PICK1/Calcineurin Suppress ASIC1-mediated Ca\(^{2+}\) Entry in Rat Pulmonary Arterial Smooth Muscle Cells

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Running Head: Regulation of ASIC1 by PICK1 in PASMC

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Acid sensing ion channel 1 (ASIC1) contributes to Ca\(^{2+}\) influx and contraction in pulmonary arterial smooth muscle cells (PASMC). ASIC1 binds the PDZ (PSD-95/Dlg/ZO-1) domain of the protein interacting with C kinase 1 (PICK1) and this interaction is important for the subcellular localization and/or activity of ASIC1. Therefore, we first hypothesized that PICK1 facilitates ASIC1 dependent Ca\(^{2+}\) influx in PASMC by promoting plasma membrane localization. Using Duolink to determine protein-protein interactions and a biotinylation assay to assess membrane localization, we demonstrated that the PICK1 PDZ domain inhibitor, FSC231, diminished the co-localization of PICK1 and ASIC1, but did not limit ASIC1 plasma membrane localization. Although stimulation of store-operated Ca\(^{2+}\) entry (SOCE) greatly enhanced co-localization between ASIC1 and PICK1, both FSC231 and shRNA knockdown of PICK1 largely augmented SOCE. These data suggest PICK1 imparts a basal inhibitory effect on ASIC1 Ca\(^{2+}\) entry in PASMC and led to an alternative hypothesis that PICK1 facilitates the interaction between ASIC1 and negative intracellular modulators, namely PKC and/or the calcium-calmodulin-activated phosphatase, calcineurin. FSC231 limited PKC-mediated inhibition of SOCE, supporting a potential role for PICK1 in this response. Additionally, we found PICK1 inhibits ASIC1-mediated SOCE through an effect of calcineurin to dephosphorylate the channel. Furthermore, it appears PICK1/calcineurin-mediated regulation of SOCE opposes PKA phosphorylation and activation of ASIC1. Together our data suggest PKA and PICK1/calcineurin differentially regulate ASIC1-mediated SOCE and these modulatory complexes are important in determining downstream Ca\(^{2+}\) signaling.
INTRODUCTION

Acid sensing ion channels (ASICs) belong to the degenerin/epithelial sodium channel (DEG/ENaC) superfamily, which includes several amiloride-sensitive cation channels. Significant progress has been made in understanding the structure and function of ASICs in the nervous system; however, several questions remain regarding their physiological importance in other tissues. There is an emerging role for both ENaCs and ASICs in vascular smooth muscle and endothelial cells from a variety of vascular beds (8, 10-12, 19, 20, 25, 33, 46). Consistent with a physiological role of ASIC in the vasculature, our laboratory has recently shown that ASIC1 is an important facilitator of G-protein coupled receptor signaling via store-operated Ca\(^{2+}\) entry (SOCE) in pulmonary arterial smooth muscle cells (PASMC) (21, 22). Furthermore, ASIC1-mediated Ca\(^{2+}\) entry in PASMC appears to be an important constituent of both the active vasoconstrictor and vascular remodeling components of chronic hypoxic-induced pulmonary hypertension (21, 31).

Although the conventional mode of ASIC activation is via extracellular acidosis, various non-proton ligands, protein kinases, and other signaling molecules have been shown to regulate ASIC function (52, 53). Despite advances in identifying these signaling molecules, the molecular mechanism(s) that govern the trafficking and activity of ASICs remain largely unknown. All ASICs contain a C-terminal PDZ (PSD-95/Dlg/ZO-1) binding motif (1, 9, 14, 15). The C-termini of ASIC1 and ASIC2 share homology with type-II PDZ binding motifs and bind protein interacting with C-kinase-1 (PICK1) (9, 15). PICK1 is a scaffolding protein that mediates the direct interaction of many proteins that contain PDZ binding motifs. In addition to the PDZ-domain, PICK1 contains a larger BAR (Bin/amphiphysin/Rvs) domain which directly binds to lipids and is important in membrane localization (23). The interaction between the PDZ domain of PICK1 and the C-terminus of ASIC1 increases surface expression and clustering of ASIC1 (9, 15, 24). Although PICK1 is expressed in many tissues (42, 49), whether it is specifically expressed in PASMC and the relative physiological importance of PICK1 in ASIC1 membrane trafficking and regulation of...
channel activity is unknown. Therefore, we initially hypothesized that PICK1 facilitates ASIC dependent Ca\(^{2+}\) influx in PASMC by promoting surface localization.

In addition to changes in the cellular localization of the channel, PICK1 may alter the activity of ASIC1 by facilitating the interaction with intracellular modulators of the channel. PICK1 was originally isolated by its ability to bind the C terminus of protein kinase C (PKC) through its PDZ domain and facilitate PKC phosphorylation of ASIC1 and ASIC2. Although PKC phosphorylation leads to potentiation of ASIC2 current, the effect of PKC on ASIC1 activity is controversial. Additionally, PICK1 can bind the calcium/calmodulin-activated phosphatase, calcineurin B, and ASIC activity has been shown to be inhibited by calcineurin. Therefore, we additionally assessed the role of PICK1 to indirectly regulate ASIC1 phosphorylation status and activity by providing a necessary scaffolding complex for PKC and calcineurin.
METHODS

Generation of Primary PASMC Culture

All protocols employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico School of Medicine (Albuquerque, NM). Male Wistar rats (~12 wk old, Harlan Industries) were anesthetized with pentobarbital sodium (200 mg/kg ip), and the heart and lungs were removed by midline thoracotomy. Intrapulmonary arteries (~2nd–5th order) were dissected from surrounding lung parenchyma and enzymatically digested in reduced-Ca²⁺ Hank’s Balanced Salt Solution (HBSS) containing papain (26 U/ml), type-I collagenase (1750 U/ml), dithiothreitol (1 mg/ml), and BSA (2 mg/ml) at 37°C for 30 min. Single smooth muscle cells were dispersed by gentle trituration with a fire-polished pipette in Ca²⁺-free HBSS. The cell suspension was plated in Ham’s F-12 media supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin for 3-4 days in a humidified atmosphere of 5% CO₂-95% air at 37°C. Cellular purity was >90%, as assessed by morphological appearance and immunofluorescence of anti-smooth muscle 22 alpha (SM-22α).

Determination of mRNA and Protein Expression of PICK1 in PASMC

Total RNA was prepared from PASMC and brain tissue using TRIzol extraction and 1 µg of total RNA was reverse transcribed to cDNA using the Transcriptor First-Strand cDNA Synthesis kit (Roche). PCR was performed on cDNA with the iCycler® PCR system (Bio-Rad) using Taq polymerase and specific primers to detect transcripts for PICK1 [forward: 5’-GGGACGTGTTCTCTGTGATT-3’; reverse: 5’-CCAGGTACTTTCTTGATGGTGAG-3’; product size: 201] and β-actin [forward: 5’-AGTGTGACGTTGACATCCGT-3’; reverse: 5’-GACTCATCGTACTCCTGCTT-3’; product size: 244]. PCR products were electrophoresed through a 3% agarose gel and stained with ethidium bromide for visualization under UV light. PICK1 protein expression was determined by western blot analysis and immunofluorescence. PASMC were homogenized in 10 mM Tris-HCl homogenization buffer [containing 255 mM sucrose, 2
mM EDTA, 12 μM leupeptin, 1 μM pepstatin A, 0.3 μM aprotinin] and centrifuged at 10,000 g for 10 min at 4°C to remove insoluble debris (21). The PASMC lysate (20 μg) was separated by SDS-PAGE (7.5% Tris/Glycine) and transferred to a polyvinylidene difluoride membrane. The blot was blocked for 1 hr with 5% milk then incubated overnight at 4°C with rabbit anti-PICK1 (1:500; Abcam). For immunochemical labeling, blots were incubated with goat anti-rabbit IgG-horseradish peroxidase (1:3,000; 1 h; Bio-Rad). Following chemiluminescence labeling (ECL, Pierce), PICK1 was detected by exposing the blot to chemiluminescence-sensitive film (GeneMate).

For immunofluorescence, PASMC were fixed in 2% paraformaldehyde, permeabilized with 0.05% Triton-X, and incubated with rabbit anti-PICK1 (1:100) and goat anti-SM-22α (1:200) overnight at 4°C. PICK1 and SM-22α were detected with donkey anti-rabbit Alexa Fluor 647 and anti-goat DyLight 549 (1:100, 1 h, Jackson ImmunoResearch) and nuclei were stained with Sytox (1:10,000). Sections were mounted with FluoroGel (Electron Microscopy Sciences), and images were acquired using a confocal microscope (TCS SP5, Leica).

**Determination of ASIC1-PICK1 Co-localization**

Protein-protein interactions in PASMC were determined using the Duolink in situ Proximity Ligation Assay (PLA) according to manufacturer’s instructions (Olink Biosciences; Sigma Aldrich). Briefly, PASMC were plated on 18-well slides (Ibidi) and grown until ~ 75% confluent. In some experiments, PASMC were pretreated with the PICK1 inhibitor FSC231 (50 μM; 30 min) prior to fixing the cells with 2% paraformaldehyde. PASMC were incubated with Duolink blocking buffer for 30 minutes at 37°C then incubated overnight with rabbit anti-PICK1 (1:100; Abcam) and goat anti-ASIC1 (1:50; Santa Cruz). We have previously determined the specificity of goat anti-ASIC1 using wildtype and knockout mice (31). PASMC were then incubated with anti-rabbit PLUS and anti-goat MINUS PLA probes (1:5) for 1h at 37°C. Negative controls were completed by 1) omission of primary antibody and 2) incubation of each primary
antibody individually. Samples were amplified with Duolink In Situ Detection Reagent Orange (Excitation/Emission: 554/579nm; Sigma Aldrich) for 100 min at 37°C. Sytox Green (1:5,000; Invitrogen) was used as a nuclear stain and actin was stained with Alexa Fluor 647 Phaillodin (1:100; Invitrogen). Samples were mounted with Duolink mounting media and Z-stack images of the PLA interaction were acquired using a confocal microscope (TCS SP5, Leica). Each puncta was considered a positive protein-protein interaction. The number and size (pixel²) of puncta per cell were determined using Image J (National Institutes of Health). In addition, colocalization of ASIC1-PICK1 was determined by traditional immunofluorescence in parafomaldehyde-fixed PASMC by incubating with rabbit anti-PICK1 (1:100) and goat anti-ASIC1 (1:50; Santa Cruz) overnight at 4°C. Nuclei were stained with Sytox (1:10,000).

Colocalization of ASIC1 and PICK1 were determined using Leica Microsystems software.

For ASIC1-PICK1 co-immunoprecipitation (co-IP) experiments, ASIC1 or PICK1 antibodies (20 µg) were incubated with M-270 Epoxy Dynabeads (1.5 mg, Life Technologies) overnight at 37°C to covalently couple the antibody to the beads. PASMC were homogenized in lysis buffer and incubated with antibody-labeled beads (1 hr @ 4°C). Captured proteins and protein complexes were separated from lysate using magnetic separation followed by elution buffer. IP samples were analyzed by western blot with either rabbit anti-PICK1 (1:500; Abcam) or rabbit anti-ASIC1 (1:500; Millipore) as described above.

**Role of PICK1 in regulation of ASIC1 surface expression**

PASMC were grown until ~90% confluent in 75cm² flasks and treated with or without PICK1 PDZ Domain Inhibitor, FSC231 (50 uM; Calbiochem) for 30 min or 24 hrs. Non–treated and treated cells were then incubated with Sulfo-NHS-SS-Biotin (Pierce) for 30 minutes at 4°C. The reaction was quenched and PASMC were harvested and lysed with 10 mM Tris·HCl homogenization buffer as mentioned previously and spun at 10,000 x g for 2 minutes. Clarified supernatant was added to NeutrAvidin Agarose resin columns for 1h RT. Surface protein was collected by eluting with 5x sample buffer and analyzed by
western blot. Surface protein (25 µl) and total protein lysates (20 µg) were separated by SDS-PAGE (7.5% Tris/Glycine) and incubated 48h at 4°C with rabbit anti-ASIC1 (1:500; Millipore). For immunological labeling, blots were incubated with goat anti-rabbit IgG-horseradish peroxidase (1:3,000; 1 h; Bio-Rad). Following chemiluminescence labeling (ECL, Pierce), ASIC1 was detected by exposing the blot to chemiluminescence-sensitive film (GeneMate). A separate blot was probed with goat anti-smooth muscle 22α (SM22α; 1:500) and donkey anti-goat IgG (1:3000) to demonstrate the specificity of biotin to label cell surface versus cytosolic proteins.

Role of PICK1 to regulate ASIC1-dependent SOCE

PASMC were incubated with fura-2 AM (2 µM and 0.05% pluronic acid in PSS, Molecular Probes) for 30 min at 32°C. Fura-2-loaded PASMC were alternately excited at 340 and 380 nm at a frequency of 1 Hz with an IonOptix Hyperswitch dual excitation light source (IonOptix LLC), and the respective 510-nm emissions were detected with a photomultiplier tube. PASMC were superfused (5 ml/min at 37°C) with Ca²⁺-free, HEPES-based physiological saline solution [(PSS; (in mM) 130 NaCl, 4 KC1, 1.2 MgSO₄, 4 NaHCO₃, 10 HEPES, 1.18 KH₂PO₄, 6 glucose, 3 EGTA; pH adjusted to 7.4 with NaOH] containing 50 µM diltiazem (Sigma-Aldrich) to prevent Ca²⁺ entry through L-type voltage-gated Ca²⁺ channel, and 10 µM cyclopiazonic acid (CPA; Calbiochem) to deplete intracellular Ca²⁺ stores and prevent Ca²⁺ reuptake through the sarcoplasmic reticulum Ca²⁺-ATPase. The changes in [Ca²⁺]i were determined upon repletion of HEPES-based PSS containing 1.8 mM CaCl₂ in the continued presence of diltiazem and CPA. Area under the curve was calculated as previously described (36). Experiments were conducted in the absence or presence of the PICK1 inhibitor, FSC231 (50 µM; Calbiochem); the ASIC1 inhibitor, PcTX1 (20 nM; Phoenix Peptides); or in PASMC transfected with the negative or PICK1 shRNA.

shRNA Transfection: PASMC (1x10⁶) were suspended in Nucleofector Solution (Lonza) with 5 µg PICK1 shRNA plasmid (Qiagen). The cell/ DNA suspension was transferred to an Ingenio cuvette (Mirus
and was electroporated using a Nucleofector Device (program P-024; Lonza). Samples were then plated onto 100mm² dishes and incubated for 24h, media was changed to reduced serum (1% FBS) and incubated an addition 24h. PASMC were then used for qPCR (RT² SYBR green; Qiagen), protein expression or [Ca²⁺], measurements. β-actin was used as the reference gene and relative quantification of gene expression was determined by the $2^{-\Delta\Delta CT}$ method. PICK1 protein expression was determined as described above using GAPDH as the house keeping protein.

Role of PKC, calcineurin, and PKA in PICK1 regulation of ASIC1-mediated SOCE

SOCE responses were determined as described above in the presence, absence, or combination of the PKC Activator VII, CGK062 (30 μM, Calbiochem); myristoylated-PKC inhibitor (myr-PKC, 10 μM, Calbiochem); calcineurin inhibitor, cyclosporin A (1 μM, Cayman Chemicals); PICK1 inhibitor, FSC231 (50 μM, Calbiochem); ASIC1 inhibitor, PcTX1 (20 nM, Phoenix Peptides); PKA activator, forskolin (10 μM, Calbiochem); or PKA inhibitor, KT 5720 (300 nM, Calbiochem).

To determine the effect of PKA on ASIC1 phosphorylation, co-IP experiments were performed in PASMC treated with or without forskolin (10 μM), KT 5720 (1 μM), FSC231 (50 μM), or cyclosporin A (1 μM) for 30 minutes at 37°C. M-270 Epoxy Dynabeads were incubated with ASIC1 antibody (20 μg) followed by PASMC lysate as described above. IP samples were analyzed by western blot for anti-rabbit phosphoserine (3 μg/ml; Abcam). Total ASIC1 levels were determined as an internal control.

Calculations and Statistics

All data are expressed as means ± SE. Values of n refer to number of animals in each group unless otherwise stated. A one sample t-test, two sample t-test, one-way ANOVA, or two-way ANOVA was used to make comparisons. The statistical test performed for each experiment is indicated in the
If differences were detected by ANOVA, individual groups were compared with the Student-Newman-Keuls test. A probability of $p < 0.05$ was accepted as significant for all comparisons.
RESULTS

PICK1 Expression in PASMC

PICK1 is widely distributed in many different tissues, with highest levels in the brain and testis (42, 49). Using brain tissue as a positive control, we show mRNA expression of PICK1 in PASMC by RT-PCR (Figure 1A). Western blot analysis of PICK1 expression in PASMC reveals a prominent single band present at the predicted molecular weight (52 kDa; Figure 1B). Based on immunofluorescence imaging, PICK1 expression is fairly diffuse throughout the cell with prominent perinuclear staining (Figure 1C), as reported previously in heterologous expression systems (9, 15, 23, 42). Taken together, these data demonstrate the presence of PICK1 in PASMC.

PICK1 Co-Localizes with ASIC1

PICK1 has been shown to interact with ASIC1 in yeast two-hybrid assays, heterologous expression systems and neuronal cells (9, 15, 16, 23, 42). Figure 2A and 2D demonstrates this interaction in PASMC using a C-terminal anti-ASIC1 and anti-PICK1 antibody via Duolink PLA. According to the manufacturer, the theoretical maximum distance between the two target proteins must be <40 nm to create a PLA signal. As a negative control, either one or both primary antibodies were omitted at a time. We observed very few puncta under these conditions (Figure 2B and C). The distribution of ASIC1-PICK1 PLA signal is diffuse throughout the cells (Figure 2A), with apparent membrane localization as well (Figure 2D-E). To confirm this association, we performed co-immunoprecipitation assays in PASMC. Immunoprecipitation of ASIC1 from PASMC lysates resulted in the co-immunoprecipitation of PICK1 (Figure 2F). The reciprocal immunoprecipitation assays of PICK1 showed that ASIC1 co-immunoprecipitated in PASMC (Figure 2G). Inhibition of PICK1 with FSC231 tended to reduce the number of puncta per cell (p=0.063) and significantly decreased the size of the puncta detected indicating diminished clustering of the proteins (Figure 3).
**PICK1 Does Not Alter ASIC1 Plasma Membrane Localization**

To determine whether PICK1 regulates trafficking of ASIC1 to and from the plasma membrane, we used cell surface biotinylation and subsequent western blot analysis. We found that inhibition of PICK1 with FSC231 for 30 min did not alter membrane localization of ASIC1 (data not shown). Since the time required for ASIC1 trafficking is unknown, we also examined ASIC1 membrane localization following 24 hr inhibition of PICK1. Again, inhibition of PICK1 did not significantly alter ASIC1 surface expression (Figure 4A) or total ASIC1 expression (Figure 4B) in PASMC. The absence of the cytosolic protein, SM22α, in the biotinylated protein samples demonstrates the specificity of the assay for cell surface versus intracellular proteins (Figure 4C).

**Store Depletion Increases Co-Localization Between ASIC1 and PICK1**

Our initial hypothesis was that PICK1 facilitates ASIC dependent Ca\(^{2+}\) influx in PASMC by promoting surface localization. However, we did not detect changes in plasma membrane localization after PICK1 was inhibited (Figure 4). We next examined the association of ASIC1 and PICK1 upon stimulation of store depletion (SD; Ca\(^{2+}\)-free plus CPA and diltiazem) and SOCE (Ca\(^{2+}\)-repletion plus CPA and diltiazem). Compared to baseline (BL) conditions (data taken from Figure 3 vehicle), Ca\(^{2+}\) store depletion largely increased the number of puncta per cell as well as puncta size (Figure 5), suggesting greater interactions between ASIC1 and PICK1. Replenishing extracellular Ca\(^{2+}\), in the presence of CPA and diltiazem, resulted in a fall in the number of puncta per cell and puncta size (Figure 5). However, the average number of puncta per cell was still elevated compared to baseline.

**PICK1 Inhibits ASIC1-Dependent SOCE**

The functional significance of the interaction between PICK1 and ASIC1 is unknown; therefore, we examined the effect of PICK1 inhibition on ASIC1-dependent SOCE. In contrast to our hypothesis,
inhibition of PICK1 with FSC231 (24 hr incubation) augmented SOCE (Figure 6A). A 30 min incubation with FSC231 produced a similar effect (Figure 6B). Inhibition of ASIC1 with PcTX1 inhibited SOCE and normalized the response between the vehicle and FSC231-treated groups suggesting the augmented SOCE response is ASIC1-dependent (Figure 6B). Similar results were obtained following knockdown of PICK1 with shRNA (Figure 7). Figure 7A shows ~65% knockdown of PICK1 mRNA (Figure 7A) and ~30% knockdown of PICK1 protein (Figure 7B) in PASMC by shRNA. Consistent with pharmacological inhibition of PICK1, shRNA knockdown of PICK1 significantly augmented SOCE (Figure 7C). Together, these findings suggest PICK1 limits ASIC1-dependent SOCE and this is independent of ASIC1 membrane trafficking by PICK1.

**PKC Inhibits SOCE**

In addition to changes in the cellular localization of the channel, PICK1 may alter the activity of ASIC1 by facilitating the interaction with intracellular modulators of the channel (26). PICK1 binds the C terminus of protein kinase C (PKC) through its PDZ domain (42) and can regulate ASIC1. However, the effect of PKC on ASIC1 activity is controversial (3, 4, 50); therefore, we first determined the effect of PKC on SOCE. Activation of PKCα significantly attenuated SOCE, whereas inhibition of PKC augmented SOCE (Figure 8A). It is possible that PICK1 facilitates PKC phosphorylation of ASIC1, thereby inhibiting ASIC1-mediated SOCE. Therefore, we repeated these experiments in the presence of the PICK1 inhibitor, FSC231 (Figure 8B). Although inhibiting PICK1 improved SOCE during PKC activation, PKC still had an inhibitory effect on SOCE in the presence of PICK1 inhibition. PKC activation resulted in ~42% inhibition under vehicle conditions and ~25% inhibition in the presence of FSC231 (Figure 8C). Although PICK1 may facilitate PKC-induced inhibition of SOCE, it is not appear to be a necessary component of this response.

**Calcineurin inhibits SOCE and ASIC1 phosphorylation**
The activity of ASICs can further be regulated by the calcium/calmodulin-activated phosphatase, calcineurin, and PICK1 has been shown to bind calcineurin B (5, 17). Therefore, we examined the role of calcineurin to regulate SOCE and the dependency of PICK1 in this response. We found that the calcineurin inhibitor, cyclosporine A (CsA) augmented SOCE similarly to FSC231 (Figure 9A). There was no further effect of combining the inhibitors suggesting these proteins function together. Moreover, SOCE responses in the presence of FSC231/CsA, were largely reduced by the ASIC1 inhibitor, PcTX1 (Figure 9A). Together, these data suggest PICK1 may facilitate inactivation of ASIC1 via calcineurin-dependent dephosphorylation of the channel.

Consistent with reports in neurons (5), we found that activation of PKA with forskolin augmented SOCE, while inhibition of PKA with KT 5720 decreased SOCE responses in PASMC (Figure 9B). To determine whether PICK1-calcineurin limits SOCE by reversing PKA-dependent ASIC1 phosphorylation, we examined the effect of PKA stimulation on SOCE following combined inhibition of PICK1 and calcineurin. The ability of forskolin to increase SOCE was further potentiated in the presence of PICK1-calcineurin inhibition; while the decrease in SOCE by KT 5720 was not enhanced in the presence of PICK1-calcineurin inhibition (Figure 9B) suggesting inhibition of PKA prevents any further regulation of ASIC1 by PICK1-calcineurin.

Leonard et al., reported that PKA-dependent phosphorylation of Ser-479 in the ASIC1 C-terminus interferes with PICK1 binding (26). Therefore, to examine serine phosphorylation of ASIC1, we treated PASMC with agents to activate or inhibit PKA, PICK1, and calcineurin. ASIC1 was immunoprecipitated from PASMC lysates and probed for phosphoserine by western blot. Consistent with other studies showing ASIC1 is a PKA substrate (26), we observed basal levels of ASIC1 phosphorylation in PASMC that are significantly enhanced by forskolin and diminished KT 5720 (Figure 10). Both FSC231 and CsA largely augmented ASIC1 serine phosphorylation (Figure 10), suggesting both PICK1 and calcineurin play a role in dephosphorylating ASIC1.
DISCUSSION

Our overall goal of this study was to determine the contribution of the scaffolding protein PICK1 to the regulation of both ASIC1 plasma membrane localization and ASIC1-mediated Ca\(^{2+}\) influx in PASMC. Our results demonstrate that PASMC express PICK1, which co-localizes with ASIC1. Inhibition of PICK1 diminished the interaction with ASIC1, but did not limit ASIC1 plasma membrane localization. Intracellular Ca\(^{2+}\) store depletion increased the interaction between ASIC1 and PICK1; however, studies using both pharmacological inhibition of PICK1 and shRNA knockdown of PICK1 suggest that PICK1 imparts a basal inhibitory effect on ASIC1 Ca\(^{2+}\) entry in PASMC. These data argue against our original hypothesis that PICK1 facilitates ASIC1 dependent Ca\(^{2+}\) influx in PASMC by promoting plasma membrane localization, but rather suggest that PICK1 negatively regulates ASIC1-mediated SOCE. Further investigation uncovered a role for PICK1 to facilitate calcineurin-dependent dephosphorylation of ASIC1. Moreover, it appears PICK1/calcineurin-mediated regulation of SOCE opposes PKA phosphorylation and activation of ASIC1 (Figure 10). Together these data demonstrate the ability of PICK1 to regulate PASMC signal transduction by providing a scaffold for multiple signaling proteins important to overall Ca\(^{2+}\) homeostasis in PASMC.

PDZ domains are well-characterized protein-protein interaction modules that play an essential role in a number of cellular processes by facilitating protein scaffolding and assembly of protein complexes. These supramolecular signaling complexes mediate signaling and regulatory cascades that can either promote or inhibit the activation of certain proteins; and thus can regulate channel activity, trafficking, and localization. PDZ domains generally consist of 80 to 100 amino acids and can be divided into three basic types based on the short C-terminal sequence it recognizes on the target proteins (51). ASIC1 and ASIC2 C-termini share homology with type-II PDZ binding motifs and bind PICK1 (9, 15). This interaction with PICK1 is known to change the subcellular clustering of ASIC1. Indeed, we observed less clustering (smaller puncta size) in PASMC in the presence of the PICK1 PDZ-domain inhibitor. Although
the interaction between PICK1 and ASIC1 is well-characterized, the functional significance of this interaction is not well understood.

PICK1 regulates the trafficking of its binding partner by altering either its subcellular targeting and/or surface expression. Both the PDZ and lipid binding BAR domains are essential for vesicular formation and protein trafficking (23, 24, 35). Moreover, PICK1 is associated with both promoting and prohibiting membrane localization of various proteins (6, 27, 34, 44, 48, 49). In terms of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), which has been the most widely studied, PICK1 promotes the intracellular retention and prolonged reinsertion of membrane proteins upon internalization (6, 27). With regards to ASIC1, PICK1 has been shown to increase ASIC1 surface levels in heterologous expressing HEK293T cells (24). However, compared to ASIC1 expression alone, co-expression of PICK1 resulted in a marginal (~2-3%) increase in ASIC1 surface levels suggesting the majority of ASIC1 trafficking to the membrane may be PICK1 independent (24). Although we did not detect an effect of PICK1 to regulate ASIC1 membrane localization in naive PASMC, we cannot rule out a role for PICK1 in regulating subcellular trafficking of ASIC1. Further investigation will determine the mechanisms involved in ASIC1 membrane trafficking, but likely involve other trafficking proteins. The current finding regarding PICK1 suppression of SOCE suggests that PICK1 binding to ASIC1 is associated with phosphorylation rather than changes in the cellular localization of the channel.

Although PICK1 potentiates the activity of ASIC2a (2, 7); the influence of PICK1 on the activity of ASIC1 is less clear. Whether PICK1 inhibition of SOCE observed in the present study is a result of direct ASIC1 interaction or a broader regulation of the SOCE complex is unknown. The scaffolding properties of PICK1 enable macromolecular complexes to form thereby assimilating molecules that might have important functions to regulate store-operated channels. PICK1 binds the C terminus of protein kinase C (PKC) through its PDZ domain (42) and can regulate ASICs. The effect of PKC on ASIC1 has been shown to be either stimulatory or inhibitory, depending on the PKC isoform and phosphorylation site (3, 4, 16, 50).
Consistent with previous data from our laboratory in isolated pulmonary arteries and pulmonary arterial endothelial sheets (32, 39), activation of PKC suppressed SOCE in PASMC. These data are in line with reports that PKC activation reduces current amplitude of human ASIC1 in glioma cells and *Xenopus* oocytes (3, 4). Although PICK1 may contribute to PKC-mediated inhibition of SOCE; it does not appear to be required, since inhibition of PICK1 did not prevent PKC-mediated attenuation of SOCE. Therefore, other PICK1-dependent mechanisms are likely involved in the regulation of ASIC1-mediated SOCE.

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 ASIC1 (5). PICK1 binds calcineurin B (17), and calcineurin-dependent dephosphorylation has been shown to be involved in the inactivation of ASICs in neuronal cells (5). Consistent with this observation, our current study showed that inhibition of calcineurin with cyclosporin A augments SOCE and increases ASIC1 serine phosphorylation similarly to FSC231 in PASMC. A recent study demonstrates calcineurin plays a role in regulation of SOCE in pulmonary artery endothelial cells; however, in contrast to our study the calcineurin inhibitory peptide increases the phosphorylation of the endoplasmic reticulum Ca\(^{2+}\) sensor, STIM1, and decreases SOCE (45). The reason for this discrepancy is currently unclear.

The ability of PICK1 and calcineurin to suppress SOCE in PASMC suggests they may be involved in a feedback mechanism that limits excessive SOCE. Furthermore, this association between ASIC1 and PICK1 appears to be largely influenced by levels of [Ca\(^{2+}\)], since Ca\(^{2+}\) store depletion resulted in a dramatic increase in co-localization of PICK1 and ASIC1. PICK1 contains two acidic stretches adjacent to both the N- and C-terminus that bind Ca\(^{2+}\) and can function as potential calcium sensors (13). Therefore, it is possible Ca\(^{2+}\) plays a significant role in regulating the functional interaction between ASIC1 and PICK1 and calcineurin. Although we currently do not know the significance of this interaction, it is additionally possible that Ca\(^{2+}\) entry through ASIC1 directly activates calcineurin. Several studies provide
evidence that Ca^{2+} store depletion triggers the dynamic recruitment of AKAP79/calcineurin to activated store-operated channel complexes (29, 37, 40, 41). This is an area that we are currently investigating in more detail.

Consistent with other reports, we also show ASIC1 is a substrate for PKA (5, 26). Concerning the role of PKA in ASIC1 function, there are conflicting data. Chai et al. show that anchoring of PKA in AKAP150 is required for the full activation of ASICs in neurons and CHO cells (5); whereas Leonard et al. report that neither activation of PKA with forskolin nor inhibition with KT 5720 altered acid-evoked currents in hippocampal neurons even though the drugs increased and decreased ASIC1 phosphorylation, respectively (26). Interestingly, Leonard et al., demonstrated that PKA-mediated phosphorylation of ASIC1 reduces ASIC1 colocalization with PICK1 and clustering of ASIC1 (26). Consistent with this, we observed that inhibition of PICK1 increased ASIC1 serine phosphorylation. Based on our current findings and those of others, it would seem possible that inhibition of PICK1 increases PKA phosphorylation and activity of ASIC1 by preventing calcineurin-dependent dephosphorylation in PASMC.

In the pulmonary circulation, ASIC1 contributes to agonist-induced vasoconstriction and increases in pulmonary vascular smooth muscle [Ca^{2+}] (21, 31). It therefore seems somewhat counterintuitive that PKA, a known vascular mediator of vasodilation, stimulates SOCE in PASMC. However, SOCE is the central mechanism involved in replenishing the level of Ca^{2+} in the sarcoplasmic reticulum (SR) and PKA is a prominent regulator of the SR Ca^{2+}-cycling proteins. When phospholamban is phosphorylated by PKA, its ability to inhibit the SR calcium ATPase (SERCA) is lost, leading to rapid increases in SR Ca^{2+} load (38). Although this will decrease [Ca^{2+}], and lead to smooth muscle relaxation, increasing the Ca^{2+} load of the SR will also lead to faster turn around and increased release of Ca^{2+} from the stores upon subsequent and/or continuous activation. Indeed, PKA increases Ca^{2+}-spark frequency mediated by SR ryanodine receptor channels (RyR) (18, 30, 47). It is possible that PKA not only regulates
SR Ca\textsuperscript{2+} through SERCA and RyR channels, but also by activating the store-operated channels responsible for replenishing the SR. PKA has recently been shown to activate the heteromeric Orai1/Orai3 channel, through phosphorylation of STIM1 in HEK-293 cells (43). These heteromeric Orai channels are arachidonic acid-regulated calcium-selective (ARC), and in contrast to the related homomeric Orai1 channel (CRAC channel), their activation can be store-independent (28). Together these studies suggest the potential for PKA to be a fundamental modulator of agonist-induced Ca\textsuperscript{2+} entry and the subsequent downstream cellular responses in a variety of cell types. Further investigation is needed to determine the functional relevance of PKA versus PICK1/calcineurin in regulating ASIC1-mediated SOCE and how these modulatory complexes facilitate either SR Ca\textsuperscript{2+} load or downstream Ca\textsuperscript{2+} signaling.

In summary, this study demonstrates a regulatory interaction between ASIC1 and PICK1 in PASMC. More specifically, we found that PICK1 binding to ASIC1 is associated with changes in the phosphorylation state of the channel, rather than plasma membrane localization. PKA phosphorylates ASIC1 and stimulates channel activity (Figure 10). This activation of ASIC1 can be counteracted by PICK1/calcineurin-mediated dephosphorylation of ASIC1. PICK1 suppression of SOCE suggests that PICK1 may be involved in a Ca\textsuperscript{2+}-sensitive feedback mechanism that limits excessive SOCE. However, we cannot presently rule out the ability of PICK1 to facilitate downstream signaling by providing the scaffold for the store-operated complex. The regulatory role of PICK1 on SOCE suggests PICK1 is an important determinant of overall Ca\textsuperscript{2+} homeostasis in PASMC and that loss of PICK1 could potentially result in the dysregulation of Ca\textsuperscript{2+} handling observed in many disease states.
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No conflicts of interest, financial or otherwise, are declared by the author(s).
REFERENCES


**FIGURE LEGENDS**

**Figure 1.** PICK1 is expressed in rat pulmonary arterial smooth muscle cells (PASMC). A) PCR analysis of PICK1 (top panel, 201 bp) and β-actin (bottom panel, 244 bp) mRNA expression in PASMC and rat brain tissue as a positive control. B) Immunoblot showing protein expression of PICK1 (~52 kDa) in PASMC. C) Immunofluorescence in PASMC showing smooth muscle α (SM22α; red), PICK1 (green) and the nuclear dye, Sytox (blue).

**Figure 2.** Co-localization of PICK1 and ASIC1 in PASMC. A) Representative confocal images of the Duolink PLA interaction between goat anti-ASIC1 and rabbit anti-PICK1 (red puncta). For negative controls, PASMC were incubated with each primary alone (B-C) and both PLA probes. Actin is labeled with Alexa Fluor 647 phalloidin (blue) and the nuclei are labeled with SyTOX (green). Zoomed Duolink (D) and traditional co-localization (E) images showing ASIC1-PICK1 localize at the cell edge. Endogenous PICK1 and ASIC1 were immunoprecipitated from PASMC lysates with anti-ASIC1 and anti-PICK1-labeled beads. Co-immunoprecipitated ASIC1 and PICK1 were detected by western blotting using either anti-PICK1 (F) or anti-ASIC1 (G) antibodies.

**Figure 3.** Inhibition of PICK1 diminishes co-localization and clustering with ASIC1. Representative confocal images (A) of the Duolink interaction between goat anti-ASIC1 and rabbit anti-PICK1 (A; red puncta) in the absence or presence of the PICK1 inhibitor, FSC231 (50 µM for 30 min). Actin is labeled with Alexa Fluor 647 Phalloidin (blue) and the nuclei are labeled with SyTOX (green). Summary data for average number of puncta per cell (B) and puncta size (C). Values are means ± SE; n = 9-11 images from 5 separate experiments/group; *p < 0.05 vs. vehicle; analyzed by unpaired t-test.

**Figure 4.** PICK1 does not alter ASIC1 plasma membrane localization in PASMC. Representative western blots (A and B) and summary data (D) showing the ratio of cell surface biotinylated (A) to total (B) ASIC1 expression following 24 hr treatment with the PICK1 inhibitor, FSC231 (50 µM). To verify specificity of biotin labeling for cell surface proteins, a separate blot of cell surface biotinylated proteins was probed for smooth muscle 22α (SM22α; 23kDa; C). The blot also contains a total protein sample (+) as a positive control. Values are means ± SE; n = 6/group; analyzed by unpaired t-test.

**Figure 5.** Store depletion increases the interaction and clustering between PICK1 and ASIC1. Representative confocal images (A) of the Duolink interaction between goat anti-ASIC1 and rabbit anti-PICK1 (A; red puncta) upon stimulation of store depletion (SD; Ca^{2+}-free plus CPA and diltiazem) and SOCE (Ca^{2+}-repletion plus CPA and diltiazem). Actin is labeled with Alexa Fluor 647 Phalloidin (blue) and
the nuclei are labeled with SyTOX (green). Summary data for average number of puncta per cell (B) and
puncta size (C). Baseline values are the same data from Figure 3 vehicle. Values are means ± SE; n = 3-5
images from 7 separate experiments/group; *p < 0.05 vs. baseline, # p < 0.05 vs store depletion (SD),
analyzed with one-way ANOVA and individual groups compared with the Student-Newman-Keuls test.

**Figure 6.** PICK1 inhibits ASIC1-dependent SOCE. A) SOCE responses as determined by area under the
curve (AUC) for PASMC pretreated with increasing concentrations of the PICK1 inhibitor, FSC231 (10-100
µM) for 24 hrs. Values are means ± SE; n = 4-6/group; *p < 0.05 vs. vehicle; analyzed with one-way
ANOVA and individual groups compared with the Student-Newman-Keuls test. B) SOCE responses (AUC)
for PASMC pretreated for 30 min with FSC231 (50 µM) with or without pretreatment with the ASIC1
inhibitor, psalmotoxin 1 (PcTX1; 20 nM). Values are means ± SE; n = 4-6/group; *p < 0.05 vs. vehicle ; # p
< 0.05 vs. control; analyzed with two-way ANOVA and individual groups compared with the Student-
Newman-Keuls test.

**Figure 7.** PICK1 inhibits SOCE. Fold change in PICK1 mRNA (A) and protein (B) expression in PASMC
following treatment with PICK1 shRNA compared to the negative (NEG) control shRNA. Values are
means ± SE; n = 4/group; *p < 0.05 vs. NEG shRNA; analyzed by one sample t-test. C) SOCE responses as
determined by area under the curve (AUC) in PASMC treated with NEG or PICK1 shRNA. Values are
means ± SE; n = 8-10/group; *p < 0.05 vs. NEG shRNA; analyzed by unpaired t-test.

**Figure 8.** PKC inhibits SOCE. SOCE responses as determined by area under the curve (AUC) for PASMC
pretreated with A) the PKC Activator VII, CGK062 (30 µM) or myristoylated-PKC inhibitor (myr-PKC, 10
µM). Responses in the presence of CGK062 were additionally assessed in the presence of FSC231 (B) and
the percent PKC-induced inhibition of SOCE under vehicle and FSC231 treatments was determined (C).
Values are means ± SE; n = 5-6/group; *p < 0.05 vs. vehicle; # p < 0.05 vs non-CGK062; analyzed with t-
test (C) one-way (A) or two-way (B) ANOVA and individual groups compared with the Student-Newman-
Keuls test.

**Figure 9.** PICK1/Calcineurin inhibit PKA-stimulated SOCE. SOCE responses were determined by area
under the curve (AUC) in PASMC pretreated with the PICK1 inhibitor, FSC231 (50 µM); calcineurin
inhibitor, cyclosporin A (CsA; 1 µM); ASIC1 inhibitor, psalmotoxin 1 (PcTX1; 20 nM); PKA activator,
forskolin (10 µM); or PKA inhibitor, KT 5720 (300 nM) as indicated on graphs. Values are means ± SE; n =
4-6/group; A) *p < 0.05 vs. vehicle; # p < 0.05 vs. FSC231/CsA, analyzed with one-way ANOVA and
individual groups compared with the Student-Newman-Keuls test. B) *p < 0.05 vs. non-FSC231/CsA
treatment and # p < 0.05 vs. non-forskolin or -KT 5720 treatment; analyzed with one-way (A) or two-way (B) ANOVA and individual groups compared with the Student-Newman-Keuls test.

**Figure 10.** PICK1 and Calcineurin Inhibit ASIC1 phosphorylation. PASMC were pretreated with vehicle; PKA activator, forskolin (10 µM); PKA inhibitor, KT 5720 (300 nM); PICK1 inhibitor, FSC231 (50 µM); or calcineurin inhibitor, cyclosporin A (CsA; 1 µM). Endogenous ASIC1 was immunoprecipitated from PASMC lysates with anti-ASIC1-labeled beads. Phosphorylation of ASIC1 was detected by western blotting using anti-phosphoserine antibody (A). PASMC lysates were additionally probed for total ASIC1 (B). Summary data showing the fold change in ratio of co-IP ASIC1-phosphoserine to total ASIC1 relative to vehicle-treated samples (dotted line; C). Values are means ± SE; n = 3/group; *p < 0.05 vs. vehicle, analyzed with one-way ANOVA and individual groups compared with the Student-Newman-Keuls test.

**Figure 11.** Summary of major findings. Ca²⁺ depletion of the sarcoplasmic reticulum (SR) stimulates ASIC1-dependent store-operated Ca²⁺ entry (SOCE; solid line) in pulmonary arterial smooth muscle cells (PASMC). ASIC1-mediated SOCE is augmented by PKA-mediated phosphorylation and inhibited by PKC-mediated phosphorylation. Store depletion also increases the association between ASIC1 and PICK1 which limits SOCE by facilitating calcineurin (Cn)-dependent dephosphorylation of ASIC1.
FIGURE 1

A. 

<table>
<thead>
<tr>
<th>bp</th>
<th>PASMC</th>
<th>brain</th>
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<tbody>
<tr>
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PICK1

β-actin

B. 

PASMC

150 -- 100 -- 50 -- 25 -- 15 --

PICK1

C.

SM22α (red) 
PICK1 (green) 
Sytox (blue) 
merged

25 µm
FIGURE 2

A. PICK1/ASIC1

B. ASIC1

C. PICK1

D. 25 µm

E. 10 µm

F. IP Ab: PICK1

G. IP Ab: ASIC1

IP Ab: PICK1

WB: PICK1 ~52 kDa

IP Ab: ASIC1

WB: ASIC1 ~65 kDa
FIGURE 3

A. Vehicle

B. Average Puncta Per Cell

C. Average Puncta Size (pixels^2)

Veh FSC

Average Puncta Size (pixels^2)

0.00
0.05
0.10
0.15
0.20
0.25

* NS

p = 0.063

Veh FSC

0 10 20 30 40 50 60 70

Average Puncta Per Cell

0 10 20 30 40 50

Average Puncta Size (pixels^2)

0.00 0.05 0.10 0.15 0.20 0.25

*
FIGURE 4

A. VEHICLE | FSC231

B. VEHICLE | FSC231

C. VEHICLE | FSC231

D. NS

$\alpha = 0.23$
A. baseline  
store depletion  
SOCE

B. Average Puncta Per Cell

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C. Average Puncta Size (pixels$^2$)

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**FIGURE 6**

A. Bar graph showing the effect of different concentrations of FSC231 on SOCE (AUC). The x-axis represents the concentration of FSC231 in µM, and the y-axis represents the SOCE (AUC). The concentrations tested are Vehicle, 10, 50, and 100 µM. The graph indicates a significant increase in SOCE (AUC) with increasing concentrations of FSC231, as indicated by the asterisks (*) above the bars. The NS (not significant) line represents the control group.

B. Bar graph comparing the SOCE (AUC) between Control and PcTX1. The bars represent Vehicle and FSC231 treatments. The asterisks (*) indicate a significant difference between the two groups, while the hash marks (#) indicate a significant difference within the PcTX1 group.
FIGURE 7

A. 
Fold Change in PICK1 mRNA

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Fold Change in PICK1 protein

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C. 
SOCE (AUC)

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FIGURE 8

A. B. C.
FIGURE 9

A. SOCE (AUC)

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<tr>
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B. SOCE (AUC)

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