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ASIC PROTEINS REGULATE SMOOTH MUSCLE CELL MIGRATION

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Abstract

The purpose of the present study was to investigate Acid Sensing Ion Channel (ASIC) protein expression and importance in cellular migration. We recently demonstrated Epithelial Na⁺ Channel (ENaC) proteins are required for vascular smooth muscle cell (VSMC) migration, however the role of the closely related ASIC proteins has not been addressed. We used RT-PCR and immunolabeling to determine expression of ASIC1, ASIC2, ASIC3 and ASIC4 in A10 cells. We used small interference RNA to silence individual ASIC expression and determine the importance of ASIC proteins in wound healing and chemotaxis (PDGF-bb) initiated migration. We found ASIC1, ASIC2, and ASIC3, but not ASIC4, expression in A10 cells. ASIC1, ASIC2, and ASIC3 siRNA molecules significantly suppressed expression of their respective proteins compared to non-targeting siRNA (RISC) transfected controls by 63%, 44%, and 55%, respectively. Wound healing was inhibited by 10, 20 and 26% compared to RISC controls following suppression of ASIC1, ASIC2, and ASIC3, respectively. Chemotactic migration was inhibited by 30% and 45%, respectively following suppression of ASIC1 and ASIC3. ASIC2 suppression produced a small, but significant, increase in chemotactic migration (4%). Our data indicate ASIC expression is required for normal migration and may suggest a novel role for ASIC proteins in cellular migration.

Keywords

vascular smooth muscle cell migration; ion channel; degenerin proteins; wound healing; interference RNA

INTRODUCTION

Cellular migration contributes to many biological processes including embryogenesis, metastasis and inflammation (Dormann and Weijer, 2003; Jones, 2000; Kassis et al., 2001). In the cardiovascular system, migration of vascular smooth muscle cells, from the media into the intima, contributes to tissue repair following vascular injury induced by hypertension, smoking, diabetes or hyperlipidemia (Libby et al., 1988; Ross, 1993; Rutherford et al., 1997). Ion transport plays a prominent role in the migratory response. Activation of transporters and ion channels facilitates changes in cell volume, cytoskeleton reorganization, and cellular

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polarization that take place during cell migration (Dormann and Weijer, 2003; Schwab, 2001a; Schwab, 2001b).

Recently, others and we have shown members of the Degenerin/Epithelial Sodium (Na^+) Channel/Acid Sensing-Ion Channel (DEG/ENaC/ASIC) protein family, contribute to cellular migration in glial, epithelial and VSM cells (Chifflet et al., 2005; Grifoni et al., 2006; Vila-Carriles et al., 2006). Members of this family share a similar structure, two membrane spanning domains separated by a large extracellular domain and intracellular N- and C-termini. Many members of this family form cation selective ion channels. Vila-Carriles et al have shown ASIC proteins are essential in glial migration (Vila-Carriles et al., 2006). While previous findings from our lab have shown ENaC proteins are required for VSMC migration, the role of ASIC proteins has not been determined. At least four different ASIC genes have been identified, named ASIC1 – 4. In rodents, ASIC1 and ASIC2 encode for two splice variants identified by the suffix a or b, with the a variant more widely studied (Akopian et al., 2000; Chen et al., 1998; Grunder et al., 2000; Lingueglia et al., 1997; Waldmann et al., 1997a; Waldmann et al., 1997b; Waldmann et al., 1996). ASIC proteins interact to form homomeric as well as heteromeric cation channels that are proton gated and amiloride blockable (150 nM – 100 μM) (Bassilana et al., 1997; Champigny et al., 1998; Lingueglia et al., 1997; Mano and Driscoll, 1999; Price et al., 2000; Price et al., 2001).

Previous reports have suggested that ASIC protein expression is limited to neuronal cells and sensory epithelial (Akopian et al., 2000; Chen et al., 1998; Grunder et al., 2000; Kellenberger and Schild, 2002; Lingueglia et al., 1997; Waldmann et al., 1997a; Waldmann et al., 1997b; Waldmann et al., 1996). However, the recent reports demonstrating expression of ASIC1a, ASIC2a, ASIC3 and ASIC4 in glial cells raised the possibility that ASIC proteins may also be expressed in other tissues (Berdiev et al., 2003; Bubien et al., 1999; Vila-Carriles et al., 2006). In our previous report, we demonstrated a significant portion of VSMC migratory responses were sensitive to amiloride/benzamil concentrations greater than 1 μM , a sensitivity range compatible with ASICs (Grifoni et al., 2006). These two findings raised the possibility that ASIC proteins may also be involved in VSMC migration.

Therefore, the main goal of this study was to determine the importance of ASIC proteins in VSMC migration. Data presented in this study demonstrate ASIC transcripts and proteins are expressed in VSMCs. Furthermore, siRNA mediated gene silencing demonstrates ASICs are required for normal chemotactic (PDGF-bb) and wound-healing VSMC migration. Results from this current study augment the body of evidence from our lab that suggests DEG/ENaC/ASIC proteins contribute to processes underlying VSMC migration.

EXPERIMENTAL PROCEDURES

Cell Culture

A10 cells (ATCC; Manassas, VA) were grown at 37°C, 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Cells were regularly passaged to maintain exponential growth. Studies were performed with cells at passage 1 to 10.

Reverse Transcriptase-Polymerase Chain Reaction

To determine if ASIC transcripts are expressed in VSMCs, we used reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from cultured A10 cells using the RNA STAT-60™ kit (Tel-Test Inc, Friendswood, TX), DNase treated using TURBO DNA-free protocol (Ambion, Austin, TX), and reverse transcribed using oligo-dT primers and AMV reverse transcriptase (Promega, Madison, WI). Primer sequences and predicted product size

are listed in Table 1. All reactions were preheated on Robocycler (Stratagene, LaJolla, CA) to 94°C for 3 min, then cycled 40 times at 94°C for 30 sec, annealed for 30 sec at 55°C, then 72°C for 1 min. PCR reactions in which reverse transcriptase was not added to RNA served as a negative control. PCR products were separated using gel electrophoresis, visualized with ethidium bromide, and sequenced to confirm identity.

Western Blotting

Cultured A10 cells were plated on T75 flasks and grown to 70% confluence. Cells were lysed by scraping into 500 µl of 2X Laemmli sample buffer and incubated at room temperature for 20 minutes. Cell lysates (40 µl) were separated using standard electrophoresis procedures as described (Grifoni et al., 2006). After transferring to nitrocellulose, blots were rinsed in PBS, blocked in Odyssey blocking solution then incubated with rabbit anti-ASIC1a (1:500, Chemicon), rabbit anti-ASIC2a (1:500, Chemicon), or guinea pig anti-ASIC3 antibodies (1:500, Chemicon). The antibodies for ASIC1 and ASIC2 recognize the “a” splice variant. We did not blot for ASIC4 since we could not detect ASIC4 message by RT-PCR. The membrane was cut just below the 50 kDa marker and probed with mouse anti β-Actin (1:5000, Abcam) as a loading control. To assess antibody specificity, blots were probed with ASIC antibodies were pre-incubated with their corresponding antigen at (1:10). Antibody binding was visualized incubating with IR700 conjugated donkey anti-rabbit IgG and IR800 conjugated donkey anti mouse IgG (1:2000, Rockland Immunologicals). Blots were scanned using the Odyssey Infrared Scanner. Blots incubated with antibody alone and antigen + antibody were scanned side-by-side under identical conditions.

Immunofluorescence Cell Staining

Cultured cells were plated on fibronectin coated glass slides, grown for 24 hours, rinsed with phosphate buffer solution (PBS), then fixed in 4% paraformaldehyde for 10 minutes. After fixation, samples were rinsed with PBS, and then blocked with 5% normal donkey serum (NDS) in PBS for 1 hour. Rabbit anti-ASIC1a (1:100, Chemicon, Temecula CA), rabbit anti-ASIC2a (1:100, Chemicon), and rabbit anti-ASIC3 antibodies (1:100, ADI, San Antonio, TX) were used for immunostaining. We did not immunolabel for ASIC4 since we could not detect ASIC4 message by RT-PCR. All samples were co-labeled with mouse anti-α smooth muscle actin (1:200, Sigma Chemicals, St. Louis MO) or C12-18 NBD Ceramide (Molecular Probes). Samples were incubated with primary antibodies plus 5% NDS in PBS overnight at 4°C. The following day, the samples were rinsed with PBS then exposed to secondary antibody [Alexa 488 conjugated donkey anti-mouse IgG (1:1000, Molecular Probes, Eugene OR), Cy-3 conjugated donkey anti-rabbit F(ab')₂ (1:100, Jackson Immunologicals, West Grove PA) or Cy-5 conjugated donkey anti-rabbit F(ab')₂ (1:100)] in 5% NDS for 1 hour. As a negative control, samples are treated as above, except the primary antibody was pre-incubated overnight at 4°C with excess antigenic peptide (Ag, 10 µg/ml). Samples were examined using a fluorescence confocal microscope (TCS-SP2, Leica Microsystems, Exton, PA) and images were prepared in PhotoShop (Adobe Systems, San Jose, CA).

We used double staining to determine if ASIC proteins co-localize. To determine if ASIC1a/ASIC3, and ASIC2a/ASIC3, samples were co-stained with rabbit anti ASIC1a or ASIC2a and ASIC3 guinea pig antibodies and visualized with Cy3 donkey anti-rabbit F(ab')₂ and Cy5 donkey anti-guinea pig F(ab')₂. Since both ASIC1a and ASIC2a antibodies were raised in rabbits, we used “species conversion” of the ASIC1a antibody to determine if ASIC1a and 2a co-localize. For these experiments, samples were labeled with rabbit anti-ASIC1a as described previously. The rabbit anti-ASIC1a antibody was “converted” to a goat antibody by incubation with goat anti-rabbit Fab (monovalent, 1 mg/ml) for 1 hour at 4°C. Samples were then incubated with rabbit anti-ASIC2a and antibody labeling was visualized with Alexa-488 labeled donkey anti-goat IgG and Cy3 labeled donkey anti-rabbit F(ab')₂. To test for complete conversion, the

second rabbit primary antibody was omitted in some samples. In these controls, no staining was observed with the Alexa-488 labeled donkey anti-goat secondary antibody, indicating a complete species conversion from rabbit to goat. The degree of co-localization was quantitated using Leica Confocal software. Regions of interest (ROI) were drawn to include only perinuclear or cytoplasmic regions.

siRNA

To determine if individual ASIC proteins contribute to VSMC migration, we used siRNA to suppress expression. We have used this approach previously to silence ENaC/ASIC expression (Drummond et al., 2006; Grifoni et al., 2006; Jernigan and Drummond, 2006). For migration assays, VSMCs were plated and allowed to grow to 90% confluence before transfection with siRNA molecules. Cells were transfected using Lipofectamine²⁰⁰⁰ (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Validated siRNA molecules, directed to ASIC1, ASIC2, and ASIC3 (ASIC1; ID # 52768, ASIC2; ID# 197232, and ASIC3; ID# 47195), were obtained from Ambion (Ambion, Austin, TX). As a negative control, we used a nontargeting siRNA control molecule that activates the RNA Induced Silencing Complex (RISC, cat.# D 001210-02, Dharmacon, Lafayette, CO). Following a 4-hour incubation, cultures were supplemented with growth media for 72 hours prior to study.

To determine extent of siRNA suppression ASIC expression, we used quantitative immunostaining, an approach that we have used previously (Drummond et al., 2006; Grifoni et al., 2006; Jernigan and Drummond, 2006). For immunostaining experiments, cells were plated on fibronectin coated glass slides, treated with siRNA molecules for 4 hours, grown 72 hours, and then prepared for ASIC immunolabeling as described before. All samples to be compared were treated identically. Using a fluorescence confocal microscope (Leica TCS SP2), images of 5 – 10 randomly chosen fields of view were obtained for each condition. Fluorescence intensity was calculated using Leica software and was normalized for cell area, background subtracted (secondary antibody only signal), and presented as relative fluorescence units (RFU/ μm^2). Values were obtained in 20–68 cells per treatment group. Samples were examined using a fluorescence confocal microscope (TCS-SP2, Leica Microsystems, Exton, PA) and images prepared in Photoshop (Adobe Systems, San Jose, CA).

Wound Healing Assay

We used two assays to evaluate migration. In the first assay, we used a wound healing, or scratch assay. For this assay, A10 cells were seeded in 24-well plates at a concentration of 30×10^4 cells/well, following trypsinization and counting using a standard grid assay. After plating, cells were grown to approximately 90% confluence and transiently transfected with siRNA molecules and maintained for 48 hours before the experiments. To omplex minimize proliferation and enhance contribution of migration to wound healing, VSMC monolayers were serum starved (0.4% FBS) 24 hours before wounding. Monolayers were manually scraped with a 200 μL pipette tip then gently washed twice with phosphate buffer solution (PBS) to remove non-adherent cells. Images of the wounded area were captured immediately after (time zero), 8 and at 24 hours after injury. Images were collected with a Nikon Eclipse TE200 microscope equipped with a Photometrics CoolSnap CCD camera (Roper Scientific, Trenton, NJ). A grid attached to the bottom of the cell culture plate was used as a reference point to capture images of the same location at each time interval. The wounded area was determined using MetaMorph software (Universal Imaging, Downingtown, PA). Healing was quantified as the percent of initial wound area that had been reinvaded with VSMCs, termed % reinvansion. The formula for determining % reinvansion follows: % Reinvansion = $(\text{Area}_I - \text{Area}_T) / \text{Area}_I \times 100\%$, where: Area_I = Initial area, and Area_T = Area at time (T) 8 or 24 hours after injury.

Chemotactic Migration Assay

The second assay evaluated A10 cell migration in response to a chemotactic stimulus using modified Boyden chambers (Costar® Transwell inserts, 6.5 mm diameter, 8.0 µm pore size). Confluent VSMC monolayers were serum starved (0.4% FBS) for 24 hours. The VSMC monolayers were rinsed with filtered PBS, detached by trypsin (3 ml per T75 flask, 5 min, 37° C), and reconstituted to 3.0×10^5 cells/mL in 0.4% FBS media. Cells were counted using a standard grid assay and plated at 3.0×10^4 cells/well in 100 µL of 0.4% FBS media. The chemoattractant Platelet Derived Growth Factor - bb (PDGF-bb, 0.05 µg/mL; RDI, Flanders NJ) was added to the lower well of the Boyden chamber to stimulate migration and the cells were incubated for 4 hours at 37° C, 5% CO₂. Following migration, inserts were rinsed with PBS and unmigrated cells on the upper surface of the insert were removed with a cotton swab. Migrated VSMCs attached to the bottom surface were fixed by treatment with -20°C methanol for 10 min and rinsed twice with PBS. Hematoxylin stain was used to visualize the cells. Inserts were examined on a Nikon Eclipse 200 inverted microscope with a 20X objective. PDGF-bb-stimulated migration was quantified as the average number of cells identified from 4 fields of view per insert. All samples were run in triplicate and each experiment was performed at least two times. In order to determine if ASIC proteins play a role on random migration, we also studied migratory responses in absence of the chemoattractant PDGF-bb. Since the absolute number of migrated VSMCs varied from experiment to experiment, the data were normalized as percent of RISC transfected control samples.

Cell Adhesion Assay

To determine if individual ASIC proteins contribute to cell adhesion, we examined cell adhesion using Innocyte™ ECM Cell Adhesion Assay, Fibronectin (Calbiochem, San Diego, CA) according to the manufacturer's instructions. At 72 hours after transfection with the siRNA molecules, the VSMC monolayers were rinsed with filtered PBS, detached by trypsin/EDTA, and resuspended in serum-free media. Cells were plated at a concentration of 2.5×10^4 cells/well, and allowed to adhere for 2 hours at 37° C in a cell culture incubator (5% CO₂). The cells were then gently washed twice with PBS and the cell attachment was quantified with the green fluorescent dye calcein-AM using a fluorescence plate reader at excitation wavelength 485 nm and emission wavelength 520 nm. All samples were run in duplicate and the data are presented as relative fluorescence units (RFU).

Statistical Analysis

All data are expressed as mean ± SEM. Groups were compared using analysis of variance (ANOVA) followed by Student-Newman-Keuls post-test. Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

ASIC expression in cultured VSMCs

To determine if ASIC transcripts and proteins are expressed in cultured VSMCs, we used RT-PCR, immunoblotting, and labeling. RT-PCR analysis detected transcript expression of ASIC1a, ASIC2a, and ASIC3, but not ASIC4 in cultured VSMCs. Brain RNA and the absence of reverse transcriptase served as positive and negative controls, respectively (Fig. 1). We used western blotting to determine if VSMCs express ASIC proteins and immunolabeling to determine ASIC localization. As shown in Figure 2, ASIC1, ASIC2 and ASIC3 were detected near the predicted molecular weights (70–100 kDa). Antibody binding was blocked or inhibited by pre-incubation with excess antigen. Punctate cytoplasmic expression of ASIC1, ASIC2, and ASIC3 proteins was detected. In addition, we observed a strong perinuclear staining in most cells, suggestive of endoplasmic reticulum or Golgi organelles (Fig. 3). The perinuclear

staining pattern was typically most intense and consistent for ASIC1, followed by ASIC2 and rarely observed for ASIC3. Although the staining patterns of the individual ASIC proteins appeared similar, results from double-labeling experiments, shown in Figure 4, revealed very little co-localization among any of the ASIC proteins in cytoplasmic regions (1–2%) and moderate co-localization in the perinuclear regions (20%).

siRNA inhibits ASIC expression

To determine if individual ASIC proteins contribute to VSMC migration and cell adhesion, we used ASIC specific siRNA molecules to silence ASIC expression. Semi-quantitative immunostaining was used to confirm the efficacy of siRNA approach. As shown in Figure 5, ASIC1, ASIC2, and ASIC3 siRNA molecules significantly suppress expression of their respective proteins when compared to RISC transfected controls by 63%, 44%, and 55%, respectively. Representative images of siRNA induced suppression of ASIC1 immunolabeling is shown in Figure 5B.

ASIC proteins are required for VSMC migration in vitro

Two independent *in vitro* cell migration assays were used in this study, wound healing and PDGF-bb directed migration. As shown in Figure 6A, 8 hours after wounding, silencing ASIC3 expression significantly inhibited wound healing by 30%. By 24 hours, wound healing was significantly inhibited by approximately 10%, 20%, and 26%, compared to RISC controls, following ASIC1, ASIC2 and ASIC3 suppression, respectively (Fig. 6B). Chemotactic migration was inhibited following ASIC1 and ASIC3 suppression by approximately 30% and 45%, respectively, when compared to RISC transfected controls (Fig. 6C). In contrast, ASIC2 suppression enhanced chemotactic migration by 4%. The random migratory response (migration in the absence of PDGF-bb) was assessed and found to be virtually non-existent (1 – 3% of PDGF-bb stimulated migration), indicating VSMCs undergo very little random migration. Furthermore, the random migration responses were unaltered following ASIC1, ASIC2, and ASIC3 suppression, compared to RISC transfected controls.

ASIC proteins are not required for VSMC adhesion in vitro

To determine if reduced cell adhesion contributes to the migratory responses, we evaluated adhesion to fibronectin following ASIC silencing. As shown in Figure 7, cell adhesion to fibronectin was not affected following ASIC1, ASIC2, and ASIC3 suppression, when compared to RISC transfected controls. This observation suggests that reduced cellular adhesion is not likely to be the basis of reduced migratory responses following ASIC silencing.

DISCUSSION

Migration of VSMCs is believed to play an important role in the pathophysiology of many vascular disorders, such as hypertension, diabetes, atherosclerosis and restenosis after coronary angioplasty. Previous studies have shown that VSMC migration, from the media into the intima, plays a significant role following arterial injury, and blocking VSMC migration can reduce the neointimal lesion size (Rutherford et al., 1997; Slepian et al., 1998). A role for DEG/ENaC/ASIC proteins in cellular migration has been suggested in recent reports (Chifflet et al., 2005; Grifoni et al., 2006; Vila-Carriles et al., 2006). Our previous study showed that DEG/ENaC/ASIC pharmacological inhibition with amiloride and its analog benzamil inhibit VSMC migration. We demonstrated a significant portion of chemotactic and wound healing migratory responses were sensitive to amiloride/benzamil inhibition at doses greater than required for ENaC, suggesting another amiloride sensitive channel or transporter also contributed to migration. We decided to evaluate the contribution of ASIC proteins in migration in light of the results of Vila-Carriles et al. and others suggesting a role for ASIC proteins in migration

(Berdiev et al., 2003; Bubien et al., 1999; Vila-Carriles et al., 2006). Therefore, the goal of the present study was to determine if ASIC proteins are required for VSMC migratory responses. Our findings suggest ASIC1, ASIC2 and ASIC3, but not ASIC4, are expressed in VSMCs and differentially participate in chemotaxis- and injury-initiated migratory responses.

ASIC1, ASIC2 and ASIC3 are expressed in VSMCs

Previous studies have suggested ASIC protein expression is limited to neuronal and sensory epithelial tissue. However, recent studies suggest ASIC molecules may be expressed in other cells such as glial and skeletal muscle cells (Gitterman et al., 2005; Vila-Carriles et al., 2006). Our studies demonstrate that ASIC proteins are also expressed in VSMCs. The perinuclear staining patterns suggest that ASIC1 and ASIC2 proteins tend to be retained in a perinuclear intracellular pool, perhaps trapped in the endoplasmic reticulum and/or Golgi complex, a finding consistent with Vila-Carriles et al. (Vila-Carriles et al., 2006). In contrast, ASIC3 protein is distributed throughout the cytoplasm. A similar perinuclear expression pattern has also been observed for ENaC proteins expressed in cultured VSMCs (Grifoni et al., 2006).

ASIC subunits can associate in a variety of combinations to form homo- and heteromeric channels, each with different kinetics, external pH sensitivity, and tissue distribution (Akopian et al., 2000; Babinski et al., 1999; Bassilana et al., 1997; Chen et al., 1998; de Weille et al., 1998; Grunder et al., 2000; Lingueglia et al., 1997; Price et al., 1996; Waldmann et al., 1997a; Waldmann et al., 1997b; Waldmann et al., 1996; Waldmann and Lazdunski, 1998). Findings from our colocalization studies revealed only 1–2% of ASIC labeling co-localized in the cytoplasm suggesting heteromeric channels may not be the predominant configuration of ASIC channels in cultured VSMCs.

Inhibition of ASIC channels on VSMC migration

In a previous report, we demonstrated low concentrations of amiloride and benzamil inhibited VSMC migration, presumably due to ENaC inhibition. However, amiloride and benzamil (1 – 10 μ M) at concentrations above the half maximal inhibitory concentrations for ENaC, produced a further inhibition of VSMC migration (up to 95%) (Grifoni et al., 2006). Therefore, we considered the possibility that inhibition of other ion channels and/or transporters also contributed to the inhibitory effects of amiloride and benzamil at these concentrations. ASIC channels are likely candidates because ASIC channels have been implicated in migration of glioma cells (Berdiev et al., 2003; Bubien et al., 1999; Vila-Carriles et al., 2006) and are also amiloride-blockable. With one exception, we found that ASIC silencing inhibited wound healing and chemotactic migration. The exception was ASIC2 in chemotactic migration, where ASIC2 silencing stimulated chemotactic migration to small extent, suggesting a potential negative regulatory role of ASIC2 specific to chemotactic migration.

A negative regulatory role for certain ASIC proteins has been demonstrated previously (Page et al., 2005; Price et al., 2001; Vila-Carriles et al., 2006). For example, in astrocyte tumor (glioma) cells, cell surface expression of ASIC2 inhibits an amiloride-sensitive Na^+ current as well as migratory ability of glioma cells (Vila-Carriles et al., 2006). In peripheral touch receptors, Price et al. demonstrated ASIC3 contributes in an inhibitory manner in some to activation of sensory neurons, but an excitatory manner in others (Price et al., 2001). While these findings provide precedence for ASIC proteins functioning in both positive and negative regulatory roles in similar cell types, it is important to note that the negative effect of ASIC2 silencing on chemotaxis was very small (4%), and thus may have little physiologic relevance. The explanation for the contrasting role of ASIC2 in chemotaxis and wound healing migration is unclear. Differences in signaling mechanisms triggering or mediating chemotactic versus wound healing migration are possible underlying factors.

Despite a moderate (44–65%) reduction in ASIC protein expression using siRNA, we observed a much smaller impact on migration responses. Multiple factors may account for the disparity between reduction in expression and function. One possibility is that cell surface levels of ASIC proteins may be stable and, therefore, not very sensitive to siRNA silencing. Alternatively, upregulation of other ASIC proteins or associations among the other ASICs could partially compensate for the reduction of a given ASIC protein. A third possible explanation is that cell proliferation during the 24-hour wound-healing assay masks the inhibitory effect of ASIC suppression. We favor the later two possibilities.

In summary, our data demonstrate that ASIC1, ASIC2 and ASIC3 are expressed in VSMCs where they contribute to migration. Although our data suggest an important role for ASIC proteins in PDGF-bb and wound initiated VSMC migration, the precise mechanisms by which ASIC proteins contribute to VSMC migration remains to be determined. Our data also suggest that ASIC protein expression is not limited to neural tissue and sensory epithelia, as has been described before. In conclusion, our data are consistent with the hypothesis that ASIC proteins are required for VSMC migration. While it is unknown exactly how ASIC proteins contribute to migration, results from the current study raise the possibility that ASIC proteins play an important role in VSMC remodeling following arterial wall injury.

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Figure 1. ASIC message expression in cultured VSMCs

Representative images of RT-PCR detection of ASIC1a, ASIC2a, and ASIC3, but not ASIC4 transcripts. The presence (RT+) and absence (RT-) of reverse transcriptase is indicated. Brain was used as positive control.

Figure 2. ASIC protein expression by immunolabeling in cultured VSMC

Representative images of ASIC1a, ASIC2a, and ASIC3 protein expression in cultured VSMC. Antibody labeling is blocked when the antibody is incubated with excess antigen. α -actin or golgi stain (NBD-ceramide) was used as a positive control. Punctate cytoplasmic and perinuclear staining were typically observed.

Figure 3. ASIC protein expression by western blotting in cultured VSMC

Western blot detection of ASIC1a, ASIC2a, and ASIC3 protein in whole cell VSMC lysates. Antibody labeling is blocked when the antibody is incubated with excess antigen. β -Actin loading control is shown below the corresponding ASIC blot.

Figure 4. Co-localization of ASIC proteins in VSMCs

Representative images shown in A–C and quantitative data shown in D. A–C. Co-labeling of ASIC proteins, as indicated, is shown in the first two panels. A merged image is shown in the third panel. Yellow coloration indicates co-localization. An enlarged image of a cytoplasmic region in the same cell is shown in the far right panel. D. Co-localization of ASIC1/ASIC2, ASIC1/ASIC3 and ASIC2/ASIC3 is approximately 20% in the perinuclear regions, but only 1–2% in cytoplasmic regions.

Figure 5. Silencing ASIC expression by siRNA approach inhibits ASIC protein expression in VSMC *in vitro*

A. Quantitative effect of siRNA on ASIC expression. Delivery of individual ASIC1, ASIC2 or ASIC3 siRNA (100 nM) reduces the respective ASIC protein expression in cultured VSMCs when compared to non-targeting siRNA (RISC) controls. B. Representative images of ASIC1 and α -actin immunostaining in cultured VSMCs following transfection with ASIC1 or RISC (negative control) siRNA molecules. Data are mean $\pm$ SEM.

* Significantly different from respective control, $p < 0.05$.

Figure 6. Silencing ASIC expression inhibits VSMC migration *in vitro*

A and B. ASIC1, ASIC2 or ASIC3 silencing on wound healing at 8 and 24 hours. At 8 hours, only ASIC3 suppression significantly inhibits wound healing. However, by 24 hours, suppression of ASIC1, ASIC2 or ASIC3 expression inhibits wound healing. C. Suppression of ASIC1 and ASIC3 inhibits migration in response to PDGF-bb, while ASIC2 suppression enhances migration. D. Random migration responses were unaltered by ASIC silencing. Data are mean $\pm$ SEM.

* Significantly different from control, $p < 0.05$.

Figure 7. Silencing ASIC expression does not affect VSMC adhesion *in vitro*

VSMC adhesion to fibronectin following ASIC silencing was not different from RISC control.

Data are mean $\pm$ SEM.

* Significantly different from RISC control, $p < 0.05$.

Table 1

RT-PCR primer sequence, predicted product size, and annealing temperature.

Target	S or AS	Primer Sequence 5' – 3'	Predicted Product Size (bp)
ASIC1a	S	GCTTTAGCCAAGTCTCCAAG	1064
	AS	AGTCAAAGAGTTCCAGCACG	
ASIC2a	S	TGCTGCCTTACTTGGTGACA	151
	AS	GTGGCTCCCTTCCTCTTCTT	
ASIC3	S	TGACATGGCACAACCTCTACG	568
	AS	TCATCGACAGCCACACTTC	
ASIC4	S	TATAGTGTGTCTGCCTGCCG	288
	AS	TGTAGGTCTCATTGCGGTTG	

S = sense

AS = antisense