the indicated length of time (5–40 s). Then, a second pH 6.0 application was used to assess recovery of ASIC1a current. *A*, representative tracings showing recovery from desensitization during 5 s and 20 s at pH 7.4 in CHO cells expressing ASIC1a alone. *B*, percentage recovery of current from desensitization in response to pH 6.0 application for 60 s. Data were compared with currents of the first pH 6.0 application. ●, ASIC1a, n = 4–9; △, ASIC1a + rod-actinin, n = 5–10; ◇, ASIC1a + α-actinin-4, n = 4–6; □, ASIC1a$_{mut}$, n = 4–5. *, $p < 0.05$ compared with ASIC1a alone.

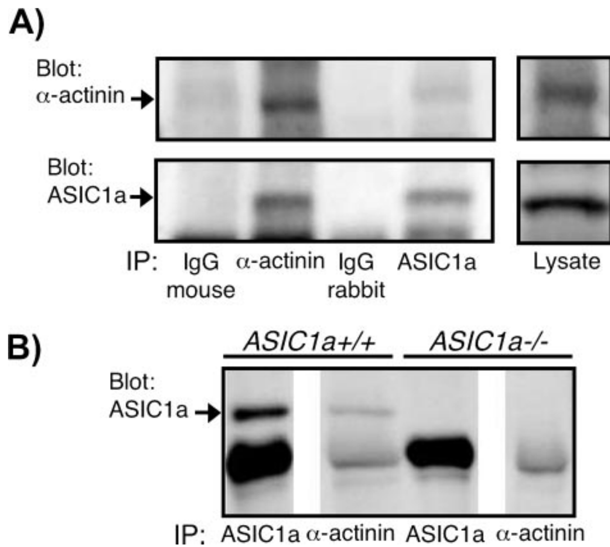

FIGURE 9. ***α*-Actinin associated with ASIC1a in the brain.** *A*, Western blot of α-actinin and ASIC1a co-immunoprecipitated (*IP*) from brain lysates. For immunoprecipitation anti-ASIC1a or anti-α-actinin antibodies (BM-75.2, which recognizes all four actinin-isoforms) were used. Western blots were probed with anti-α-actinin antibody (*upper panels*) or anti-ASIC1a antibody (*lower panels*). Note that anti-ASIC1a antibody co-precipitated α-actinin, and conversely, anti-α-actinin antibody co-precipitated ASIC1a. The *right panels* show blots of total cell lysates used for co-immunoprecipitation experiments and were probed with anti-ASIC1a and anti-α-actinin antibody. *B*, brain lysates from wild-type and *ASIC1a*$^{-/-}$ mice (5) were immunoprecipitated with anti-ASIC1a or anti-α-actinin antibody as indicated. The immunoprecipitates were probed with ASIC1a antibody.

ASIC1a and α-Actinin Associate in Brain—To test the hypothesis that ASIC1a and α-actinins associate in neurons, we used immunoprecipitation from mouse brain. We found that ASIC1a co-precipitated α-actinin, and α-actinin co-precipitated ASIC1a (Fig. 9*A*). α-Actinin failed to co-immunoprecipitate ASIC1a from ASIC1a$^{-/-}$ mouse brain (Fig. 9*B*). These results suggest that ASIC1a and α-actinin reside in close proximity in brain.

α-Actinin Regulates Hippocampal Acid-activated Currents—In the brain ASIC1a has been implicated in learning and memory, and an association of ASIC1a with the cytoskeletal linker protein α-actinin could be one mechanism underlying synaptic plasticity. Our data suggested that in heterologous cells endogenous α-actinins regulate ASIC currents; therefore, we asked if this was the case in neurons. We used RT-PCR to learn which α-actinin genes are expressed in the hippocampus and found α-actinin-1, -2, and -4 but not α-actinin-3 (Fig. 10*A*). To reduce their expression, we constructed vectors encoding siRNAs directed against each α-actinin isoform. The efficacy and specificity of the RNA interference were shown by Western blotting (Fig. 10*B*). With this information, for subsequent studies we reduced α-actinin expression by co-transfecting primary cultures of hippocampal neurons with a mix of the siRNAs against α-actinin-1, -2, and -4 (RNAi to α-actinin-1, -2, and -4). As controls, we used either siRNA directed against α-actinin-3 or dsRed.

Acid-evoked currents in hippocampal neurons are produced by a mixture of ASIC1a, -2a, and -2b subunits (7, 53, 54). RNAi against α-actinin-1, -2, and -4 increased endogenous ASIC current density in hippocampal neurons (Fig. 10*C*). In addition, it reduced the pH sensitivity of hippocampal acid-activated currents (Fig. 10*D*). Although the effects were small, it also slowed the rate of recovery from desensitization within the first 2 s (Fig. 10*E*). These results are consistent with our findings in a heterologous system expressing ASIC1a alone. In contrast to our results with ASIC1a homomultimers studied in CHO cells, τ_{des} of hippocampal ASIC currents were not affected by reducing endogenous α-actinins (Fig. 10*F*).

DISCUSSION

Previous studies localized α-actinin to dendritic spines and the postsynaptic density (29–35). Likewise, ASIC1a localized to dendritic spines and was enriched in the postsynaptic membrane (5, 14, 15). Those earlier studies positioned α-actinin-4 and ASIC1a where they could interact. In this study, we now show physical and functional links between these two proteins both in heterologous cells and in neurons. The data also indicate that the ASIC1a-α-actinin interaction occurred through a motif in the ASIC1a C terminus. This interaction appears to be specific, as the α-actinin binding motif is not conserved in ASIC2 or -3. In addition, ASIC1a associated with α-actinin-1 and -4 but not with α-actinin-2 or -3. These results suggest specificity in the interaction between these two families of proteins.

The association with α-actinins likely incorporates ASIC1a into a postsynaptic macromolecular signaling complex. α-Actinins bind actin and may thereby link ASIC1a to the predominant cytoskeletal element of dendritic spines (55, 56). The C termini of α-actinins also contain PDZ binding motifs that attach to several scaffolding proteins, including densin-180 (21, 33, 57). Thus, α-actinin may tie ASIC1a to multiple other proteins to form a signaling complex in the postsynaptic membrane (21). A potential example (25) is the functional coupling between ASIC1a and NMDA receptors; NMDA receptors are also regulated by α-actinin (38, 58). Gao *et al.* (25) suggested that during acidosis, Ca^{2+}/calmodulin-dependent protein kinase II is recruited to NMDA receptors where it phosphorylates ASIC1a, thereby increasing its pH sensitivity. Interestingly, ASIC1a (5), α-actinin (37), NMDA receptors (58), actin (59), and many of their associated components contribute to synaptic plasticity.

The mechanism by which α-actinin-4 reduced current density appears to be a direct effect on channel function. This conclusion is supported by the finding that α-actinin-4 altered multiple characteristics of the current without changing the amount of ASIC1a on the cell surface. In CHO cells, overexpressing α-actinin-4 increased H^+-evoked current density and increased pH sensitivity, whereas we saw the opposite effect when we expressed the dominant-negative rod-actinin or mutated the α-actinin interaction motif in ASIC1a. Likewise, in neurons, knockdown of α-actinin increased current density and reduced pH sensitivity. However, there were some differences between H^+-gated currents in CHO cells expressing ASIC1a and H^+-gated currents in neurons. In CHO cells α-actinin-4 accelerated and rod-actinin and $ASIC1a_{mut}$ slowed recovery from desensiti-

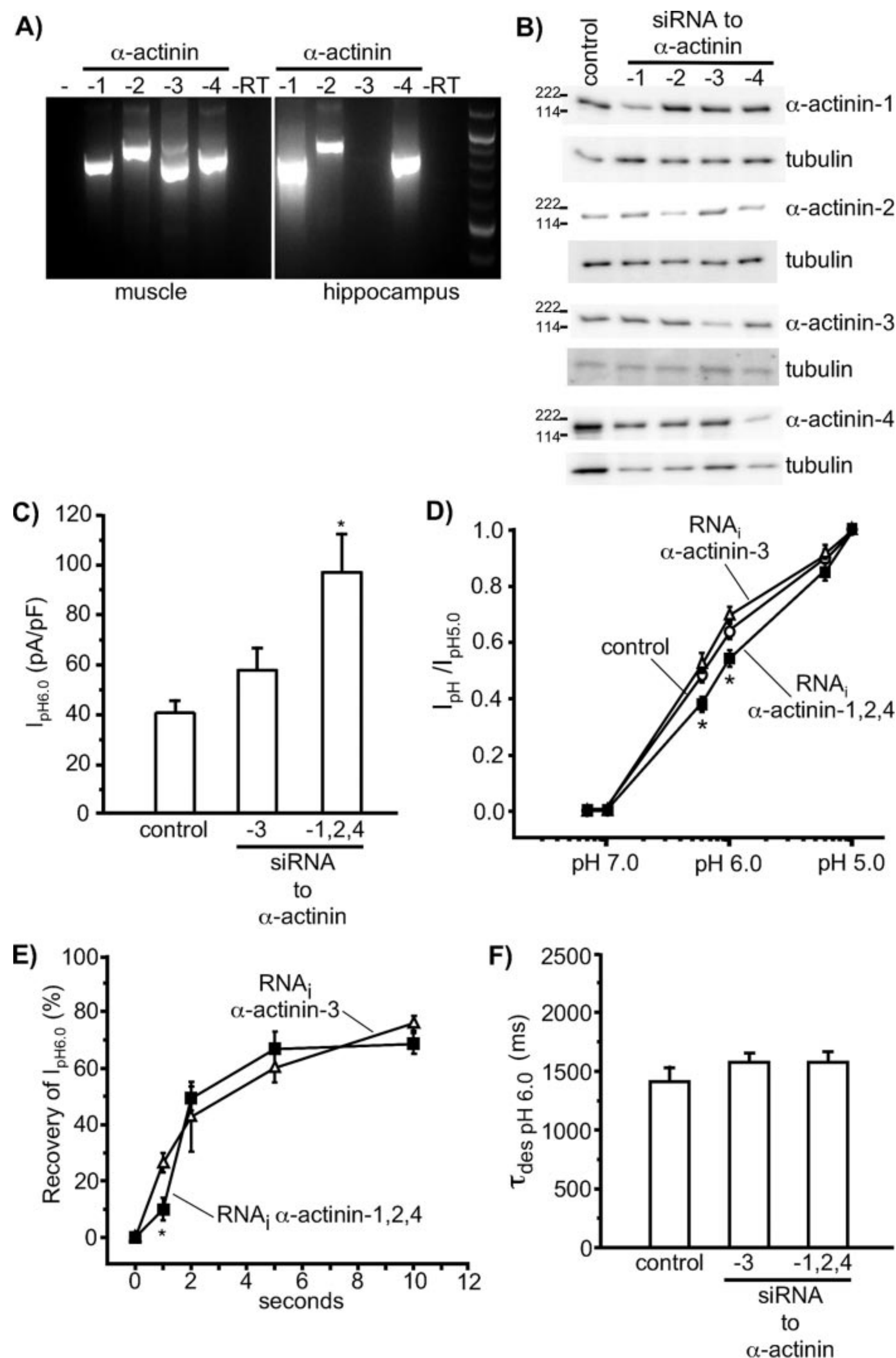

FIGURE 10. **α-Actinin regulated H^+-gated current in hippocampal neurons.** *A*, rat hippocampal neurons were tested for the expression of endogenous α-actinins. RT-PCR with isoform-specific primer pairs revealed expression of α-actinin-1, -2, and -4, whereas transcription of α-actinin-3 was not detected. Muscle from the same donor contained transcripts for all four α-actinin isoforms including α-actinin-3. In negative control experiments (−*RT*) reverse transcriptase was omitted, but the specific primers pairs for all four α-actinin isoforms were present. *B*, to assess the efficacy and specificity of siRNA, CHO cells were co-transfected with EGFP-tagged α-actinin-1,-2,-3, or -4 and isoform-specific siRNAs. Western blot was probed with either anti-GFP or anti-tubulin antibody. Each of the four siRNAs attenuated expression of its corresponding α-actinin isoform. *C–F*, reduction of endogenous α-actinin affected properties of acid-activated current in hippocampal neurons. For controls, neurons were transfected either with vector encoding dsRed or siRNA directed against α-actinin-3. *C*, current density was increased by simultaneous knockdown of α-actinin-1, -2, and -4 ($n = 23–47$, *, $p < 0.02$). *D*, pH sensitivity was decreased by knockdown of endogenous α-actinin-1, -2, and -4 (■, $pH_{50} = 6.16$, $n = 9–36$; *, $p < 0.05$) compared with controls (dsRed (○), $pH_{50} = 6.30$, $n = 7–21$ and RNAi to α-actinin-3 (△), $pH_{50} = 6.36$, $n = 17–28$). *I* indicates peak current amplitude at given pH, and $I_{pH5.0}$ indicates peak current amplitude at pH 5.0. *, $p < 0.05$. *E*, time-dependent recovery of desensitized current evoked by pH 6.0 (RNAi to α-actinin-1, -2, and -4 (■), $n = 5–11$ and RNAi to α-actinin-3 (△), $n = 3–11$; *, $p < 0.05$). *F*, time constants of desensitization of currents evoked by pH 6.0 ($n = 18–43$).

zation, whereas α-actinin siRNA had minor effects on recovery from desensitization in neurons. Moreover, the interaction with α-actinin altered the desensitization rate of ASIC1a currents in CHO cells, but α-actinin siRNA had no effect on H^+-activated current in neurons. We suspect these differences result from the fact that in CHO cells we studied ASIC1a homomeric channels, whereas in neurons much of the ASIC1a exists in heteromultimers (7). The conclusion that α-actinin-4 increased pH sensitivity in both cases is interesting, because pH sensitivity is a property usually associated with the ASIC extracellular domain (4, 60–62). Therefore, we speculate that the α-actinin binding motif in the ASIC1a intracellular C terminus somehow links ASIC1a to structures that control gating and pH sensitivity.

α-Actinin is not the only protein that interacts with ASIC1a; PICK1 (protein interacting with C kinase), stomatin, and annexin II light chain p11 also bind the channel. In PICK1, a PDZ domain interacts with the ASIC1a C terminus, and the two proteins resided in dendrites, probably in postsynaptic membranes (15, 22). Stomatin associated with ASIC1a in heterologous systems, although the site of interaction, remains unknown (63). Neither PICK1 nor stomatin altered the amplitude or properties of ASIC1a currents. The annexin II light chain p11 associated with an undefined site in the ASIC1a N terminus (24). In heterologous cells, it increased cell surface expression of ASIC1a and increased ASIC1a current amplitude without altering the characteristics of the current. Thus, of the proteins known to associate with ASIC1a, only α-actinin affected its channel function.

The connections between α-actinin and ASIC1a and their previously established distribution in the postsynaptic membrane position these proteins at a key site for influencing neuronal activity. Together, these observations sug-

gest that α-actinins may have an important impact on ASIC1a function at this location.

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