Interaction of the synaptic protein PICK1 (protein interacting with C kinase 1) with the non-voltage gated sodium channels BNC1 (brain Na^+ channel 1) and ASIC (acid-sensing ion channel)

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Neuronal members of the degenerin/epithelial Na^+ channel (DEG/ENaC) family of cation channels include the mammalian brain Na^+ channel 1 (BNC1), acid-sensing ion channel (ASIC) and dorsal-root acid-sensing ion channel (DRASIC). Their response to acidic pH, their sequence similarity to nematode proteins involved in mechanotransduction and their modulation by neuropeptides suggest that they may function as receptors for a number of different stimuli. Using the yeast two-hybrid assay, we found that the PDZ domain-containing protein PICK1 (protein interacting with C kinase) interacts specifically with the C-termini of BNC1 and ASIC, but not DRASIC or the related αENaC or βENaC. In both the yeast two-hybrid system and mammalian cells, deletion of the BNC1 and ASIC C-termini abolished the interaction with PICK1. Likewise, mutating the PDZ domain in PICK1 abolished its interaction with BNC1 and ASIC. In addition, in a heterologous expression system PICK1 altered the distribution of BNC1 channels; this effect was dependent on the PDZ domain of PICK1 and the C-terminus of BNC1. We found crude synaptosomal fractions of brain to be enriched in ASIC, suggesting a possible synaptic localization. Moreover, in transfected hippocampal neurons ASIC co-localized with PICK1 in a punctate pattern at synapses. These data suggest that PICK1 binds ASIC and BNC1 via its PDZ domain. This interaction may be important for the localization and/or function of these channels in both the central and peripheral nervous systems.

Key words: DEG/ENaC channel, mechanosensation, mechanotransduction, nociception, PDZ domain.

INTRODUCTION

The brain Na^+ channel 1 (BNC1, also called MDEG, BNaC1 and ASIC2) [1–4], the acid-sensing ion channel (ASIC, also called BNaC2 and ASIC1) [3–5] and the dorsal-root acid-sensing ion channel (DRASIC, also called ASIC3) [4,6], and their differentially spliced isoforms, are mammalian members of the degenerin/epithelial Na^+ channel (DEG/ENaC) superfamily. These channels are expressed in the central nervous system and in peripheral sensory neurons. There are at least two hypotheses about their function in sensory neurons. First, it has been proposed that in the periphery they form a central part of a mechanoreceptor complex [7]. This hypothesis is based on their similarity to the nematode DEG/ENaC channels, mechanosensory (MEC)-4 and MEC-10, that are involved in mechanotransduction in the worm. Evidence supporting this hypothesis came from studies of BNC1-knockout mice; the reduced sensitivity of rapidly adapting mechanoreceptors suggested that BNC1 may be a central component of a mechanoreceptor complex [8]. Secondly, it has been proposed that they may contribute to acid-evoked nociception [4]. This hypothesis is based on the finding that a reduction in extracellular pH activates current in cells heterologously expressing BNC1, ASIC or DRASIC [5,6,9,10]. Moreover, FMRFamide (Phe-Met-Arg-Phe-amide)-related neuropeptides, which can be induced by inflammation, modulate the response of ASIC and DRASIC to acid [11]. Although these DEG/ENaC channels share biophysical properties with H^+-gated channels in peripheral sensory and central neurons, their function at these sites remains to be determined with certainty. DEG/ENaC channels share a common architecture, with intracellular N- and C-termini, two transmembrane domains and a large extracellular domain containing highly conserved cysteine residues [12–14]. Individual DEG/ENaC subunits associate as homo- or hetero-multimers to form voltage-insensitive, amiloride-inhibited cation channels.

Numerous recent studies have shown that association of neuronal ion channels and receptors with adapter and cytoskeletal proteins influences their localization and regulation (reviewed in [15,16,16a]). Although no proteins have been reported to associate with BNC1, ASIC or DRASIC, their proposed neuronal functions and the importance of such connections to other channels suggest that binding partners may exist. Such associations may be particularly important for DEG/ENaC channels that function as mechanosensors. Several models of mechanoreceptor function propose a tethered structure in which a channel is linked to the intracellular cytoskeleton and the extracellular matrix; these elements are thought to transmit mechanical tension to open the channel. Genetic studies of the *Caenorhabditis elegans* DEG/ENaC proteins MEC-4 and MEC-10 support this hypothesis [7]. The intracellular domains of these channels may link to microtubules made up of the α-tubulin MEC-12 and the β-tubulin MEC-7 via the stomatin-related MEC-2 [17–19]. At the extracellular surface, these

Abbreviations used: BNC1, brain Na^+ channel 1; ASIC, acid-sensing ion channel; DRASIC, dorsal-root acid-sensing ion channel; DEG/ENaC, degenerin/epithelial Na^+ channel; PICK1, protein interacting with C kinase 1; MEC, mechanosensory; AMPA, α-amino-3-hydroxy-5-methyl-isoxazole-4-proprionic acid; mGluR7a, metabotropic glutamate receptor 7a; CSF, crude synaptosomal fraction; GluR2/3, glutamate receptor 2/3; PKC, protein kinase C; AD, activation domain; DB, DNA-binding domain; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; h, human.

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channels may interact with an extracellular matrix collagen, MEC-5, and/or MEC-9, which contains epidermal growth factor repeats, which are known interaction domains [20].

These observations suggested that BNC1, ASIC and DRASIC may interact with other proteins through their intracellular N- and C-termini. Such interacting proteins might localize the channels to specific regions of the cell membrane, might control the amount of protein at the cell surface, and in the case of mechanosensory function might influence channel gating. Thus identification of proteins that interact with BNC1, ASIC and DRASIC may help us understand their function and regulation. To identify interacting proteins, we used the yeast two-hybrid system and screened a human brain cDNA library.

EXPERIMENTAL

Yeast two-hybrid assay

Subcloning by PCR was used to amplify and insert the cytoplasmic C-terminal tail of human BNC1 (residues 470–512) into the unique *Eco*RI and *Bam*HI sites of the GAL4 DNA-binding domain (DB) vector pAS2-1 (Clontech, Palo Alto, CA, U.S.A.). This bait construct was transformed into the PJ69-2A yeast strain using the lithium acetate procedure and mated with yeast strain Y187 pretransformed with the human brain Matchmaker cDNA library (Clontech). The mRNA source was a normal, whole brain from a 37-year old Caucasian male as reported on Clontech's product-analysis certificate. Mated cells were plated on $Leu^-/Trp^-/His^-$ plates supplemented with 5 mM 3-amino-1,2,4-triazole and grown for 10 days at 30 °C before positive colonies were picked and streaked on to plates lacking adenine, His, Leu and Trp. Library plasmids from clones that grew in the absence of adenine, His, Leu and Trp and that tested positive for β-galactosidase expression were isolated. They were co-transformed with either the bait vector or the original pAS2-1 vector into PJ69-2A to confirm the interaction. Those that were specific for the bait were sequenced.

DNA constructs

The BNC1-C-GAL4(DB) plasmid was constructed as described for the yeast two-hybrid assay. BNC1 has two splice variants, BNC1a and BNC1b (also called MDEG1 and MDEG2), which have different N-termini and first transmembrane domains [8,9]. Subcloning by PCR was used to amplify and insert the cytoplasmic N-termini of human (h) BNC1a (residues 1–38) and hBNC1b (residues 1–82), or the C-termini of hASICα (residues 459–528), hDRASIC (residues 476–531), hαENaC (residues 589–669) and hβENaC (residues 559–640) into the unique *Eco*RI and *Bam*HI sites of the GAL4(DB) vector pAS2-1. Similar cloning techniques were used to generate BNC1Δ4 (containing residues 470–508), BNC1-C1/2 (residues 470–485), ASICΔ4 (residues 459–524), ASIC-C1/2 (residues 459–493) and ASIC-N1/2 (residues 494–528), which were subcloned into pAS2-1.

PICK1-GAL4(DB) was cloned by excising protein interacting with C kinase 1 (PICK1) from PICK1-GAL4(AD) (where AD indicates activation domain) with *Eco*RI and *Xho*I and ligating it into pAS2-1 cut with *Eco*RI and *Xho*I. This plasmid construct was then cut with *Nde*I, treated with mung-bean nuclease and blunt-end ligated to bring PICK1 in frame with the GAL4(DB) in pAS2-1. mutPICK1-GAL4(AD) was made by PCR mutagenesis using the primers 5′-TCCGAATTCGCATGTTTGCAGACTTGGATTATGACATC-3′ and 5′-GATCAGGTTCTGAGCAGCCGCCTGC-3′. The PCR product was cut with *Eco*RI and *Alw*NI and ligated in a triple ligation reaction with the PICK1 *Alw*NI/*Xho*I fragment and pACT-2 (Clontech) cut with *Eco*RI and *Xho*I. Expression vectors for COS-7 cell transfections were obtained by cloning cDNA into pMT3. The BNC1bΔ4-pMT3 plasmid was constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). PICK1 and mutPICK1 cDNAs were subcloned into the pMT3 vector with a FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) at the C-terminus. Expression vectors for hippocampal neuron transfections were obtained by cloning hASICα into pcDNA3.1 and hPICK1 into pCMVmyc (Clontech). All plasmid constructs were confirmed by DNA sequencing.

Antibodies

Anti-FLAG M2 monoclonal antibody was purchased from Sigma (St. Louis, MO, U.S.A.). Portions of hBNC1a (residues 466–512) and hASIC (residues 459–528) were expressed as thioredoxin fusion proteins, and part of mouse DRASIC (residues 79–153) was expressed as a glutathione S-transferase fusion protein. Polyclonal antibodies made against these fusion proteins were raised in rabbits (Pocono Rabbit Farm, Canadensis, PA, U.S.A.). The specificity of the antisera was evaluated in COS-7 cells transfected with BNC1a/b, ASIC or DRASIC. Anti-PSD95 monoclonal antibody and anti-glutamate receptor 2/3 (GluR2/3) antibody were purchased from Sigma. Goat PICK1 (C-20) polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), and mouse monoclonal anti-Myc (clone 9E10) was from the Developmental Studies Hybridoma Bank (Iowa City, IA, U.S.A.).

COS-7 cell transfections and co-immunoprecipitations

COS-7 cells were maintained in culture with Dulbecco's modified Eagle's medium plus 10% fetal calf serum in a humidified atmosphere of 5% CO_2 in air. The cells were transfected by electroporation using 10^7 cells with 30 μg of plasmid DNA at a ratio of BNC1a/b/Δ4, ASIC, DRASIC or empty vector to $PICK1^{flag}$, $mutPICK1^{flag}$ or empty vector of 1:5. Cells were lysed 24–48 h post-transfection at 4 °C in lysis buffer (50 mM Tris, pH 7.4/150 mM NaCl/1% Triton X-100) as described previously [21]. We allocated 5% of the lysate to be used for Western blot and the remainder was incubated with 1 μl of anti-FLAG M2 antibody overnight at 4 °C. Proteins were separated on 8% polyacrylamide gels using SDS/PAGE. Western blots were blocked with 5% BSA, and incubated first with primary antibody (1:5000 dilution of anti-FLAG, anti-BNC1, anti-ASIC or anti-DRASIC), then with a horseradish peroxidase-coupled secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) at a 1:5000 dilution. Proteins were detected by enhanced chemiluminescence (Pierce, Rockford, IL, U.S.A.).

Crude synaptosomal fraction (CSF)

The CSF was prepared by modifications of published protocols [22,23]. One adult mouse brain was homogenized in 3.6 ml of homogenization buffer (320 mM sucrose/4 mM Hepes, pH 7.4/1 mM EGTA/0.4 mM PMSF/20 μg/ml aprotinin/20 μg/ml leupeptin/10 μg/ml pepstatin A) with 10 strokes of a tight-fitting glass Dounce tissue grinder (7 ml; Wheaton). The homogenate was centrifuged at 1000 ***g*** for 10 min. The supernatant was collected and centrifuged at 12000 ***g*** for 15 min, and the second pellet was resuspended in 2.5 ml of homogenization buffer and centrifuged at 13000 ***g*** for 15 min. The resulting pellet representing the CSF was resuspended in 0.5 ml of homogenization buffer. Samples of the homogenate and the CSF (10 μg of protein

for the PSD95 and GluR2/3 blots, 20 μg of protein for the PICK1 blot and 200 μg of protein for the ASIC blot) were separated on SDS/PAGE gels, and Western-blot analyses were performed with the indicated antibodies.

Immunofluorescence

COS-7 cells were grown on chamber slides coated with collagen. Cells were fixed with 4 % formaldehyde in PBS, permeabilized with 0.1 % Triton X-100 in PBS, blocked with SuperBlock (Pierce), and incubated with primary antibodies anti-BNC1 (1:600 dilution) and anti-FLAG (1:250 dilution), followed by the secondary antibodies donkey anti-mouse Cy2 (1:500 dilution; Jackson Immuno Research, West Grove, PA, U.S.A.) and goat anti-rabbit Alexa 568 (1:1250 dilution; Molecular Probes, Eugene, OR, U.S.A.). Staining was visualized using a Bio-Rad 1024 confocal microscope (BioRad, Hercules, CA, U.S.A.).

Hippocampal neuron transfection and immunofluorescent staining

Rat E18 hippocampal neurons were purchased from Dr Gregory Brewer (Southern Illinois University School of Medicine, Springfield, IL, U.S.A.) [24,25]. Neurons were plated at 3500–4000 cells/well on CC2 (cell-culture second generation)-treated 8-well glass chamber slides (Fisher Scientific, Pittsburgh, PA, U.S.A.) in B27/Neurobasal supplemented with 0.5 mM glutamine/25 μM glutamate. Neurons were transfected 3–4 days after plating with 1 μg of plasmid DNA/well using the calcium phosphate method [26]. After transfection (2–3 days), neurons were fixed in 4 % formaldehyde/4 % sucrose in PBS for 10 min and permeabilized for 30 min with PBST (0.1 % Triton X-100 in PBS). The cells were blocked for 30 min in SuperBlock and incubated for 2 h at 37 °C in primary antibodies diluted in SuperBlock. The primary antibodies used were mouse monoclonal anti-Myc (clone 9E10) and affinity-purified hASIC, diluted 1:100. Cells were then incubated with goat anti-mouse Alexa488 (1:1250) and goat anti-rabbit Alexa568 (1:2000) secondary antibodies. Staining was visualized using a Bio-Rad 1024 confocal microscope.

RESULTS

Identification of PICK1 using the yeast two-hybrid screen

We began the search for interacting proteins using human BNC1 because of its neuronal pattern of expression and its sequence and potential functional similarity to the nematode DEG/ENaC proteins involved in mechanotransduction. We used the intracellular C-terminus of BNC1 as bait in a yeast two-hybrid screen of a human brain cDNA library. The BNC1 C-terminal screen produced a clone containing the entire open reading frame of the human homologue of a previously reported mouse protein named PICK1. PICK1 contains a single PDZ domain [a protein domain named after PSD95, Discs large and ZO-1] at its N-terminus, a coiled-coil domain at amino acids 139–166 and a stretch of acidic residues at its C-terminus (residues 382–389) [27,28]. The PDZ domain in PICK1 interacts with the C-terminal residues of protein kinase C (PKC) α [27], GluR2/3/4c subunits of the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors [28,29], Eph receptor tyrosine kinases and ephrin ligands [23], metabotropic glutamate receptor 7a (mGluR7a) [30,31] and monoamine transporters [32].

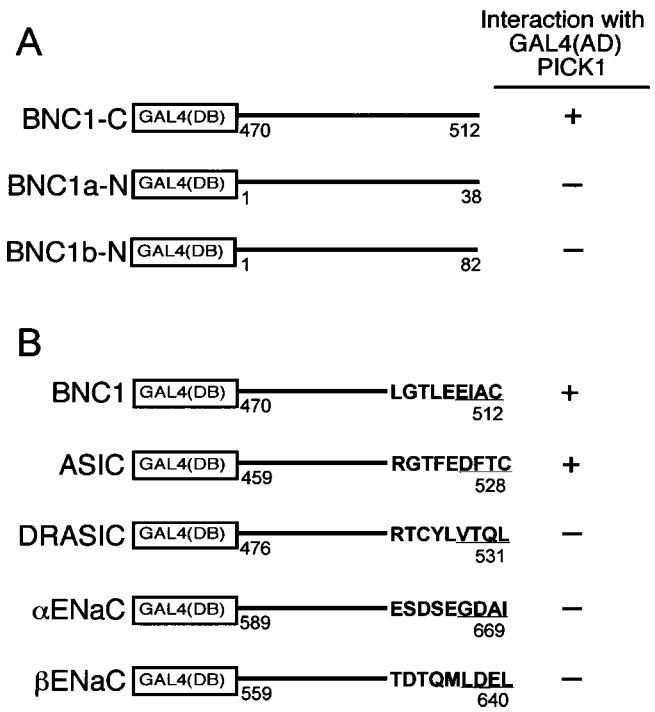

Figure 1 Specificity of PICK1 for interaction with the C-termini of BNC1 and ASIC in the yeast two-hybrid system

(**A**) PICK1 interacted specifically with the intracellular C-terminus, but not the intracellular N-termini of BNC1a or BNC1b. (**B**) PICK1 interacted with the C-termini of BNC1 and ASIC, but not those of DRASIC, αENaC or βENaC. The intracellular C-terminal regions of BNC1, ASIC, DRASIC, αENaC and βENaC were fused to the GAL4(DB) and tested for interaction with PICK1 in the yeast two-hybrid system.

PICK1 binds specifically to the C-terminus of BNC1 and ASIC

The BNC1 gene produces two alternatively spliced transcripts, BNC1a and BNC1b (also called MDEG1 and MDEG2), that have different N-termini but share an identical C-terminus [1,2,9]. To determine whether the PICK1 interaction with BNC1 was specific for the C-terminus, we tested the intracellular N-termini of BNC1a and BNC1b for binding to PICK1. Neither N-termini interacted with PICK1 in a yeast two-hybrid assay (Figure 1A). We also tested the specificity of the interaction between PICK1 and the C-terminus of BNC1 by testing for binding between PICK1 and the C-termini of other human DEG/ENaC proteins using the yeast two-hybrid assay. PICK1 interacted with the C-termini of ASIC, but not with those of DRASIC, αENaC or βENaC (Figure 1B).

Identification of the PICK1 binding domain in BNC1 and ASIC

To identify the sites in BNC1 and ASIC that interact with PICK1, we made deletions in the C-terminal BNC1 and ASIC constructs and tested their interaction with PICK1 in the yeast two-hybrid assay. Figure 2(A) shows that deletion of the N-terminal half of the ASIC C-terminus (ASIC-N1/2) had no effect on the interaction. In contrast, deletion of the C-terminal half of the BNC1 (BNC1-C1/2) or ASIC (ASIC-C1/2) C-terminus eliminated the interaction. Moreover, deletion of the last four amino acids of the BNC1 and ASIC C-terminus abolished the interaction. These data suggest that PICK1 binds to the C-terminal ends of BNC1 and ASIC. This result was interesting because the four C-terminal residues of BNC1 (Glu-Ile-Ala-Cys)

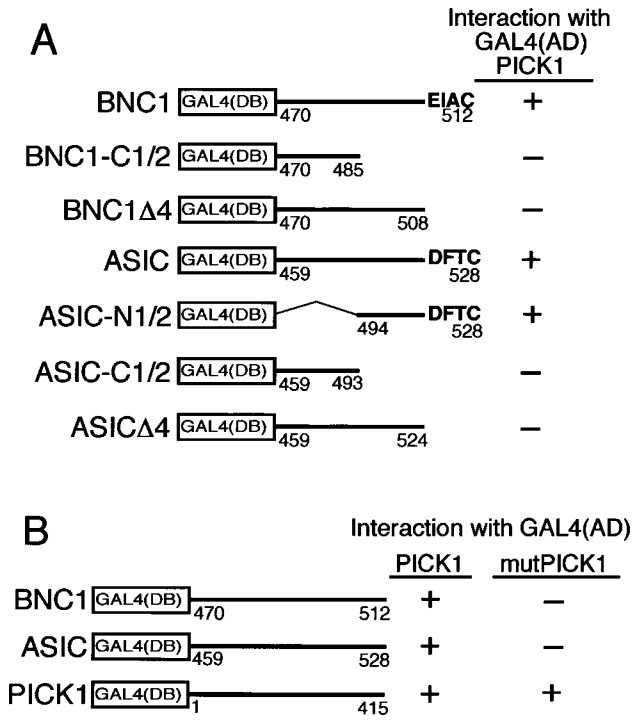

Figure 2 BNC1 and ASIC interact with PICK1 via its PDZ domain

(**A**) The C-terminal ends of BNC1 and ASIC are necessary for interaction with PICK1. C-terminal deletions of BNC1 and ASIC abolished their interaction with PICK1. However, the intact C-terminal half of ASIC allowed its interaction with PICK1. (**B**) Mutations in the PDZ domain of PICK1 abolished its interaction with BNC1 and ASIC. PICK1 was mutated at residues 27 and 28, from Lys-Asp to Ala-Ala (mutPICK1), fused to the GAL4(AD) and tested for interaction with the C-termini of BNC1 or ASIC, or full-length PICK1 fused to the GAL4(DB) in the yeast two-hybrid system. As a control, wild-type PICK1 was still able to interact with the mutPICK1.

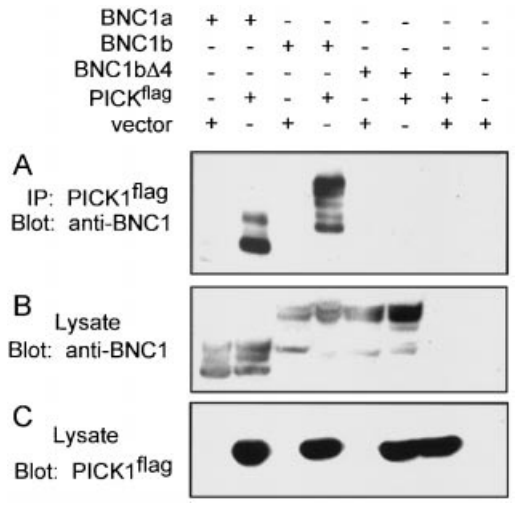

Figure 3 Detection of PICK1 interactions with both BNC1a and BNC1b, but not BNC1bΔ4 in cell extracts

COS-7 cells were transfected with expression vectors encoding BNC1a, BNC1b or BNC1bΔ4 alone or with PICK1^{flag} and then immunoprecipitated (IP) with anti-FLAG antibody. (**A**) The immunoprecipitates were resolved by SDS/PAGE and probed with anti-BNC1 antibodies. (**B**, **C**) Total lysates from the same samples were resolved by SDS/PAGE and probed with either anti-BNC1 (**B**) or anti-FLAG antibodies (**C**) to confirm expression.

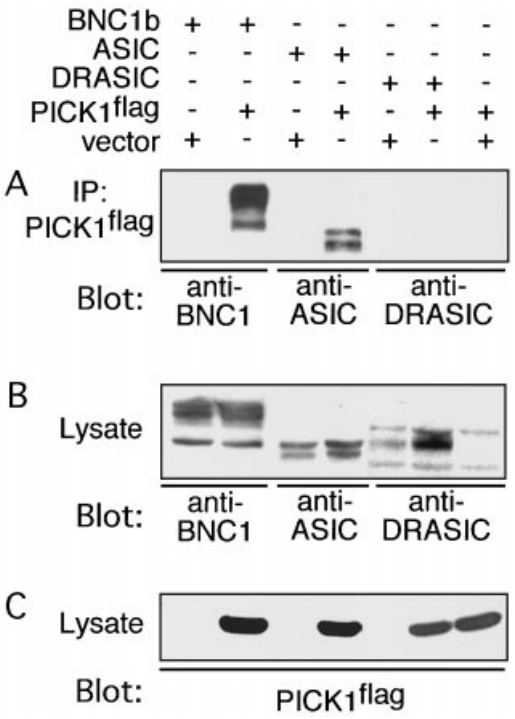

Figure 4 Detection of PICK1 interactions with BNC1b and ASIC, but not DRASIC in cell extracts

COS-7 cells were transfected with expression vectors encoding BNC1b, ASIC or DRASIC alone or with PICK1^{flag} and then immunoprecipitated with anti-FLAG antibody. (**A**) The immunoprecipitates were resolved by SDS/PAGE and probed with the indicated antibodies. (**B**, **C**) Total lysates from the same samples were resolved by SDS/PAGE and probed with the indicated antibodies to confirm expression.

show some similarity to those of ASIC (Asp-Phe-Thr-Cys). PDZ domains are known to interact with the C-terminal ends of proteins. Thus binding through the C-termini of BNC1 and ASIC suggested that the PDZ domain of PICK1 may be the site of interaction.

Identification of the BNC1- and ASIC-binding site in PICK1

PDZ domains contain a conserved amino acid, typically a lysine or arginine, that is required for interaction with the free carboxyl group at the C-terminus of target proteins [33–35]. To determine whether the PDZ domain of PICK1 is involved in the interaction with BNC1 and ASIC, we mutated the putative carboxylate-binding loop of the PICK1 PDZ domain by replacing Lys^{27} and Asp^{28} with alanine residues [27,28]. We found that this Lys^{27}-Asp^{28} → Ala-Ala mutation of the PDZ domain of PICK1, mutPICK1, abolished the interaction with the C-terminus of BNC1 and ASIC in the yeast two-hybrid assay (Figure 2B). These results provide further evidence that the interaction between PICK1 and BNC1 or ASIC is via the PDZ domain of PICK1. As reported previously [27,28], we found that mutPICK1 interacts with wild-type PICK1 in the yeast two-hybrid system. This result indicates that the mutPICK1 protein is produced and can interact functionally with another protein in the two-hybrid assay.

PICK1 interacts with BNC1a, BNC1b and ASIC in mammalian cells

We asked whether the full-length BNC1 channel interacted with PICK1 in mammalian cells. Although the C-termini of the splice

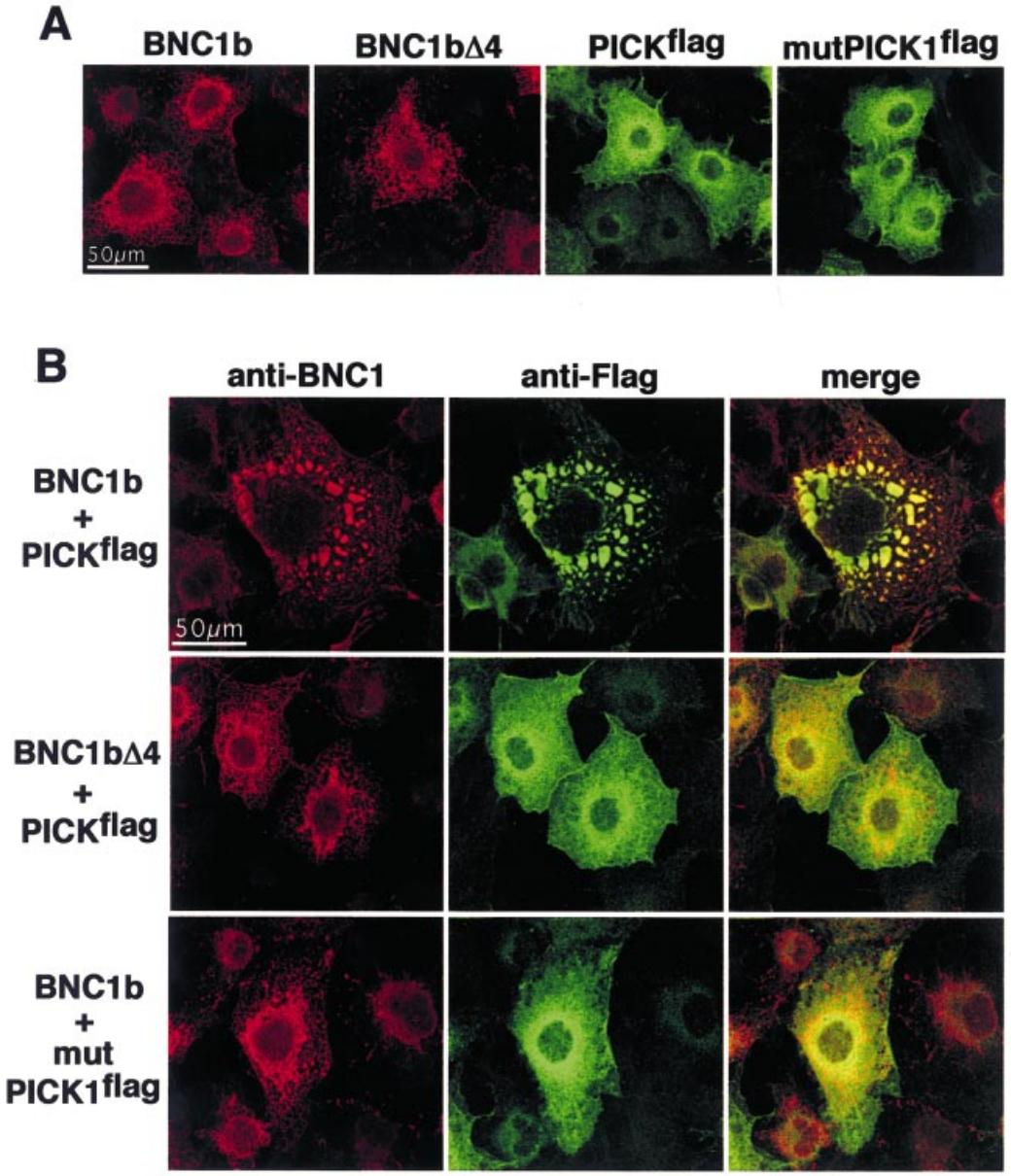

Figure 5 PICK1 alters the distribution of BNC1b via its PDZ domain in COS-7 cells

(**A**) COS-7 cells were transfected with BNC1b, BNC1bΔ4, PICK1^{flag} or mutPICK1^{flag} as indicated. (**B**) Co-expression of wild-type BNC1b and PICK^{flag} led to an altered distribution of both proteins. PICK1^{flag} and BNC1b lacking the last four amino acids (BNC1bΔ4) or BNC1b and mutPICK1^{flag} did not lead to redistribution of the two proteins.

variants BNC1a and BNC1b are identical, their N-termini are very different and thus could affect the interaction with PICK1. Therefore, BNC1a or BNC1b were expressed in COS-7 cells with and without a FLAG-tagged PICK1 construct, PICK1^{flag}. Figure 3(A) shows that immunoprecipitation of PICK1 precipitated BNC1a and BNC1b. In the absence of PICK1^{flag} or BNC1a or BNC1b, no co-precipitation was observed. These results indicate that both BNC1a and BNC1b interact with PICK1 in mammalian cells despite their different N-termini. To confirm that it is the last four amino acids in BNC1 that are required for binding, we expressed PICK1 with BNC1b without the last four residues (BNC1bΔ4). Consistent with the yeast two-hybrid assays, BNC1bΔ4 did not co-immunoprecipitate with PICK1^{flag} (Figure 3A), even though both BNC1bΔ4 and PICK1^{flag} proteins were present (Figures 3B and 3C).

The yeast two-hybrid experiments demonstrated that PICK1 also interacts with ASIC. To confirm this interaction, COS-7 cells were transfected with ASIC and PICK1^{flag}. Figure 4(A) shows that PICK1^{flag} co-immunoprecipitated ASIC. In contrast, DRASIC did not interact with PICK1^{flag}, even though both proteins were expressed (Figures 4B and 4C). Moreover, as observed with BNC1bΔ4, deletion of the last four amino acids of ASIC abolished the interaction with PICK1 (results not shown), again confirming the results of the yeast two-hybrid assay.

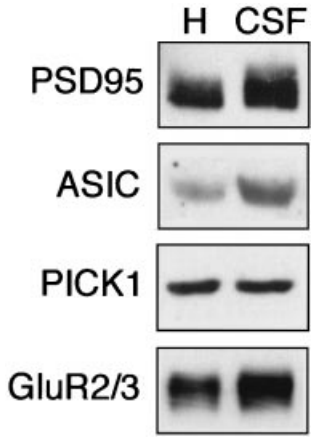

Figure 6 ASIC is enriched in the CSF

Homogenate (H) and CSF were immunoblotted for PSD95 and GluR2/3, synaptic membrane markers that are enriched in the CSF. ASIC was highly enriched in the CSF. PICK1 was present in both fractions, indicating its presence within as well as outside synapses in the adult mouse brain.

Co-expression alters the distribution of PICK1 and BNC1b channels in heterologous cells

Previous studies have shown that PICK1 can change the localization of AMPA receptors, Eph receptors and ligands, mGluR7a and monoamine transporters in heterologous expression systems [23,28,29,31,32]. To determine whether PICK1 alters the distribution of the BNC1 channel, we transfected PICK1^{flag} and BNC1b into COS-7 cells and analysed their cellular distribution by immunofluorescence microscopy. When expressed separately, PICK1 staining was diffuse throughout the cell, whereas BNC1b showed a reticular pattern (Figure 5A). However, expression together in the same cell altered the distribution of both proteins (Figure 5B). To test the requirement for the interaction between BNC1b and PICK1, we co-expressed BNC1bΔ4 and PICK1^{flag}. Deletion of the last four amino acids of BNC1b eliminated the PICK1-induced redistribution of BNC1b and prevented co-localization of PICK1 (Figure 5B, middle row). In addition, we examined the role of the PICK1 PDZ domain by co-transfecting the PICK1 PDZ domain mutant mutPICK1 (Lys^{27}-Asp^{28} → Ala-Ala). Figure 5(B) (bottom row) shows that mutation of the PDZ domain also eliminated the redistribution of both BNC1b and PICK1.

ASIC co-localizes with PICK1 at synapses

PICK1 is present at synapses where it co-localizes with AMPA receptors, Eph receptors and mGluR7a receptors [23,28,29,31]. To determine whether BNC1 or ASIC were associated with synapses, we isolated crude synaptosomes from adult mouse brain using differential centrifugation [22,23]. PSD95, a protein that is highly concentrated in the post-synaptic density [23,36], was enriched in crude synaptosomes (Figure 6). The GluR2/3 subunits of the AMPA receptor were also enriched in the CSF, but to a lesser extent than PSD95 (Figure 6). ASIC was highly enriched in the CSF, suggesting that it is localized at synapses. PICK1 was not enriched in the CSF (Figure 6). This finding is consistent with earlier results showing PICK1 at synapses, but also throughout the brain [23]. Similar studies with BNC1 were not possible because current antibodies were not sufficiently sensitive for immunoblotting the endogenous protein.

To further test whether PICK1 and ASIC co-localize at synapses, we expressed ASIC and a Myc-epitope-tagged PICK1 in cultured hippocampal neurons and immunostained the protein

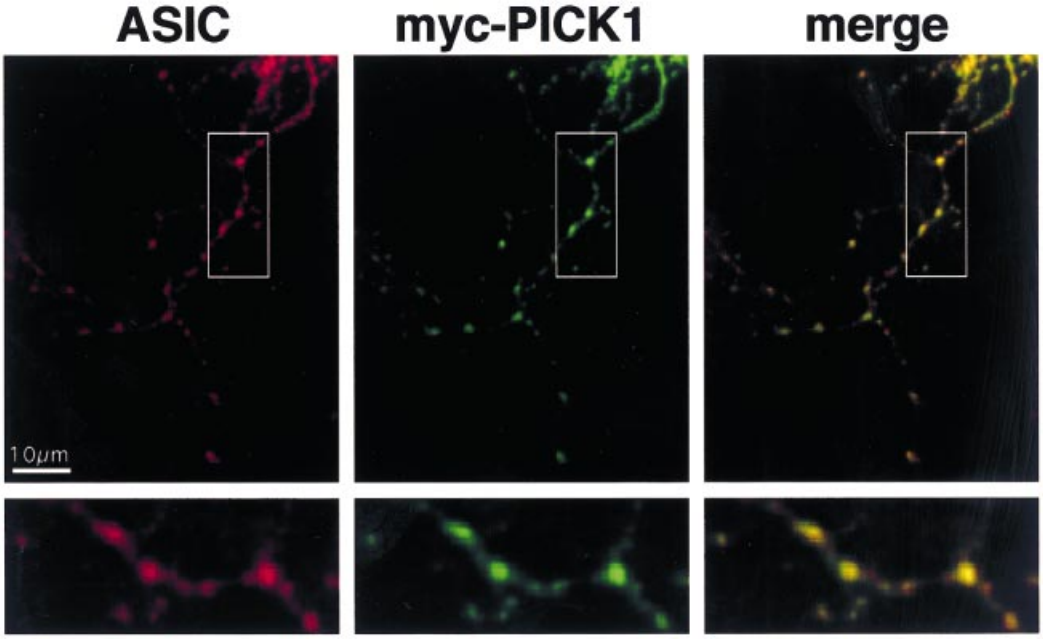

Figure 7 Co-localization of ASIC and PICK1 in transfected hippocampal neurons are found at synapses

Neurons were co-transfected with ASIC and Myc-PICK1 cDNAs and stained with anti-ASIC and anti-Myc antibodies 3 days later. ASIC staining is red and Myc-PICK1 staining is green. The boxed areas were enlarged and are displayed in the bottom panels.

2–3 days after transfection. Recombinant Myc-PICK1 has been shown previously to selectively cluster at excitatory synapses in cultured hippocampal neurons, as reported for the endogenous protein [37]. Consistent with earlier reports, we found Myc-PICK1 localized in a highly punctate pattern along the neurites (Figure 7). Importantly, co-expressed wild-type ASIC showed the same pattern; it co-localized with PICK1 in the nerve processes (Figure 7). In transfected hippocampal neurons, ASIC also co-localized in a synaptic pattern with PSD95-GFP (green fluorescent protein; J. A. Wemmie, unpublished work), which has a punctate expression pattern identical with endogenous PSD95 [38]. Co-localization of ASIC with Myc-PICK1 and PSD95-GFP in rat hippocampal neurons indicates that ASIC localizes at synapses.

DISCUSSION

The results indicate that PICK1 interacts with the C-termini of BNC1a, BNC1b and ASIC through its PDZ domain. PDZ domains have recently been divided into classes based on the C-terminal sequences with which they interact. Interestingly, the PDZ domain of PICK1 can be sorted into more than one class. The C-terminus of PKCα (Gln-Ser-Ala-Val) falls into class I (Ser/Thr-Xaa-ϕ, where ϕ indicates a hydrophobic residue) of PDZ binding domains, whereas the AMPA and Eph receptors fall into the more loosely defined class II, where the amino acids at the C-terminal 0 or -2 position can be either hydrophobic or aromatic [35]. The C-terminal amino acids in BNC1 (Glu-Ile-Ala-Cys) and ASIC (Asp-Phe-Thr-Cys) conform to class II. It is interesting that the PDZ domain in PICK1 can be classified into more than one class of PDZ domains. This suggests a unique structure for the PICK1 PDZ domain. It also raises the question of how interactions between PICK1 and other proteins are controlled. Perhaps the binding affinity determines the interaction partners of PICK1. Alternatively, the localization of PICK1 within the cell or during biosynthesis may determine which proteins bind.

A general function of PDZ domains may be to translocate and/or localize their binding partners to specific plasma-membrane domains. For example, PKCα resides primarily in the cytoplasm of unstimulated cells, and upon activation it translocates to the plasma membrane [39]; binding of PICK1 only to the activated form of PKCα suggests a potential mechanism for targeting activated PKCα. More recently, the PDZ domain in PICK1 was found to be required for the presynaptic localization of mGluR7a [37]. Protein localization is especially important in polarized cells. An example in epithelia is that the PDZ-domain protein Na^+/H^+ exchanger regulatory factor (NHERF) binds the C-terminus (Asp-Thr-Arg-Leu) of the cystic fibrosis transmembrane conductance regulator and localizes it in the apical membrane [40,41]. PICK1 has been found recently in the post-synaptic density of excitatory synapses, co-localizing with the GluR2/3 AMPA receptor subunits, Eph receptor tyrosine kinases and PKCα [23,28]. PICK1 has also been localized presynaptically with mGluR7a [30,31]. The synaptic localization of PICK1 raised the question of whether BNC1 and/or ASIC might also be localized there. Interestingly, we found ASIC enriched in CSFs from mouse brain. Moreover, PICK1 and ASIC transfected into cultured rat hippocampal neurons co-localized at synapses. The presence of these channels at the synapse raises the interesting question of their function at that site. BNC1 and ASIC currents are activated by protons [5,9,10,42], and ASIC current is enhanced by FMRFamide and related peptides [11]. We speculate that these, or as yet undiscovered ligands, might regulate BNC1 and ASIC function at synapses.

Earlier work indicates that DEG/ENaC channels link to cytoplasmic proteins via several mechanisms in addition to PDZ-domain interactions. Biochemical evidence has shown that subunits of another DEG/ENaC channel, the ENaC, bind Nedd4 (neuro precursor expressed developmentally down-regulated 4), a ubiquitin protein ligase [43]. The WW domains of Nedd4 (domains containing two highly conserved tryptophan residues that are spaced 20–22 amino acids apart) bind to a conserved proline-rich motif in the intracellular C-termini of ENaC subunits [43]. This binding decreases Na^+ current by increasing the rate of channel endocytosis and degradation [44]. A second proline-rich region in the rat αENaC subunit interacts with the Src homology 3 domain of α-spectrin, a cytoskeletal protein localized to the apical membrane of epithelial cells [45]. In addition, syntaxin 1A binds ENaC and reduces the number of channels at the membrane [46]. Our studies showing association of recombinant PICK1 with ASIC and BNC1, and the presence of both PICK1 and ASIC in the synaptosomal fraction, suggest an important association *in vivo*. However, future studies will be required to document association of the endogenous proteins and the physiological consequences.

As we described in the Introduction, the mechanosensory complex in *C. elegans* involves the DEG/ENaC channels MEC-4 and MEC-10 linked to intracellular cytoskeletal proteins and the extracellular matrix [7,17–19]. It has been postulated that the mammalian DEG/ENaC channels BNC1 and ASIC may also be involved in a mechanosensory complex [7,47]. Moreover, recent studies indicate that the BNC1 channel is located in specialized mechanosensory structures and is required for normal touch sensation, indicating that BNC1 may be a central component of a mechanosensory complex [8,48]. The finding that PICK1 interacts with BNC1 suggests it may play a role in a mammalian mechanosensory complex similar to that of the intracellular MEC-2 in the nematode complex. We speculate that PICK1 may link BNC1 and ASIC channels to the cytoskeleton. Consistent with this possibility, PICK1 contains a coiled-coil domain, and such protein–protein-interaction domains are found in many proteins that associate with actin filaments, microtubules and intermediate filaments [49]. PICK1 also contains an acidic region, another candidate for a protein-interaction domain that can influence synaptic localization in hippocampal neurons [37]. Studies to identify proteins that interact with PICK1 outside its PDZ domain should shed additional light on its function in neurons and its regulation of BNC1 and ASIC.

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