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# A Kinase-anchoring Protein 150 and Calcineurin Are Involved in Regulation of Acid-sensing Ion Channels ASIC1a and ASIC2a†

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## Abstract

Acid-sensing ion channel (ASIC) 1a and ASIC2a are acid-sensing ion channels in central and peripheral neurons. ASIC1a has been implicated in long-term potentiation of synaptic transmission and ischemic brain injury, whereas ASIC2a is involved in mechanosensation. Although the biological role and distribution of ASIC1a and ASIC2a subunits in brain have been well characterized, little is known about the intracellular regulation of these ion channels that modulates their function. Using pulldown assays and mass spectrometry, we have identified A kinase-anchoring protein (AKAP)150 and the protein phosphatase calcineurin as binding proteins to ASIC2a. Extended pulldown and co-immunoprecipitation assays showed that these regulatory proteins also interact with ASIC1a. Transfection of rat cortical neurons with constructs encoding green fluorescent protein- or hemagglutinin-tagged channels showed expression of ASIC1a and ASIC2a in punctate and clustering patterns in dendrites that co-localized with AKAP150. Inhibition of protein kinase A binding to AKAPs by Ht-31 peptide reduces ASIC currents in cortical neurons and Chinese hamster ovary cells, suggesting a role of AKAP150 in association with protein kinase A in ASIC function. We also demonstrated a regulatory function of calcineurin in ASIC1a and ASIC2a activity. Cyclosporin A, an inhibitor of calcineurin, increased ASIC currents in Chinese hamster ovary cells and in cortical neurons, suggesting that activity of ASICs is inhibited by calcineurin-dependent dephosphorylation. These data imply that ASIC down-regulation by calcineurin could play an important role under pathological conditions accompanying intracellular $Ca^{2+}$ overload and tissue acidosis to circumvent harmful activities mediated by these channels.

Acid-sensing ion channels (ASIC)[3] are amiloride-sensitive $Na^+$ channels and constitute one member of the epithelial $Na^+$ channel/degenerin superfamily (1–4). Although epithelial $Na^+$ channels are constitutively active in kidney epithelia, ASICs are proton-gated ion channels that are activated in response to reduction in extracellular pH. In mammalian brain and sensory neurons, isoforms of ASICs and their splice variants (ASIC1a, ASIC1b, ASIC2a, ASIC2b, and ASIC3) form various functional ASICs that are composed of homo- or heteromultimeric subunits. Homomultimeric ASICs display different sensitivities in response to external acidification, and assemblies of heteromeric subunits have acid-

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[3]The abbreviations used are: ASIC, acid-sensing ion channel; AKAP, A kinase-anchoring protein; PKA, cAMP-dependent protein kinase (protein kinase A); PKC, protein kinase C; PICK1, protein interacting with C kinase 1; CHO cells, Chinese hamster ovarian cancer cells; GST, glutathione *S*-transferase; YFP, yellow fluorescent protein; HA, hemagglutinin; HEK cells, human embryonic kidney cells; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; ANOVA, analysis of variance; pF, picofarad; NMDA, *N*-methyl-D-aspartate; GFP, green fluorescent protein.

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responsive properties that are distinct from their parental homomultimeric ASICs (5–8). Homomultimeric ASIC1a channels are permeable to $Na^+$ as well as to $Ca^{2+}$ (9–11). ASIC1a generates depolarizing currents that are implicated in hippocampal long term potentiation, an important physiological function of synaptic plasticity in brain and amygdala-dependent behavior in the context of fear conditioning (12–14). However, the acid-evoked currents associated with tissue acidosis and the $Ca^{2+}$-influx/intracellular $Ca^{2+}$ accumulation could result in detrimental consequences as occur after seizures and cerebral ischemia (11, 15). A mechanotransduction role of ASIC2 has been found in sensory neurons (16–19). The central nervous system expresses ASIC1a, ASIC2a, and ASIC2b. ASIC1a is activated below pH 7.0, and ASIC2a is activated below pH 5.5; ASIC2b generates no currents in response to low pH (7). Proton-activated currents in hippocampus are largely contributed by homo- and heteromultimers composed of ASIC1a and ASIC2a (20). ASIC2 knock-out mice have shown enhanced pH sensitivity and slow desensitization of proton-induced currents with almost no changes in current amplitude in hippocampal neurons, implying a modulatory role of ASIC2a in ASIC1a/ASIC2a heteromultimers in pH sensitivity and desensitization (20).

ASICs share a common structural architecture with members of the epithelial $Na^+$ channel/degenerin super family, which have two transmembrane domains with a large extracellular loop and two short intracellular N and C termini (for review, see Ref. 21). A number of regulatory proteins for ASICs have been identified. Protein interacting with C kinase 1 (PICK1) is a synaptic protein with a PDZ domain and interacts with the C termini of both ASIC1a and ASIC2a, which mediates the regulation of these channels by protein kinase C (PKC) (22–24). Similar to the involvement of MEC-2 in mechanosensation in *Caenorhabditis elegans*, stomatin, the mammalian homolog of MEC-2, has been shown to interact with and modulate the activity of ASIC1a, ASIC2a, and ASIC3 (25). These interacting proteins have been shown to influence the function of ASICs. Although PICK1 binding to ASIC1a is associated with phosphorylation and changes in the cellular localization of the channel (26), the interaction between stomatin and ASICs has been suggested to modulate channel gating (25). Accordingly, we sought proteins that participate in regulation of brain-expressed ASICs by protein-protein interaction. Pulldown assays in combination with mass spectrometric analyses identified A kinase-anchoring protein 150 (AKAP150) and the $Ca^{2+}$/calmodulin-dependent protein phosphatase 2B, also called calcineurin, as proteins interacting with ASIC2a. These regulatory proteins also interacted with ASIC1a. AKAP150, the neuron specific rat ortholog of human AKAP79, is present in the postsynaptic density in association with cAMP-dependent protein kinase (PKA), PKC, and calcineurin (27–30). These enzymes are associated with AKAP79/150 in inactive states, and the interaction with the anchoring protein has different consequences for the activity of these enzymes. In the case of PKA, only the regulatory subunit (RII) is anchored to AKAP and the catalytic (C) subunit, a serine/threonine kinase, interacts with RII forming active enzyme (29, 31, 32). PKC and calcineurin, on the other hand, are inactive when associated with AKAP79/150 until they are released from the anchoring protein (28, 33, 34). In the present work we have shown interaction of ASICs with AKAP150 and calcineurin. Disruption of the RII binding to AKAP150 by synthetic peptide Ht-31 decreased acid-evoked currents in mouse cortical neurons and Chinese hamster ovarian cancer (CHO) cells expressing homomeric ASIC1a or ASIC2a function, implying involvement of AKAP-anchored PKA in the function of ASIC1a and ASIC2a. Furthermore, inhibition of calcineurin by cyclosporin A significantly increased acid-evoked current in neuronal cells as well as in CHO cells. Taken together, our results suggest that both AKAP150 and calcineurin are involved in regulation of ASIC1a and ASIC2a, possibly by changing the phosphorylation status of these channels.

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# EXPERIMENTAL PROCEDURES

## Plasmid Constructs and Purification of Fusion Proteins

The rat cDNA clone of ASIC1a (GI: 13162348) was a gift from Dr. E. W. McCleskey (Vollum Institute, Oregon Health and Science University) and Dr. M. J. Welsh (Department of Internal Medicine and Physiology and Biophysics and Howard Hughes Medical Institute, University of Iowa), and ASIC2a (GI: 1280440) was from Dr. M. Lazdunski (Institut de Pharmacologie Molecularire et Celluaire, CNRS-UNSA, Institut Paul Hamel). The GST fusion to cDNA clone of AKAP150 (pGEX: :GST-AKAP150) was a gift from Dr. J. D. Scott (Vollum Institute and Howard Hughes Medical Institute, Oregon Health and Science University). From this clone the cDNA encoding AKAP150 was amplified by the PCR method and inserted into the mammalian expression vector pCDNA3.1 (Invitrogen), resulting in pCDNA-AKAP150. For GST fusion proteins of ASIC1a and ASIC2a, the coding regions of the intracellular N and C terminus were PCR-amplified from the original cDNA clones and subcloned into the plasmid pGEX-6P-3 (Amersham Biosciences). The resulting fusion proteins were designated as GST-ASIC1aN (44 amino acids from the N terminus), GST-ASIC1aC (74 amino acids from the C terminus) GST-ASIC1aE (68 amino acids from Asn-119 to Val-186), GST-ASIC2aN (41 amino acids from the N terminus), and GST-ASIC2aC (59 amino acids from the C terminus). The N-terminal fusion of YFP to ASIC1a and ASIC2a was constructed using the plasmid pCDNA6.2-N-YFP (Invitrogen), resulting pCDNA6.2-YFP-ASIC1a and pCDNA6.2-YFP-ASIC2a. For the hemagglutinin (HA) epitope tagging in the extracellular domain of ASIC1a, the nucleotide sequence encoding HA (YPYDVPDYA) was incorporated into the oligonucleotide primer sequence that was used to amplify the first 690-base pair fragment from the 5′-end, allowing the HA insertion at position 230 amino acid. The 891-base pair fragment of the ASIC1a from the 3′-end was also PCR-amplified and subcloned together with the 5′ fragment into the plasmid pCDNA3.1, resulting in full-length ASIC1a (pCDNA-ASIC1a-eHA). The HA epitope was inserted at the C terminus of the ASIC2a using PCR methods, which was then subcloned into the plasmid pCDNA3.1 (pCDNA-ASIC2a-cHA). The GFP fusion constructs of ASIC1a and ASIC2a were carried out using the plasmid pCDNAJM1-EGFP (a gift from Dr. A. Merz, Department of Biochemistry, University of Washington), allowing GFP fusion at the C terminus of ASIC1a (pCDNA-ASIC1a-EGFP) and ASIC2a (pCDNA-ASIC2a-EGFP). The overexpression and purification of the GST fusion proteins were carried out as recommended by the manufacturer (Amersham Biosciences). GST fusion proteins were affinity-purified from the bacterial lysates using GS-4B beads (supplemental Fig. S1).

## Cell Culture and Transfection

CHO cells and HEK293 were cultured in F-12K medium (ATCC) supplemented with 10% FBS in humidified 5% $CO_2$ incubator. Cortical neurons from neonatal Sprague-Dawley rat and neonatal E16 Swiss mice were cultured in neurobasal medium supplemented with B-27 and GlutaMax (Invitrogen). Neurons were cultured for 5 days before immunocytochemistry and 12 days before electrophysiological recordings. Transient transfection of neurons, CHO cells, and HEK293 cells was carried out using FuGENE 6 lipid-based transfection reagent (Roche Applied Science). For electrophysiological recordings, CHO cells were transfected with pCDNA-ASIC1a-GFP or pCDNA-ASIC2a-GFP for 48 h and then re-plated in 35-mm dishes at a lower cell density. After incubation overnight, GFP-positive cells were selected for the measurement of ASIC currents.

## Immunocytochemistry

After transfection with pCDNA-ASIC1a-GFP and pCDNA-ASIC2a-HA, neuronal cells were washed 3 times in PBS, fixed in 10% formalin, PBS, washed three times in PBS, and permeabilized with 3% Triton X-100/PBS. For immunochemical staining with anti-

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AKAP150 antibody alone, cells were incubated first in blocking buffer containing 2% donkey serum and 1% bovine serum albumin in PBS. For double detection with anti-AKAP150 and anti-HA antibody, blocking buffer containing 2% donkey serum, 2% goat serum, and 1% bovine serum albumin in PBS was used. Cells were then incubated with goat anti-AKAP 150 C-20 IgG (Santa Cruz Biotechnology) with or without rabbit anti-HA probe (Y-11) IgG (Santa Cruz Biotechnology). After extensive wash, cells were incubated for 2 h with donkey anti-goat IgG-Cy3 (Jackson Immuno Research) and/or goat anti-rabbit IgG-fluorescein isothiocyanate (Jackson Immuno Research). Both primary and secondary antibodies were diluted in the appropriate blocking buffer. After extensive washing, coverslips were mounted on glass slides. Images were collected with an epifluorescence microscope (Leica DM LB) in 40×objective/10×ocular. HEK293 cells expressing AKAP150 together with GFP, YFP-ASIC1a, or YFP-ASIC2a were immunolabeled with anti-AKAP150 antibody as described above. YFP-ASICs were visualized using a primary antibody that detects both GFP wild type and its variants (BD Living colors A.v. monoclonal antibody JL-8, Clontech) and goat anti-mouse IgG-fluorescein isothiocyanate.

## Pulldown Assays

For mass spectrometric analyses, ~0.5 mg of purified GST fusion proteins bound to GS-4B beads were used to incubate with lysate from one rat brain. Rat brain lysate was prepared by Dounce homogenization in 10 ml of HEN buffer (50 mM HEPES, 10 mM EDTA, 25 mM NaCl, pH 7.4) and subsequently diluted with 10 ml of HEN buffer containing 2% Triton X-100. After incubation at 4 °C with rotation, insoluble material was separated from the lysate by centrifugation for 15 min at 14,000×*g*. To preclear, supernatant was incubated with 0.5 ml of GS-4B beads at 4 °C for 30 min. The beads were removed by centrifugation for 15 min at 14,000 × *g*. The GST fusion proteins bound to GS-4B were added to the supernatant and incubated for 2 h at 4 °C with shaking. The complexes of bead-protein(s) were washed 6 times with PBS buffer containing 1% Triton X-100 and finally resuspended in SDS-PAGE loading buffer and stored at −20 °C. In smaller scale pulldown experiments, the same procedure was applied as described above, except only half-amount of all the reagents were used.

## Co-immunoprecipitation Assay

HEK293 cells were co-transfected with pCDNA-AKAP150 and pCDNA-GFP, pCDNA-AKAP150 and pCDNA6.2-YFP-ASIC1a, or pCDNA-AKAP150 and pCDNA6.2-YFP-ASIC2a in 6-well plates. After 24 h of transfection, cells were harvested and lysed in 300 μl of ice-cold HEN buffer supplemented with 1% Triton X-100 and complete protease inhibitor mixture (Roche Applied Science). The insoluble material including nuclei (pellet) was removed by centrifugation at 760 × *g* for 10 min, and supernatant was precleared with 5 mg of protein A-Sepharose (Amersham Biosciences) and 1 μg of anti-cascade blue antibody (Molecular Probes) at 4 °C. Beads and antibody were removed from the lysate by centrifugation at 10,000 rpm for 15 min. For co-immunoprecipitation, lysates were first incubated overnight at 4 °C with 2 μg of affinity-purified anti-GFP antibody and then with 5 mg of protein A-Sepharose for an additional 3 h. After centrifugation at 760 × *g* for 5 min, the pellet containing beads bound to antibody-protein(s) complex was washed 5 times with HEN buffer containing 0.5% Triton X-100 and resuspended in SDS-PAGE sample loading buffer for Western blot analysis.

## Sample Preparation for Mass Spectrometry

Bead-bound protein samples from pulldown assays were denatured at 95 °C for 5 min and separated by one-dimensional SDS-PAGE. One whole lane was cut in 5 gel slices. As described previously (35), the trypsin digestion of the protein samples in the gel slices and

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liquid chromatography/electrospray ionization/tandem mass spectroscopy were carried out by Proteomics facility, Fred Hutchinson Cancer Research Center (Seattle, WA).

### Western Blot Analysis

Protein samples from pulldown assays and co-immunoprecipitation assays were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in PBS containing 5% milk (blocking buffer) and probed with primary antibody diluted in blocking buffer for 2 h. After an extensive wash in PBS, membranes were probed with appropriate secondary antibody-horseradish peroxidase conjugates, donkey anti-goat IgG (Santa Cruz Biotechnology), goat anti-rabbit IgG (Sigma), and goat anti-mouse IgG (Sigma). Horseradish peroxidase bound to immunoblot was visualized with enhanced chemiluminescence (ECL, Amersham Biosciences) and Eastman Kodak BioMax Chemiluminescence film.

### Electrophysiology

ASIC currents were recorded with the conventional whole-cell patch clamp technique at room temperature (20–22 °C). In general, cells were voltage-clamped at holding potentials of −60 mV. Data were acquired using an AXOPATCH 200B amplifier with pCLAMP 8.2 software (Axon Instruments, CA). Data were filtered at 2 KHz and digitized at 5 Hz using Digidata 1322A (Axon Instruments). For rapid changes of extracellular solutions, a multibarrel perfusion system (SF-77, Warner Instruments, Hamden, CT) was used. Low pH extracellular solutions were applied at 2-min intervals to allow for complete recovery of ASICs from desensitization. The neutral extracellular solutions contained 140 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, and 10 mM glucose, 320–335 mOsm. In the extracellular solutions with pH ≤ 6.0, 20 mM HEPES was replaced by 10 mM MES for more reliable pH buffering. The pH of the extracellular solution was adjusted with NaOH/HCl. Patch pipettes were pulled from borosilicate glass (1.5-mm diameter; WPI, Sarasota, FL) on a two-stage puller (PP83, Narishige, Tokyo, Japan). Pipettes had a resistance of 2–4 megaohms when filled with the intracellular solution, which contained 140 mM CsF, 2 mM triethylammonium chloride, 11 mM EGTA, 10 mM HEPES, 1 mM $CaCl_2$, 4 mM $MgCl_2$ in pH 7.3 adjusted with CsOH/HCl, 290–300 mOsm. Cyclosporin A (Fluka) and FK-506 (LC laboratories) were first dissolved in 100% ethanol to make a 10 mM stock solution and diluted to the final concentration of 30 μM and 10 μM in the pipette solution, respectively. The AKAP inhibitor peptide Ht-31 and Ht-31P (Promega) were dissolved in water to make a 10 mM stock solution and then diluted to the final concentration of 20 μM in the pipette solution. To ensure high quality voltage-clamp, only the recordings with an access resistance of less than 10 megaohms and a leak current less than 100 pA at −60 mV were included for analysis. Data are presented as the mean ± S.E. Statistical significance was determined using two-way ANOVA and Student *t* test where appropriate. Differences were considered significant when $p < 0.05$.

## RESULTS

### Identification of AKAP150 and Calcineurin B as Binding Partners with ASIC2a N Terminus

Like the other members of the degenerin/epithelial $Na^+$ channel superfamily, N and C termini of ASIC2a are the only cytoplasmic domains of this channel (36–39). Homomeric ASIC1a and ASIC2a channels or ASIC2b in heteromeric ASIC3/ASIC2b have been shown to interact with the PICK1 and, thereby with PKC via their C terminus (8, 23, 24, 26). We questioned whether the N terminus of these channels is also involved in the regulation of function or surface expression. To identify potential regulatory proteins in brain, we first used *in vitro* protein-protein binding assays using the N terminus (41 amino acids) of ASIC2a fused to GST protein (GST-ASIC2aN). The fusion protein was affinity-purified

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using GS-4B beads from bacterial lysate and mixed with rat brain lysate to pull down any interacting proteins. The bead-protein complexes were purified and separated by one-dimensional SDS-gel electrophoresis (Fig. 1*A*). In parallel, a control experiment was performed with GST protein bound to GS-4B to identify nonspecific interaction. Both samples were analyzed by tandem mass spectrometry, and identified proteins were searched in the rat protein data base of international protein index. Of particular interest were a peptide fragment from AKAP150 and one peptide fragment from A subunit isoform α of protein phosphatase 2B (also called calcineurin). The identified peptides of AKAP150 and calcineurin A subunit were nine amino acids long (TPGSEKEAK) and 33 amino acids long (VTEMLVNVLNICSDDELGSEEDGFDGATAAARK), respectively. AKAP150/79 is a neuronal protein enriched in postsynaptic fractions (31). A number of reports indicated the involvement of AKAP150/79 in trafficking and function of channels and receptors in neuronal cells (for review, see Ref. 41). Moreover, AKAP150/79 associates with PKA, PKC, and calcineurin (28, 29, 31). To verify the mass spectrometry data, we repeated the pulldown assay with GST and GST-ASIC2aN protein from rat brain lysates. Western blot analyses with anti-AKAP150 antibody showed the presence of this protein in the GST-ASIC2aN- but not in the GST- pulldown probes, indicating that the interaction of these proteins is specific to the N terminus of ASIC2a (Fig. 1*B*). The calcineurin B subunit was detected in high amounts in GST-ASIC2aN pulldown probes (Fig. 1*B*). A negligible amount of calcineurin was also detected in the GST pulldown probes. This might be considered as an artifact, possibly resulting from high expression levels of calcineurin in brain and nonspecific binding to GST.[4]

## Interaction of AKAP150 and Calcineurin with the Intracellular Termini of ASIC1a and ASIC2a

Both ASIC1a and ASIC2a are expressed throughout brain. Interaction between the N terminus of ASIC2a with AKAP150 and calcineurin led to questions of whether both ASIC1a and ASIC2a are regulated by AKAP150 and calcineurin, and only the N terminus of the channel(s) is involved in the interaction. To answer these questions, we tested the protein-protein interaction by pulldown assay using GST fusion proteins to the N terminus (GST-ASIC1aN) and the C terminus (GST-ASIC1aC) of ASIC1a as well as to the C terminus of ASIC2a (GST-ASIC2aC) as described above. In parallel, GST and GST-ASIC1aE, which contains the extracellular domain of ASIC1a, were used as negative controls in this assay. Western blot analyses showed that AKAP150 binds, in addition to N terminus, also to the C terminus of ASIC2a as well as to the C terminus of ASIC1a (Fig. 1*C*). Although the N terminus of ASIC2a was the initial protein to identify AKAP150 in the pulled-down complex, the N terminus of ASIC1a showed no clear binding to the anchoring protein. The amount of pulled-down AKAP150 with the N terminus was slightly higher than that with the extracellular domain of ASIC1a, indicating that the C terminus of ASIC1a, but not the N terminus, interacts with AKAP150. Overall, the amount of AKAP150 protein pulled down was significantly higher with the C terminus than with the N terminus of both ASICs (Fig. 1*C*). These data suggested that that the C terminus of these channels exhibits a stronger interaction and plays a role as a major binding site for the anchoring protein. Calcineurin also interacted with the intracellular termini of ASICs (Fig. 1*D*). Similar to AKAP150, the C terminus of ASIC1a and the both termini of ASIC2a showed a higher binding affinity to this phosphatase than the N terminus of ASIC1a. Different from the interaction with AKAP150, the N and C termini of ASIC2a seem to bind equally well to calcineurin, since smaller differences were observed in the amount of calcineurin detected. On the other hand, the C terminus of ASIC1a seems to be the major interaction site for calcineurin.

[4]S. Chai, M. Li, J. Lan, Z.-G. Xiong, J. A. Saugstad, and R. P. Simon, our observation.

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## Full-length ASIC Proteins Bind to AKAP150

The purified GST fusion proteins of both intracellular termini from ASIC1a and ASIC2a showed interactions with AKAP150 and calcineurin. To verify that this protein-protein binding is not an artifact generated from the recombinant proteins that could harbor changes in protein folding, we tested the binding ability of the full-length proteins ASIC1a and ASIC2a by co-immunoprecipitation assays. The interaction between ASIC proteins and AKAP150 was tested by expressing YFP-ASIC1a or YFP-ASIC2a together with AKAP150 in HEK293 cells by co-transfection. Immunoprecipitation of protein complex was carried out using anti-GFP antibody. AKAP150 was co-immunoprecipitated only in the presence of YFP-ASIC1a or YFP-ASIC2a in the cell lysates and not in the absence of YFP-ASICs (Fig. 2*A*). To examine whether YFP-ASICs co-localize with AKAP150, the co-transfected HEK293 cells were double labeled with anti-GFP antibody and anti-AKAP150 (Fig. 2*B*). The reason for immunolabeling YFP with anti-GFP antibody was to verify the recognition of YFP by this antibody shown in the co-immunoprecipitation assay. The HEK293 cells expressed YFP-ASICs and AKAP150 in the plasma membrane and other intracellular locations. A substantial co-localization of ASICs and AKAP150 was observed in the plasma membrane, whereas cells expressing GFP and AKAP150 did not show such distinct overlap (Fig. 2*B*).

We further tested whether the heterologous expression of AKAP150 could promote interaction between ASICs and AKAP150 that could be detected by pulldown of full-length ASIC proteins. Purified GST-AKAP150 was used as bait to pull down the HA-tagged ASICs expressed in CHO cells. As shown in Fig. 2*C* and supplemental Fig. S2, ASIC1a and ASIC2a were pulled down in the presence of GST-AKAP150 and not in the presence of GST protein. Previously, the activity of GST-AKAP150 has been demonstrated, suggesting the proper folding of this recombinant protein (42). Thus, the interaction between GST-AKAP150 and ASICs is plausible. This pulldown assay also showed that the interaction between full-length ASICs and the anchoring proteins does not require brain specific factor(s) for binding, since the assay was carried out without brain lysates. However, it is yet to be determined if the interaction is direct, since this interaction does not rule out involvement of cellular component(s) provided by HEK293 or CHO cells.

## ASIC1a and ASIC2a Co-localize with AKAP150 in Rat Cortical Neurons

Previous studies have shown ASIC1a in neuronal cell bodies and dendrites of hippocampal neurons as well as in the synaptosome-containing brain fraction (14). Similarly, AKAP150 was detected in postsynaptic fractions and at the periphery of neuronal cell body and dendritic regions in hippocampal neurons (29–32). We used cultured rat cortical neurons to find out whether ASICs and AKAP150 co-localize in neurons. Transfection of neurons showed overexpression of ASIC1a-GFP fusion protein in the soma and distinctive punctate and clustering pattern in dendrites, which is consistent with previous reports (Fig. 3, *A* and *B*; Refs. 14 and 26). The ASIC1a clustering pattern is almost identical to and overlaps with the distribution pattern of endogenous AKAP150. When the ASIC2a-GFP fusion protein was expressed in neurons by transfection, we did not find any ASIC2a-GFP-expressing cells. Therefore, we used ASIC2a-HA expressing plasmid for transfection. Neurons expressing ASIC2a-HA also showed clustering and a punctate pattern in the dendrites, although such expression patterns were far less prevalent in these neurons than in ASIC1a-GFP-expressing neurons (Fig. 3, *C* and *D*). In addition, the distribution of AKAP150 in the ASIC2a-HA-expressing neurons was different from that in neurons untransfected or transfected with ASIC1a-GFP, suggesting that the targeting of AKAP150 was affected by overexpression of ASIC2a-HA (supplemental Fig. S3). Although AKAP150 was detected in small clusters, few of the AKAP150 clusters showed co-localization with ASIC2a-HA. Despite the minor differences in expression pattern, these results suggested that ASIC1a and

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ASIC2a are expressed in the same cluster region in the dendrites where AKAP150 is located. When cells were transfected with plasmids expressing GFP alone, the expression level of endogenous AKAP150 was affected in these cells. Because we could not detect AKAP150 in these cells either because the protein was not expressed or was expressed below the level of detection for the assay, GFP did not function as a suitable negative control (supplemental Fig. S3).

## Cyclosporin A Induces Increased Amplitude of Acid-evoked Currents in CHO Cells Expressing ASIC1a-GFP and ASIC2a-GFP

After the binding studies between ASICs and calcineurin, we further tested whether calcineurin is involved in regulation of ASIC function. Cyclosporin A is a calcineurin-specific immunosuppressant and has been used in many studies to inhibit this ubiquitous protein phosphatase. We, therefore, tested the effect of cyclosporin A on the acid-evoked currents in CHO cells expressing either ASIC1a-GFP or ASIC2a-GFP. CHO cells transfected with plasmid expressing ASIC-GFP fusion proteins were used for whole-cell patch clamp experiments; acid-evoked currents were recorded at a holding potential of −60 mV. When homomeric channels of the ASIC1a-GFP and ASIC2a-GFP recombinant proteins were evoked by acid, current traces were not distinguishable from that of the ASIC1a or ASIC2a wild-type channels expressed in CHO cells, indicating that GFP fusion to C terminus of these ASIC subunits did not seem to affect the electrophysiological properties. Similar to the ASIC1a homomeric channels, application of an acidic solution, pH 5.5, to the ASIC1a-GFP-expressing cells elicited a transient and fast desensitizing inward current (Fig. 4*A*). In the control cells recorded with an intracellular solution containing ethanol, the amplitude of ASIC1a currents remained relatively stable during the 16–20-min recording time. However, in cells recorded with an intracellular solution containing 30 μM cyclosporin A, the amplitude of ASIC1a-GFP currents ran up gradually with time. The current density, measured by normalizing the peak current amplitude to the cell capacitance, was significantly higher in cyclosporin A-treated cells than the mock-treated cells with respect to time, which was confirmed by two-way ANOVA ($p < 0.01$; Fig. 4*B*). Sixteen minutes after the establishment of whole-cell configuration, the current density for the mock-treated group of cells was −43.2 ± 6.8 pA/pF, whereas it was −125.8 ± 57.5 pA/pF for cyclosporin A-treated cells ($n = 10$ and 12, $p < 0.01$; Fig. 4*C*).

The effect of cyclosporin A was also tested on ASIC2a-GFP expressed in CHO cells. When evoked by a pH 5 extracellular solution, the ASIC2a-GFP-expressing cells elicited a transient but slow desensitizing inward current (Fig. 4*D*). Similar to ASIC1a channels, intracellular application of 30 μM cyclosporin A potentiated ASIC2a-GFP currents during the 16-min recording time ($p < 0.01$, two-way ANOVA; Fig. 4*E*). At 16 min, the density of ASIC2a currents in mock-treated control cells was −95 ± 20 pA/pF, whereas it was −268 ± 40 pA/pF in the 30 μM cyclosporin A-treated group ($n = 6$ for both groups, $p < 0.01$; Fig. 4*F*). Taken together, these results strongly suggest that cyclosporin A induces significant increase in both ASIC1a and ASIC2a currents.

## Cyclosporin A Induces an Increase in ASIC Current Amplitude in Cultured Mouse Cortical Neurons

Based on the effect of cyclosporin A in ASIC homomeric channels expressed in CHO cells, we then tested the effect of cyclosporin A on ASIC-like currents in cultured mouse cortical neurons. In all neurons voltage-clamped at −60 mV, large transient inward currents were activated upon a reduction of extracellular pH to 5.5 (Fig. 5*A*). As previously reported, homo- and heteromultimeric channels composed of ASIC1a and/or ASIC2a subunits are activated in these neurons by pH 5.5 external solution (15). After establishment of whole-cell configuration, the peak amplitude of ASIC currents runs down gradually to about 60%

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that of its initial value in neurons (Fig. 5, *B* and *C*). However, the amplitude of the ASIC currents remained relatively stable in neurons recorded with 30 μM cyclosporin A in intracellular solution ($p < 0.01$, two-way ANOVA). Consistent with the findings in CHO cells, the amplitude of the ASIC currents was significantly higher in the presence of cyclosporin A. At 20 min, the current density of ASICs from the mock-treated control group of neurons was −16.4 ± 3.6 pA/pF, but it was −33.9 ± 6.2 pA/pF in the cyclosporin A-treated group of neurons ($n = 6$ and 7, $p < 0.05$; Fig. 5*C*). To ensure that the cyclosporin A-induced potentiation of the ASIC currents is due to inhibition of calcineurin, we also tested the effect of FK-506, another inhibitor of calcineurin. In agreement with cyclosporin A, intracellular application of 10 μM FK-506 potentiated the ASIC currents in mouse cortical neurons ($p < 0.01$, two-way ANOVA; Fig. 5, *D*–*F*). At 20 min, the current density of ASICs in the FK-506 treated group was −25.9 ± 2.8 pA/pF, whereas it was −16.4 ± 3.6 pA/pF in the control group of neurons ($n = 5$ and 6, $p < 0.05$; Fig. 5*F*). These data suggest once more that inhibition of calcineurin induces a significant increase in ASIC currents.

### Inhibition of PKA Binding to AKAP by Ht-31 Induces a Decrease in ASIC Current Amplitude in Cultured Mouse Cortical Neurons and CHO Cells

After interaction and co-localization analyses of ASICs and AKAP150, we questioned whether AKAP150 is involved in the function of ASICs in cerebral neurons. The AKAP-derived peptide Ht-31 is the PKA binding site of human thyroid AKAP, and its binding to PKA inhibits the anchoring of PKA to AKAPs (31). The Ht-31 peptide has no effect on PKA activity despite its binding to PKA, but rather it inhibits the anchoring of this kinase in all AKAPs including AKAP150. We measured the effect of Ht-31 and the corresponding control peptide Ht-31P, which is not able to bind to PKA due to an amino acid change in the PKA binding site, on the ASIC currents in mouse cortical neurons. Upon activation by reduction of extracellular pH to 5.5, the amplitude of ASIC currents was smaller in cells with intracellular application of 20 μM Ht-31 compared with cells with the 20 μM Ht-31P control peptide in the intracellular solution or control cells without any treatment (Fig. 6*A*). Current density at 20 min of recording showed no significant difference between untreated neurons and that of Ht-31P-treated cells (−42.5 ± 7.9 pA/pF and −32.7 ± 2.0 pA/pF). However, the Ht-31-treated cells had a current density of −15.0 ± 5.8 pA/pF, which was significantly smaller than the control groups of neurons ($n = 6$, $p < 0.01$; Fig. 6, *B* and *C*). These data suggested that anchoring of PKA to AKAP is necessary for the full activation of ASICs in cortical neurons. To determine which of the ASICs were involved in reduction of currents in the presence of Ht-31 in neurons, we measured acid-evoked currents in CHO cells. CHO cells have been reported to express an endogenous Chinese hamster ortholog of AKAP150 (42). ASIC currents were measured using CHO cells expressing either ASIC1a-GFP or ASIC2a-GFP, as described above. Currents of both ASIC1a and ASIC2a were significantly lower in the presence of Ht-31 than Ht-31P (supplemental Fig. S4). These results indicated that both ASICs require PKA anchoring to AKAP for their full activation. Although neurons and CHO cells contain AKAP150 or its ortholog, we do not rule out involvement of any other AKAPs together with its associated PKA at the site of ASIC function. Nevertheless, considering the binding and co-localization of ASICs with AKAP150, involvement of this anchoring protein is a strong candidate for ASIC activation by delivering PKA for channel phosphorylation.

## DISCUSSION

In this study we have identified the proteins AKAP150 and calcineurin as regulators of ASIC1a and ASIC2a. *In vitro* assays including co-immunoprecipitation and pulldown assays showed interactions between the ASICs, the anchoring protein, and calcineurin. Immunostaining showed a co-localization of ASIC1a-GFP and ASIC2a-HA with

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endogenous AKAP150 in cultured rat cortical neurons. These results were interesting, since both the neuronal-specific anchoring protein AKAP79/150 and calcineurin have been shown to be involved in the regulation of synaptic receptors such as NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (27, 43). Like these glutamate receptors, ASIC1a and ASIC2a are expressed abundantly in hippocampus where ASIC1a is implicated in long-term potentiation and in formation of learning and memory. Furthermore, ASIC1a has been isolated from postsynaptic density and synaptosomal fractions (23). The anchoring protein is also a postsynaptic protein, and the expression pattern in hippocampal neurons showed a concentration in the periphery of the cell body and along the neutrites (29, 31, 32).

Our results further show that cortical neurons and CHO cells expressing ASICs have a significantly lower acid-evoked current conductance when the association of PKA with AKAP is disrupted by the Ht-31 peptide. This indicates the functional involvement of AKAP150 in ASICs, since the anchoring of PKA in AKAP is required for the full activation of ASICs in neurons and CHO cells. PICK1 has been the best characterized regulatory scaffold protein for ASICs so far. A number of reports showed the interaction between PICK1 and ASIC1a and ASIC2a, in which the PDZ domain of PICK1 interacts with the C terminus of ASICs, and PICK1 co-localizes at synaptic sites together with ASICs (22–24, 26). This scaffolding protein has been proposed to recruit PKCα to its target proteins. Both the phosphorylation status and current amplitude of ASIC2a have been shown to increase upon PKC activation in the presence of PICK1 in COS cells (24). The function of PKC on ASIC1a appears to be more complex. Although PICK1 is crucial for the function of ASICs, ASIC1a is not a substrate for the PICK1-associated enzyme PKCα (26, 44). Instead, PKC holoenzyme and the other isoforms, βI, βII, δ, ε, and ζ, have been shown to phosphorylate ASIC1a *in vitro* (44). Nevertheless, PKA has also been shown to phosphorylate the C terminus of this channel protein. Leonard *et al.* (26) reported that ASIC1a, but not ASIC2a, is a substrate for PKA. However, treatment with the PKA agonist forskolin or inhibitor KT5720 has shown no or little effect on acid-evoked currents in hippocampal neurons but does have an effect upon distribution of ASIC1a clustering. Based on the observation that the PKA-dependent phosphorylation inhibited PICK1 binding to the C terminus, the authors proposed a role for PKA as a modulator of PICK1 binding to ASIC1a. Our whole-cell patch-clamp experiments, however, consistently showed a decrease in ASIC-like current density when measured at pH 5.5 in the presence of the AKAP inhibitor peptide Ht-31 in cortical neurons. The rationale for this pH setting was to activate both homo- and heteromultimers of ASIC1a and ASIC2a. Homomultimers of these channels have been shown to interact with AKAP150, and thus, it is plausible that heteromultimers also interact with the anchoring protein. Furthermore, the currents of both ASIC1a and ASIC2a were lower in the presence of Ht-31 than Ht-31P in CHO cells, indicating both ASICs are regulated by AKAP-associated PKA. Concerning the role of PKA in ASIC1a function, there are apparent discrepancies between our data and the data by Leonard *et al.* (26). These differences could, however, result from the parameters used in measuring the PKA effect on ASICs. Although we tested for the PKA effect that is associated with AKAPs, others have measured a broad effect of PKA by applying an activator or inhibitor of this kinase in whole cells. It is not fully understood if any PKA-related signal pathway(s) other than phosphorylation of ASICs by PKA could influence the activity of ASICs in an indirect way. Therefore, it is possible that inhibition of PKA in one or multiple signal pathways would generate different outcomes in ASIC activity.

Although the N terminus of ASIC2a interacted with AKAP150, the C termini of ASIC1a and ASIC2a rendered a significantly higher affinity to the anchoring protein. This suggests that the C termini could be the major interaction site for both ASICs. It still remains to be determined how binding of PICK1 and AKAP150 to the C termini of ASICs is regulated.

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Taking into consideration that PICK1 has a role in translocation of synaptic proteins to the plasma membrane and that it is required for distribution of ASICs, it would be an interesting question if this scaffolding protein is involved in trafficking of ASICs to the plasma membrane, where AKAP150 is located. AKAP150 also requires scaffolding proteins for its proper location in the plasma membrane. For example, association of AKAP79/150 with membrane-associated guanylate kinase (MAGUK) proteins and/or postsynaptic density-95 (PSD-95/SAP90) is crucial for the optimal position of the anchoring protein to locate its associated enzymes to the immediate vicinity of the target glutamate receptors (27, 45). Taken together, the interaction between AKAP150 and ASICs suggests a new level of the scaffolding complex that extends the ASICs-PICK1 signaling complex to a larger multiple protein complex by connection to AKAP150 with other scaffolding protein(s). Such broad and multiple levels of protein interactions seem plausible, since the C termini of ASIC1a and ASIC2a contain PDZ domains that can interact with many other scaffolding proteins containing PDZ domains.

Another interesting finding in our study is the regulatory function of calcineurin in ASIC1a and ASIC2a activity. Cyclosporin A, a specific inhibitor of calcineurin, increased significantly the activity of ASIC1a and ASIC2a. This suggests that calcineurin-dependent dephosphorylation is involved in the inactivation of these ASICs. Cyclosporin A increased acid-evoked current amplitude in CHO cells expressing ASIC1a-GFP or ASIC2a-GFP. This implies that the homomeric ASICs are regulated by calcineurin. Cyclosporin A-induced increase in acid-evoked amplitude was also shown in cultured mouse cortical neurons, where homo- and heteromultimers of ASICs are present. Similar to AKAP binding to ASICs, we presumed that heteromeric ASICs also bind to calcineurin and, thus, exhibit calcineurin-dependent modulation. These results are intriguing, since calcineurin is a $Ca^{2+}$/calmodulin-activated enzyme, and therefore, our results imply the involvement of intracellular $Ca^{2+}$ in ASIC activity, in which $Ca^{2+}$-influx, *e.g.* caused by activation of NMDA receptors, inactivates homo- or heteromeric channels of ASIC1a and ASIC2a. Such $Ca^{2+}$-calcineurin-dependent inactivation was also reported for NR2A, a regulatory subunit of NMDA receptors, whereby these channels exhibited reduced ion gating and enhanced desensitization (43, 46, 47). Although our data show calcineurin-dependent inactivation of ASICs, a recent study by Gao *et al.* (48) reported activation of ASIC1a by $Ca^{2+}$/calmodulin-dependent protein kinase II. They showed that in hippocampal neurons ASIC1a is phosphorylated by this kinase within 1 h after global ischemia, and ischemia increased ASIC1a currents *in vitro*. These processes have been shown to require the activation of NR2B subunit of NMDA receptor. Thus, it would be interesting to find out whether different subunits of NMDA receptors are involved in modulation of ASIC1a function. ASIC1a activates at higher pH compared with ASIC2a and is permeable to $Ca^{2+}$ in addition to $Na^{+}$. Therefore, calcineurin-mediated inhibition of ASICs could be critically important to prevent cytotoxicity caused by $Ca^{2+}$ overload in ischemia and stroke. Moreover, calcineurin-dependent dephosphorylation could play an important role for ASICs, since these channels have been reported to be in a highly phosphorylated state at basal level (26). Calcineurin is a ubiquitous enzyme that is present at high levels in hippocampus, especially at synapses (49). About half of the calcineurin in the cell was proposed to be associated with the plasma membrane, and the other half cytosolic (for review, see Ref. 40). Hence, the membrane-associated calcineurin plays an important regulatory role for many postsynaptic ion channels. It still remains to be established whether calcineurin-dependent changes in ASIC current amplitude result from the surface expression of the channel, *i.e.* trafficking, or gating. It will be intriguing to further discover the mechanisms by which membrane-bound AKAP150, calcineurin, PKA, and PKC interact with ASICs in a manner dependent on pH and intracellular $Ca^{2+}$ levels.

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## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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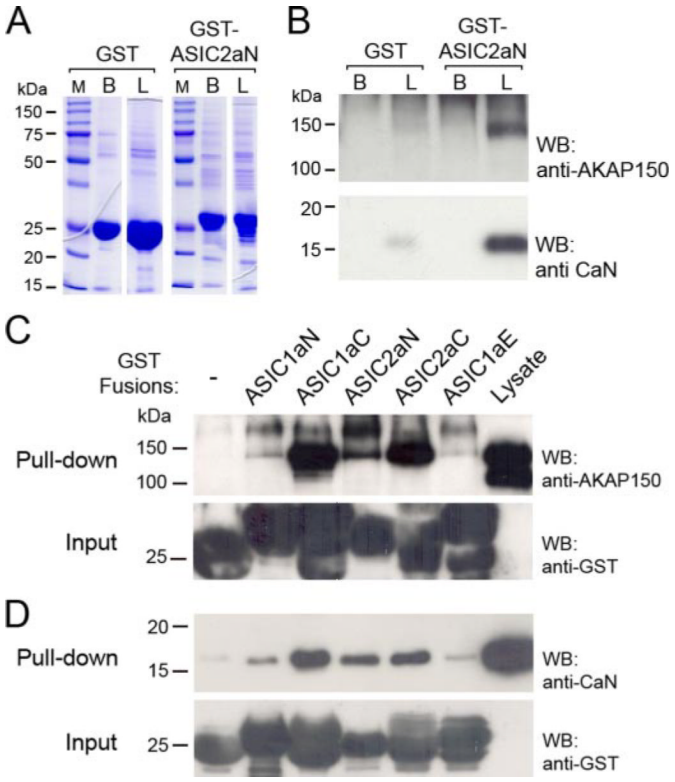


**FIGURE 1. AKAP150 and calcineurin bind to the N and C terminus of ASIC1a and ASIC2a**

*A*, pulldown assay with GST and GST-ASIC2aN with rat brain lysate. Separation of pulldown samples in one-dimensional SDS-PAGE (12%) is shown by Coomassie Blue staining. GST alone and GST fusion proteins were affinity-purified before pulldown assay with rat brain lysate (*L*) or with buffer (*B*) as mock treatment. *M*, molecular mass standards. *B*, Western blot analyses of the pulled-down samples using antibodies anti-AKAP150 C and anti-calcineurin B subunit are shown. *C* and *D*, pulldown assays with GST-ASIC1aN, GST-ASIC1aC, GST-ASIC2aN, and GST-ASIC2aC. In parallel, GST and GST-ASIC1aE fusion protein are used as negative controls. Western blot (*WB*) analyses of the pulled-down samples with antibodies anti-AKAP150 C (*C*) and anti-calcineurin B subunit (*CaN*, *D*) are shown (*Pulldown*). For loading control, the membranes are re-probed with anti-GST antibody and shown below the respective figure (*Input*).

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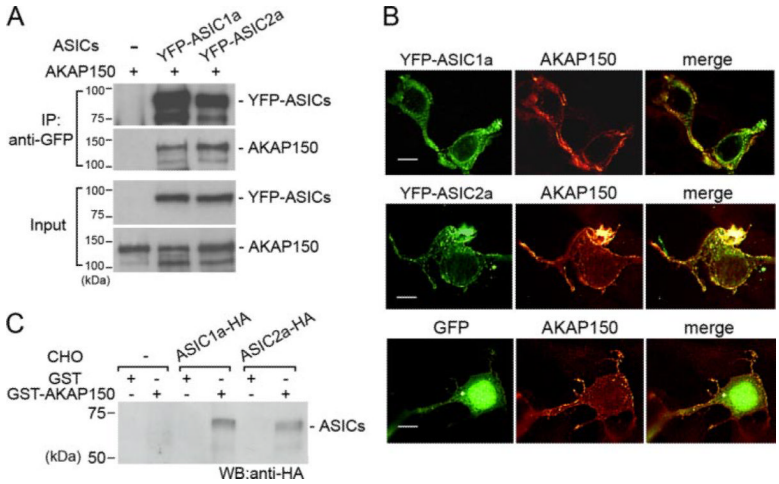


**FIGURE 2. AKAP150 interacts with the full-length proteins ASIC1a and ASIC2a**

*A*, co-immunoprecipitation of ASIC1a and ASIC2a with AKAP150. HEK293 cells were co-transfected with plasmids expressing AKAP150 and GFP, AKAP150 and YFP-ASIC1a, or AKAP150 and YFP-ASIC2a. From the cell lysates, proteins were immunoprecipitated (*IP*) with anti-GFP antibody. For both immunoprecipitated samples and total cell lysates (*Input*), Western blot analyses were carried out using anti-GFP antibody to detect the YFP-tagged ASICs, and then the blots were stripped and reprobed with anti-AKAP150 antibody. *B*, co-localization of YFP-ASICs with AKAP150 in HEK293 cells. HEK293 cells were co-transfected with plasmids expressing AKAP150 and GFP, AKAP150 and YFP-ASIC1a, or AKAP150 and YFP-ASIC2a. Immunocytochemistry was carried out using anti-GFP antibody and fluorescein isothiocyanate-labeled secondary antibody to visualize YFP-tagged ASICs. AKAP150 was visualized using anti-AKAP150 antibody and Cy3-labeled secondary antibody. Co-expression of GFP and AKAP150 in HEK cells are used as a negative control. *Scale bars*, 20 μm. *C*, pulldown assay of HA-tagged ASICs with GSTAKAP150. The affinity-purified GST-AKAP150 was incubated with CHO cell lysates containing either ASIC1aeHA or ASIC2a-cHA. GS-4B beads were used to pull down GST alone or GST-AKAP150 and the interacting proteins from CHO cell lysates. Western blot analysis (*WB*) was carried out with anti-HA antibody to detect ASICs. The equal amount of GST and GST-AKAP150 as well as HA-tagged ASICs in the binding assay is shown in supplemental Fig. S2.

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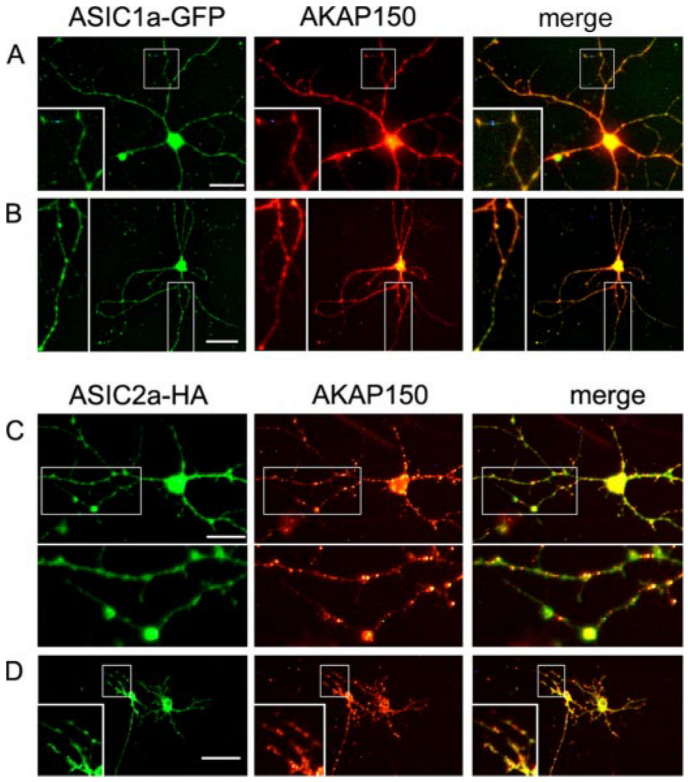


**FIGURE 3. Subcellular distribution of ASIC1a, ASIC2a, and AKAP150**

Primary cultures of rat cortical neurons were transfected with ASIC1a-GFP (*A* and *B*) and ASIC2a-HA (*C* and *D*). The endogenous AKAP150 was detected with anti-AKAP150c antibody (Cy3) and the HA-tagged ASIC2a with anti-HA antibody (fluorescein isothiocyanate). Two representative images obtained from the multiple neurons transfected with ASIC expressing plasmids are shown. Punctate and clustering pattern of ASICs and AKAP150 are visible through out the cell. Dendrites showing co-localization of ASICs with AKAP150 in the *boxed areas* in the main images are depicted in *insets* and *smaller panels* with higher magnification. *Scale bars*, 20 μm.

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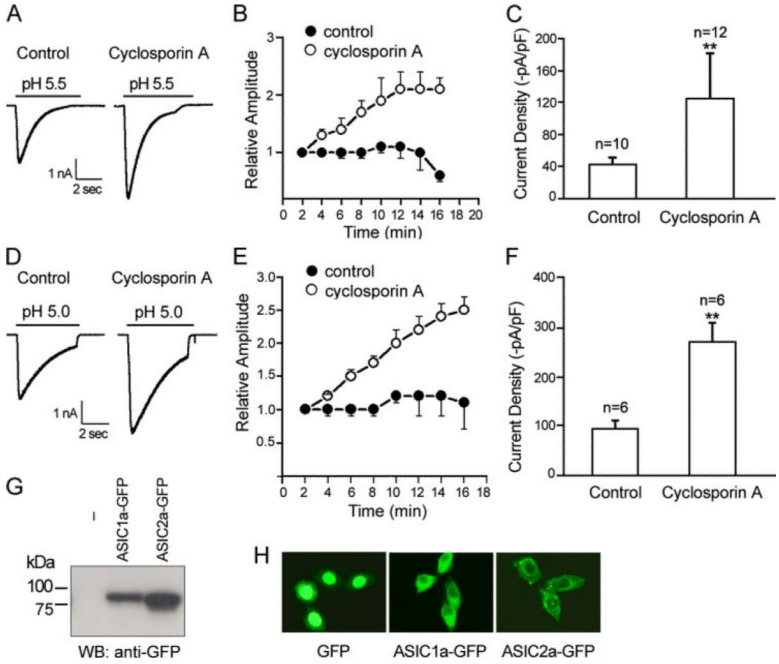


**FIGURE 4. Inhibition of calcineurin by cyclosporin A induces increase in ASIC1a and ASIC2a current amplitude in CHO cells**

ASIC1a and ASIC2a currents were induced by external solutions with pH 5.5 and 5.0 the CHO cells expressing ASIC1a-GFP (*A*–*C*) and ASIC2a-GFP (*D*–*F*), respectively, and recorded at a holding potential of −60 mV. Representatives of ASIC1a (*A*) and ASIC2a (*D*) current traces show the effect of 30 μM cyclosporin A at the 16-min time point. In control cells ethanol was used instead of cyclosporin A. *B* and *E*, peak current amplitudes were measured every 2 min and the relative amplitudes from cyclosporin A-(○) or ethanol-treated cells (●) were plotted as a function of time. *C* and *F*, peak current amplitudes are normalized by cell capacitance. The resulting current density of the ethanol-treated (control) and cyclosporin A-treated group at the 16-min time point is shown. Statistical analysis was carried out using two-way ANOVA (*B* and *E*) and Student's *t* test (*C* and *F*); for both analyses the *double asterisk* indicates $p < 0.01$ between control and cyclosporin A-treated groups. *G* and *H*, CHO cells transfected with ASIC1a-GFP and ASIC2a-GFP are shown by Western blot (*WB*) analysis with anti-GFP antibody (*G*) and fluorescence microscopy (*H*).

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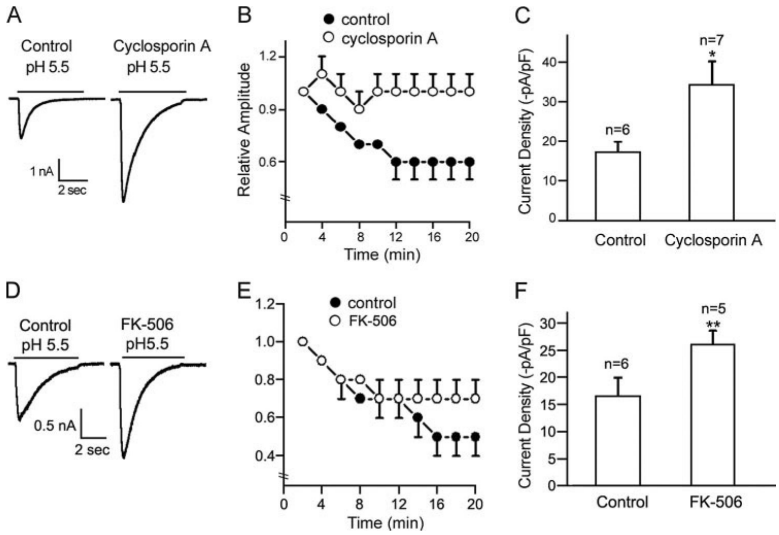


**FIGURE 5. Inhibition of calcineurin by cyclosporin A induces increased ASIC current amplitude in cultured mouse cortical neurons**

ASIC currents were induced by pH 5.5 external solution and recorded at a holding potential of −60 mV. Effect of cyclosporin A (*A–C*) and FK-506 (*D–F*) on ASIC currents are shown. *A* and *D*, representatives of ASIC current traces at the 20-min time point show increase in amplitude upon intracellular application of 30 μM cyclosporin A and 10 μM FK-506 in neurons, respectively. *B* and *E*, peak current amplitudes were measured every 2 min, and the relative amplitudes from cyclosporin A (○)-, FK-506 ((○)-, or ethanol-treated cells (●) are plotted as a function of time. *C* and *F*, peak current amplitudes are normalized by cell capacitance and shown by *bar graph* summarizing potentiation of ASIC currents in the absence and presence 30 μM cyclosporin A (*C*) or 10 μM FK-506 at 20-min time point (*F*). Statistical analysis was carried out using two-way ANOVA ($p < 0.01$ in *B* and *E*) and Student's *t* test ($p < 0.05$ in *C* and *F*). *Single* and *double asterisks* indicate $p < 0.05$ and $p < 0.01$, respectively.

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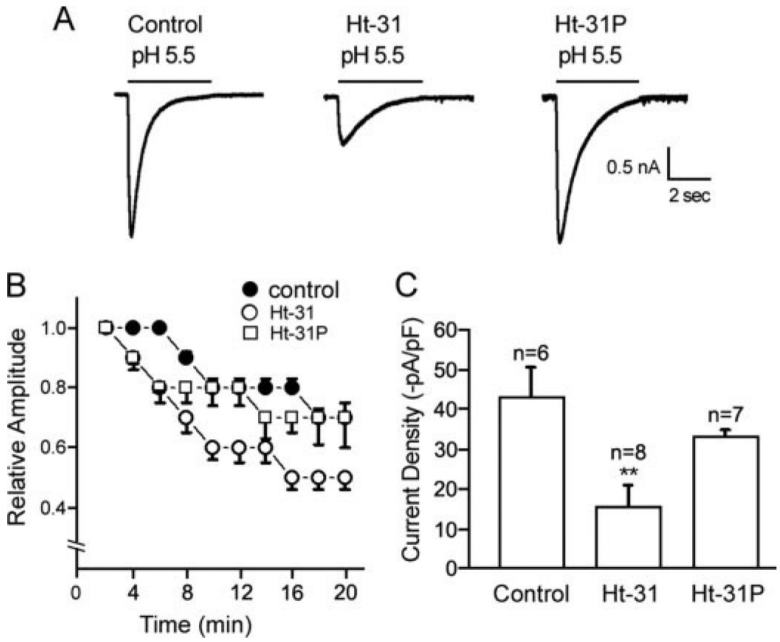


**FIGURE 6. Disruption of PKA anchoring to AKAPs by Ht-31 decreases ASIC currents in cultured mouse cortical neurons**

ASIC currents were induced by pH 5.5-external solution and recorded at a holding potential of −60 mV. *A*, representative traces of ASIC currents showing changes in peak current amplitude in untreated cells (*Control*), cells treated with 20 μM inhibitory peptide Ht-31, and 20 μM control peptide Ht-31P at the 20-min time point. *B*, peak current amplitudes were measured every 2 min and the relative amplitudes from control (●), Ht-31 (○), and Ht-31P (□)-treated cells are plotted as a function of time. *C*, *bar graph* summarizes changes in ASIC current density in the absence and presence of the peptides. Statistical analysis was carried out using two-way ANOVA (*B*) and Student's *t* test (*C*); for both analyses, the *double asterisk* indicates $p < 0.01$.