# Regulation of ASIC1 by $Ca^{2+}$/calmodulin-dependent protein kinase II in human glioblastoma multiforme

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**Abstract.** Recent studies have implicated the acid-sensing ion channel 1 (ASIC1), a proton-gated cation channel that belongs to the epithelial sodium channel (ENaC)/Degenerin family, plays an important role in glioma cell migration. Among the ASIC subunits, only ASIC1a has been found be calcium permeable. However, it has not been determined whether $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) regulates ASIC1 in glioblastoma multiforme (GBM). Herein, we report that ASIC1 and CaMKII assemble to form a functional complex at the plasma membrane of GBM cells. We found that migration ability was significantly attenuated in GBM cells that were pre-treated with autocamtide-2-related inhibitory peptide (AIP), a CaMKII-specific inhibitor, or psalmotoxin 1 (PcTX-1), a selective ASIC1 blocker. Furthermore, the inhibitory effect of AIP or PcTX-1 on migration was diminished when ASIC1 was knocked down in GBM cells; when ASIC1 knockdown GBM cells were concurrently treated with these two inhibitors, cell migration was slightly but significantly decreased. Using whole-cell patch-clamp recordings, we detected an amiloride-sensitive current in GBM cells, and this current was significantly inhibited by both PcTX-1 and AIP. Moreover, the magnitude of this current was dramatically decreased when ASIC1 was knocked down in GBM cells. The addition of AIP failed to further decrease the amplitude of this current. Taken together, these data suggest that ASIC1 and CaMKII form a functional complex in GBM cells. Furthermore, it can be concluded that CaMKII regulates the activity of ASIC1, which is associated with the ability of GBM cells to migrate.

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## Introduction

Gliomas are the most common primary tumors of the central nervous system (CNS). They are aggressive, highly invasive, and neurologically destructive tumors. Among these, glioblastoma multiforme (GBM) is the most malignant phenotype. Several studies on a variety of cancers have suggested that the activity of ion channels is closely related to a tumor cell's ability to migrate and proliferate (1-3). It has been reported that glioma cells express higher levels of potassium, sodium and chloride channels compared to normal astrocytes (4-6), suggesting these ion channels may play a role in the progression of glioma. Several members of the ENaC/Degenerin family are expressed in a variety of GBM cell lines, including U251-MG (7). The ENaC/Degenerin superfamily includes multiple members of the ENaC and ASICs (ASIC1-4) subfamilies (8). The ENaC/Degenerin superfamily members can be specifically inhibited by amiloride (9). Previous studies report that glioma cells are unable to regulate their volume subsequent to cell shrinkage by hyperosmolar solutions when treated with either amiloride or PcTX-1, a specific ASIC1 blocker (40-amino acid peptide, purified from the venom of the West Indies tarantula) (10,11). These studies suggest that ASIC1 may be involved in the regulation of glioma cell volume, thereby affecting the migration ability of glioma cells.

Unlike ENaC, ASICs are non-selective cation channels permeable to both monovalent and divalent cations, including $Ca^{2+}$ (12,13). Four ASIC genes (ASIC1, ASIC2, ASIC3 and ASIC4) and splice variants for ASIC1 (ASIC1a and ASIC1b) have been identified in a variety of cell types (14,15). ASIC channels can be transiently activated by extracellular acidosis (14). Although a constitutively activated, amiloride-sensitive $Na^+$ current was reported in GBM cells, this current is not seen in normal astrocytes or low-grade gliomas (4). Later studies suggest that this amiloride-sensitive, constitutively-activated cation current is mediated by a cross-clade channel composed of ASIC1 and two subunits of ENaC, αENaC and

γENaC. Knockdown of any of the three ENaC/Degenerin subunits (ASIC1, αENaC and γENaC) eliminates this current and attenuates migration of GBM cells (11). However, no typical extracellular acidosis-activated, whole-cell current was detected in GBM cells (16).

CaMKII catalyzes the phosphorylation of ASIC1a to activate ASIC1a channels and subsequently increase cell death in rodent ischemic CNS neurons (17). This $Ca^{2+}$-sensitive kinase may also play an important role in glioma biology, particularly because glioma cells require $Ca^{2+}$, which acts as a second messenger to support cell migration (18). Although these functional data suggest the importance of ASIC1 and CaMKII in ischemic CNS, the functional interaction of the two within GBM cells remains unclear. We hypothesized that ASIC1 is constitutively activated in GBM cells, and that CaMKII regulates this process. Furthermore, we hypothesized that the activity of ASIC1 is associated with the ability of glioma cells to migrate.

## Materials and methods

*Cell culture*. The glioma cell line U251-MG (GBM, derived from a World Health Organization grade IV tumor) (ATCC, Rockville, MD, USA) was routinely cultured in 1:1 Dulbecco's modified Eagle's/F-12 medium (Hyclone, Logan, UT, USA) supplemented with 15% fetal calf serum (Hyclone) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were incubated in an atmosphere containing 5% $CO_2$ and 95% air at 37˚C. The medium was changed every three days. Cells were split 48 h prior to electrophysiological recording onto 35-mm dishes containing flame-sterilized coverslips. Cells were split and starved 24 h and 4-6 h before transfection, respectively.

*Immunofluorescence*. U251-MG cells were grown on 25-mm glass coverslips and processed for indirect immunofluorescence after reaching confluence. Cells were fixed and subsequently incubated for 2 h with the following antibodies: a goat polyclonal anti-ASIC1 (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a rabbit monoclonal anti-CaMKII (1:200 dilution; Abcam, Cambridge, MA, USA). Following incubation with the primary antibodies, the cells were then visualized using either Alexa Fluor 594 Donkey Anti-Goat IgG (H+L) or Alexa Fluor 488 Donkey Anti-Rabbit IgG (H+L) (Invitrogen). Nuclei were stained with DAPI (1:1000 dilution, Invitrogen) for 15 min. Images were captured at room temperature using a 63x/1.2 oil immersion objective on a confocal microscope (Fluoview1000, Olympus).

*Patch clamp electrophysiology*. The whole-cell patch-clamp technique was used to record the amiloride-sensitive whole-cell current. The steady-state current-voltage relationships were determined using a holding potential of -60 mV. The membrane potential was stepped from -160 mV to +100 mV in 20 mV increments. The peak current at each voltage was measured. Resistance of patch pipettes ranged from 1.5 to 2 MΩ when filled with the pipette solution. Whole-cell recordings were performed with an Axopatch 200B amplifier operated with the pCLAMP software, filtered at 10K Hz, acquired at 2 kHz with pCLAMP, and analyzed with the Clampfit 9.0.

*Solutions*. Amiloride-sensitive currents were recorded in an external solution of RPMI-1640 medium containing the following (in mM): 133 $Na^+$, 5.3 $K^+$ and 108.3 $Cl^-$. The pipette solution contained (in mM) 100 potassium gluconate, 30 KCl, 20 HEPES, 0.5 EGTA, 4 ATP and 10 nM free calcium at a pH of 7.2 (adjusted by HCl).

*ACCN2 genes silencing*. Using the BLOCK-iT pol II miR RNAi expression vector kit from Invitrogen, we designed miRNA mimics that were 21 nt in length. The miRNA target and negative control sequences were defined as 5'-TCAGGATGTAGCCTACAGCAC-3' and 5'-AAATGTACTGCGCGTGGAGAC-3', respectively. The miRNAs were cloned into the pcDNA 6.2-GW/EmGFP-miR vector by inserting them into the 3'-UTR of the EmGFP gene. The U251-MG cells were seeded at 40-60% confluency in 6-well plates a day before transfection. The cells were transfected using lipofectamine 2000 (Invitrogen). After transfection, cells were continuously cultured in the presence of 10 ng/ml blasticidine S hydrochloride (Sigma, St. Louis, MO, USA). The cells were used for other experiments three weeks after transfection.

*Western blotting*. Cells were lysed and equal concentrations of proteins were separated on 10% SDS-polyacrylamide gels (Invitrogen) and transferred onto polyvinylidene difluoride (PVDF) membranes. The blots were incubated in anti-ASIC1 (1:500 dilution; Abcam), anti-CaMKII (1:1000 dilution; Abcam), and anti-β-actin (1:5000 dilution; Santa Cruz Biotechnology) antibodies for 2 h followed by incubation with a peroxidase-conjugated secondary antibody (1:5000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature. Labeled proteins were visualized with ECL (Invitrogen).

*Immunoprecipitation*. Cells were lysed and centrifuged at 10,000 x g for 10 min to remove cellular debris. The supernatant was removed and aliquots of proteins (500 $\mu$g) were incubated with 5 $\mu$g of anti-CaMKII antibody or rabbit IgG overnight at 4˚C. Immunoprecipitates were captured with 40 $\mu$l of protein A/G beads (Santa Cruz Biotechnology) at 4˚C for 1 h. Samples were centrifuged and then washed three times with 1 ml of lysis buffer. The proteins were separated by SDS-PAGE electrophoresis and transferred to PVDF membrane. Blots were incubated in anti-ASIC1 or anti-CaMKII antibodies overnight at 4˚C.

*Migration assay*. *In vitro* migration assays were performed using Costar transwell inserts (Costar, NY, USA; pore size 8-$\mu$m) in 24-well plates. A total of ~1-1.5x$10^5$ cells in 200 $\mu$l DMEM were seeded on the upper chamber of a 6.5 mm-transwell with 8.0 $\mu$m-pore polycarbonate membrane inserts. DMEM (600 $\mu$l) with 10% fetal bovine serum was added into the lower chamber as a chemoattractant. Cells were allowed to adhere for 30 min before treatment with 10 nM PcTX-1 (Abcam) to block ASIC1 or 1 $\mu$M myristoylated AIP (Enzo Life Sciences, Farmingdale, NY, USA) to block CaMKII. The plates were incubated for 12 h at 37˚C in 5% $CO_2$, followed by fixation in 4% buffered paraformaldehyde for 15 min and staining with DAPI for 15 min. Migrated cells

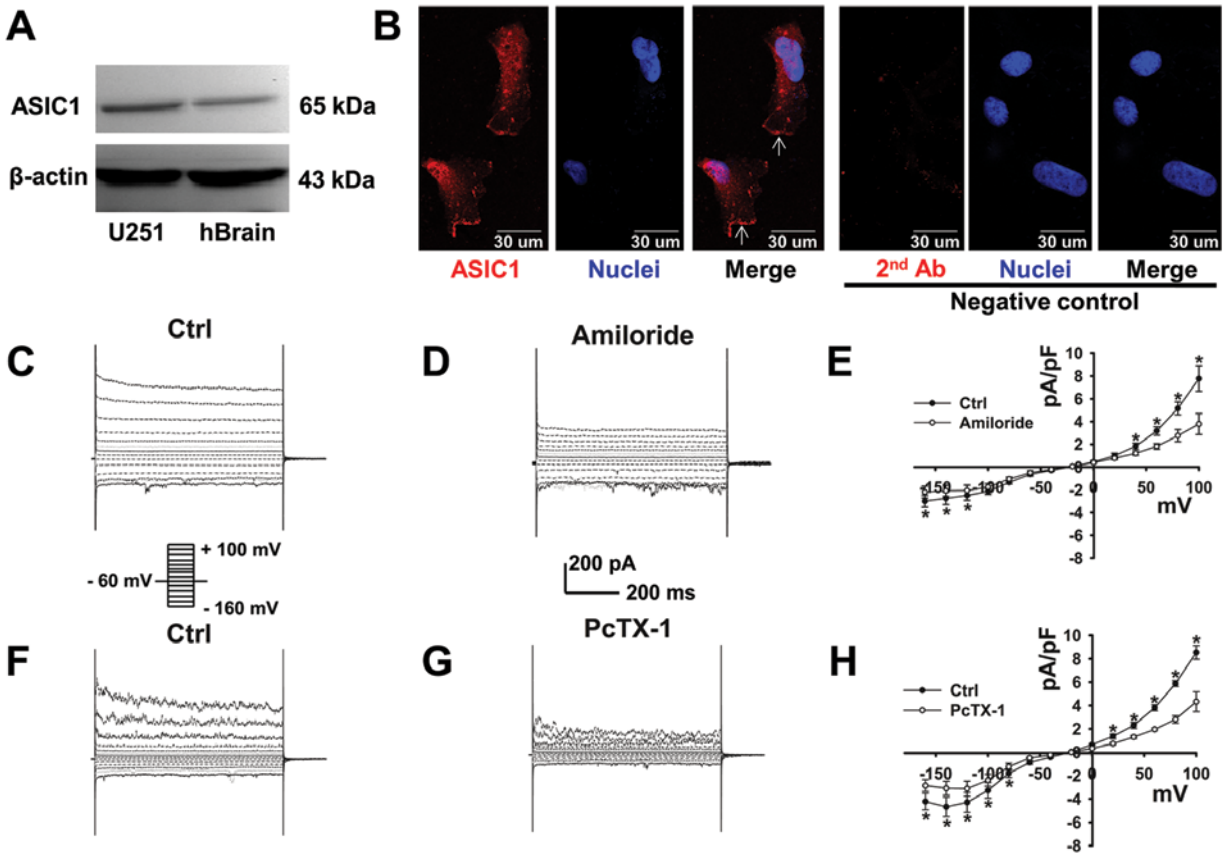


Figure 1. U251-MG cells express functional ASIC1. (A) Representative western blot demonstrating ASIC1 is expressed in U251-MG cells and human brain tissue. (B) Representative immunofluorescence staining images indicate that ASIC1 is localized at the plasma membrane of U251-MG cells (the white arrow heads), since staining the cells with the secondary antibody alone shows negative results. Red, ASIC1; blue, nuclei. (C) A constitutively activated current was recorded in a U251-MG cell using a whole-cell patch-clamp configuration, and this current was inhibited by 100 $\mu$M amiloride (D). (E) Average current and voltage (I-V) relationships were constructed by measuring average currents in the absence (the filled circles) or in the presence (the open circles) of 100 $\mu$M amiloride, as a function of voltages (n=4 paired patches). $^{*}p<0.05$. Constitutively activated currents were recorded in U251-MG cells under control condition (F) or in the presence of 10 nM PcTX-1 in the bath solution (G). (H) I-V relationships were constructed as described in E, in the absence (the filled circles) or in the presence (the open circles) of 10 nM PcTX-1 (n=6 paired patches). $^{*}p<0.05$.

were counted in five random areas of the membrane using a confocal microscope.

## Results

*ASIC1 is expressed in U251-MG cells*. We first attempted to use western blotting to determine whether U251-MG cells express ASIC1. As shown in Fig. 1A, protein bands of ~65 kDa were detected in U251-MG cells and human brain tissue. These data indicate that ASIC1 is present in U251-MG cells. The cellular distribution of ASIC1 was determined by immunofluorescence staining. Our results clearly show that ASIC1 is localized at the plasma membrane (the white arrow heads) of U251-MG cells (Fig. 1B).

*A constitutively activated, amiloride-sensitive current was detected in U251-MG cells*. Using a whole-cell patch-clamp configuration, we detected a constitutively activated current in U251-MG cells (Fig. 1C). This current was significantly inhibited by 100 $\mu$M amiloride as previously described (11) (Fig. 1D). The current densities measured at different voltages in the absence or presence of amiloride were plotted as a function of voltage; the data show that the current density was significantly reduced (Fig. 1E; n=4 paired experiments).

To confirm whether this amiloride-sensitive current is carried by ASIC1 in U251-MG cells, an ASIC1 specific blocker, PcTX-1, was applied to the bath solution after a control current was generated (14). This current was significantly inhibited by treatment with 10 nM PcTX-1 (Fig. 1F and G), which is shown by the summarized I-V relationships (Fig. 1H; n=6 paired experiments). These data are consistent with the notion that this constitutively activated current in U251-MG cells is most likely mediated by ENaC/Degenerin subunits, and that ASIC1 may constitute the central core of the channel (11).

*ASIC1 mediates the constitutively activated current in U251-MG cells*. To confirm that this constitutively activated current in U251-MG cells is mediated by ASIC1, we used a gene-silencing technique to knock down ASIC1 and performed patch-clamp experiments in these cells (knockdown efficiency shown in Fig. 4C). Upon knockdown of ASIC1, the constitutively activated current in U251-MG cells was dramatically decreased (Fig. 2A, B and D; n=6), strongly suggesting that this current is carried by ASIC1.

Since it was previously reported that CaMKII regulates ASIC1a in rats (17), we reasoned that CaMKII might regulate ASIC1 currents in U251-MG cells. We tested whether AIP, a highly specific and potent inhibitor of CaMKII, could inhibit

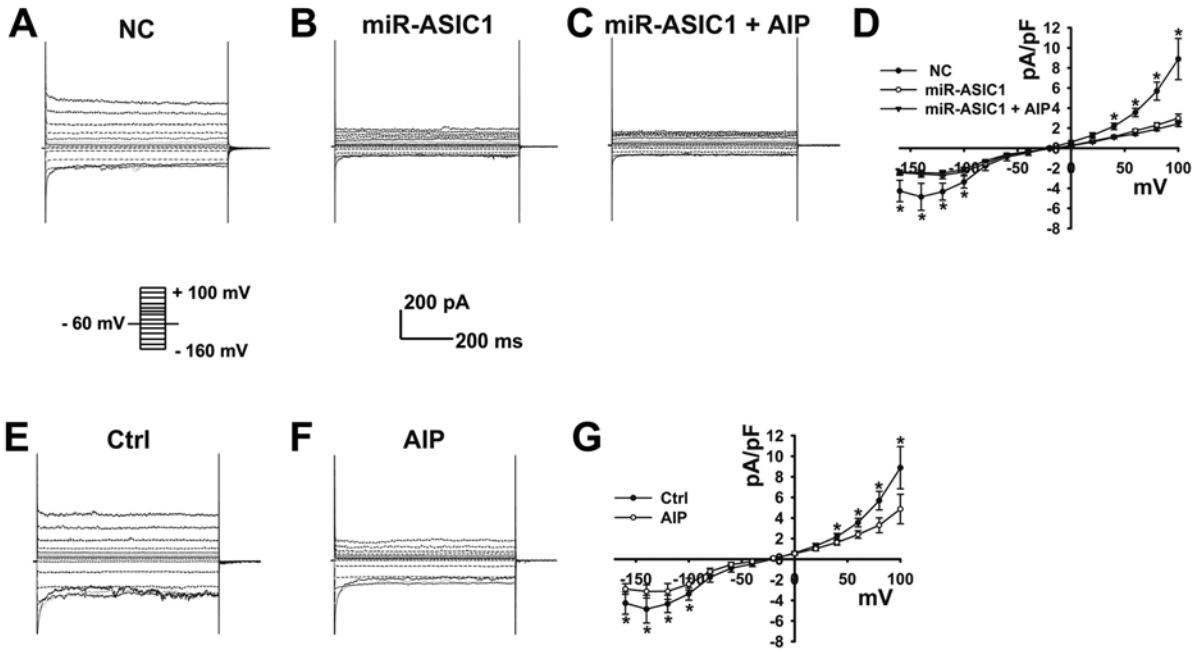


Figure 2. The constitutively activated current in U251-MG cells is carried by ASIC1, and CaMKII regulates ASIC1 channels. (A) Representative whole-cell current recorded from a U251-MG cell under control conditions. (B) The current was significantly decreased in ASIC1-knockdown U251-MG cells. (C) AIP, a specific CaMKII inhibitor, did not inhibit the remaining current in ASIC1 knockdown cells. (D) I-V relationships were constructed as described in Fig. 1E, control (the filled circles) or ASIC1 knockdown (the open circles), or AIP applied in ASIC1 knockdown cells (the filled triangles) (n=6 for each condition). $^{*}p<0.05$ compared with the ASIC1-transfected group and the specific miRNA against ASIC1-transfected group treated with AIP. Representative whole-cell currents recorded from a U251-MG cell under control conditions (E) and in the presence of 1 $\mu$M AIP to the bath solution of patches (F). (G) I-V relationships were constructed as described in 1E, control (the filled circles) and in the presence of AIP (n=6 paired patches). $^{*}p<0.05$.

this current in U251-MG cells. As seen in Fig. 2F, the ASIC1 currents were significantly decreased upon treatment with AIP (Fig. 2E-2G; n=6 paired experiments). Moreover, AIP failed to further decrease ASIC1 currents when ASIC1 was knocked down in U251-MG cells, suggesting the specificity of CaMKII-mediated regulation of ASIC1 currents (Fig. 2A-D).

*CaMKII physically associates with ASIC1 and regulates ASIC1 in U251-MG cells*. Since we found that AIP inhibits ASIC1 (Fig. 2E-G), we performed immunofluorescence staining and co-IP experiments to determine whether these proteins functionally interact. Our co-IP results show that ASIC1 and CaMKII associate in U251-MG cells (Fig. 3A). The immunofluorescence staining shows that CaMKII and ASIC1 co-localize predominantly at the plasma membrane of U251-MG cells (Fig. 3B; the white arrow head). These data, along with the data shown in Fig. 2D and G, suggest that CaMKII and ASIC1 assemble to form a functional complex in U251-MG cells, and that CaMKII regulates ASIC1.

*CaMKII-mediated activation of ASIC1 contributes to the ability of GBM cells to migrate*. Previous studies have demonstrated that ASIC1 plays a role in the malignant behavior of glioma cells (11,16,19,20), and that CaMKII regulates many ion channels (21). Therefore, we hypothesized that inhibiting CaMKII and/or ASIC1 activity would reduce the ability of GBM cells to migrate. Therefore, we used a transwell migration assay in combination with a CaMKII inhibitor and ASIC1 blocker to test this hypothesis. Fig. 4A shows representative images of migrated U251-MG cells under different experimental conditions. It appeared that treatment with PcTX-1, AIP, or PcTX-1 + AIP was able to significantly attenuate cell migration (Fig. 4A and B). Under control conditions 343±25 cells migrated through the pores; in the presence of PcTX-1, AIP, or PcTX-1 + AIP the number of migrated cells was reduced to 247±23, 190±11, and 178±11, respectively (Fig. 4A and B; n=3 for each condition).

We also performed migration assays when ASIC1 was knocked down in U251-MG cells (knockdown efficiency shown in Fig. 4C); our results indicate that the number of migrated cells was significantly reduced in these cells compared with the negative control (NC) (Fig. 4D). Of note, we found that neither PcTX-1 nor AIP treatment further reduced cell migration in U251-MG cells when ASIC1 was knocked down (188±15 and 171±11 cells vs. 213±16 cells); however, PcTX-1 + AIP treatment slightly but significantly reduced the number of migrated cells (151±12 vs. 213±16 cells) (Fig. 4A and D; n=3 for each condition). Taken together, our data suggest that CaMKII-mediated activation of ASIC1 affects the ability of GBM cells to migrate, and that inhibition of ASIC1 or CaMKII can attenuate this migration.

## Discussion

The aim of current study was to determine whether CaMKII-mediated activation of ASIC1 contributes to the ability of GBM cells to migrate. The major findings of the current study are as follows: i) ASIC1 and CaMKII physically interact and co-localized at the plasma membrane in U251-MG cells; ii) CaMKII regulates ASIC1 in U251-MG cells; iii) CaMKII-mediated activation of ASIC1 affects the ability of U251-MG cells to migrate.

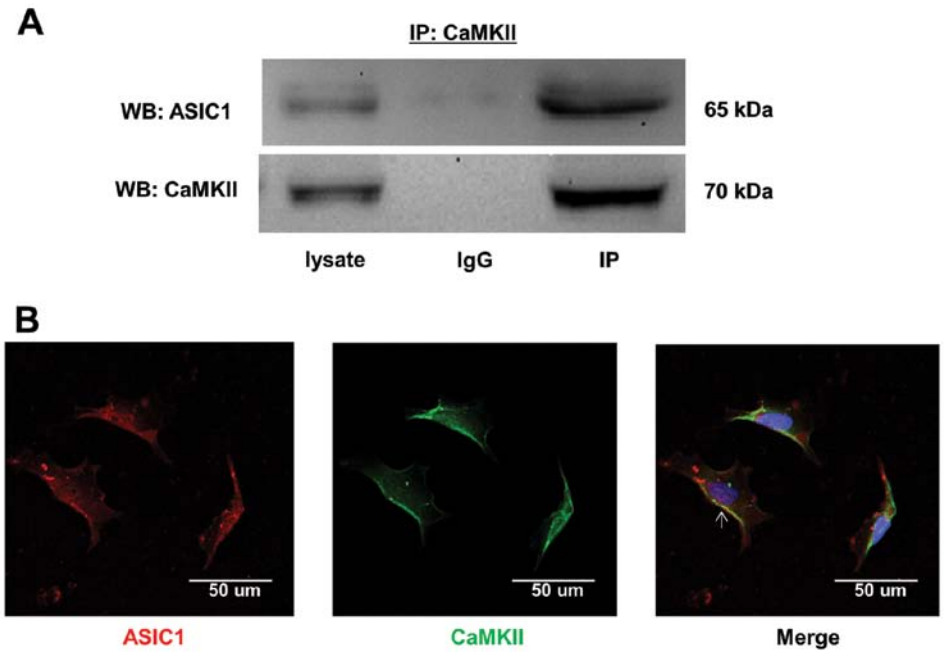


Figure 3. CaMKII physically associates and co-localizes with ASIC1 in U251-MG cells. (A) Co-immunoprecipitation (co-IP) experiments demonstrating CaMKII physically associates with ASIC1. (B) Immunofluorescence staining images suggest ASIC1 and CaMKII co-localize at the plasma membrane in U251-MG cells (the white arrow heads). Red, ASIC1; green, CaMKII; blue, nuclei. All images were taken at x60 view of a field.

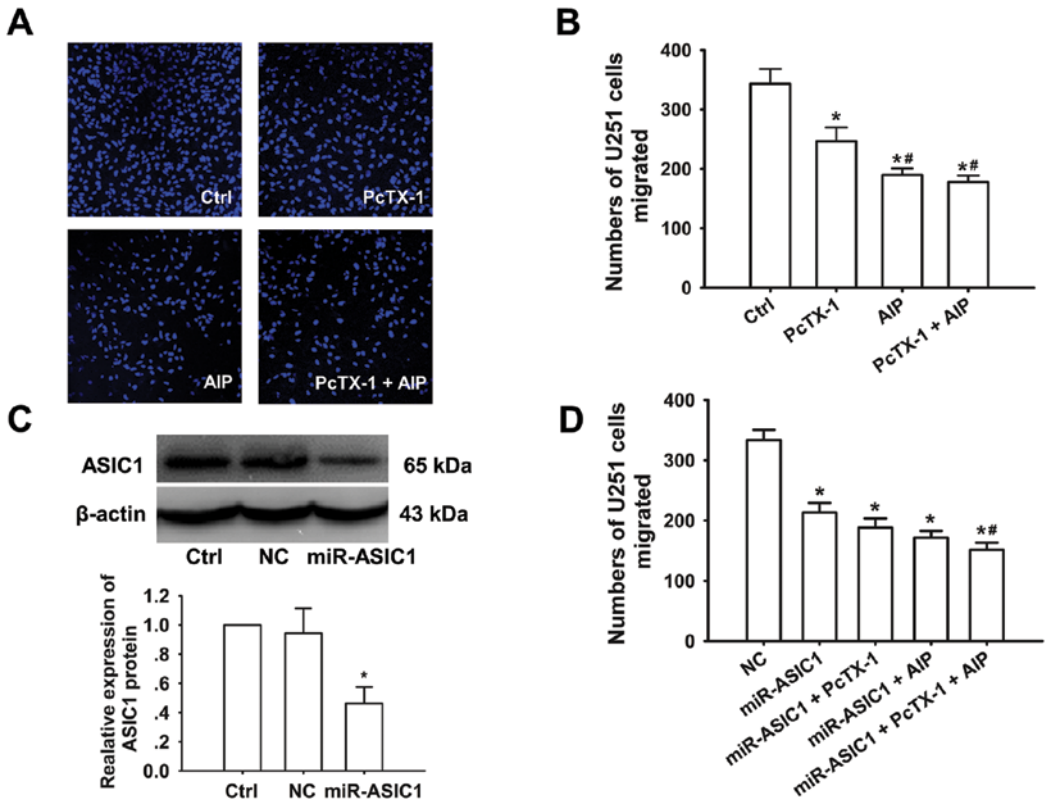


Figure 4. CaMKII mediated activation of ASIC1 plays a role in U251-MG cell migration. (A) Representative images of migrated U251-MG cells under control conditions, in the presence of 10 nM PcTX-1, 1 $\mu$M AIP, and 10 nM PcTX-1 + 1 $\mu$M AIP, respectively. (B) Summarized numbers of migrated U251-MG cells before and after application of PcTX-1, AIP and PcTX-1 + AIP (n=3). Ctrl represents normal U251-MG cells; PcTX-1, AIP, PcTX-1 + AIP indicate normal U251-MG cells treated with PcTX-1, AIP, and AIP + PcTX-1, respectively. $^{*}$p<0.05 compared with control cells. $^{\#}$p<0.05 compared with PcTX-1 treated group (n=3 for each condition). (C) Representative western blot demonstrating the knockdown efficiency of ASIC1 by miRNA (top), and the expression level of ASIC1 was reduced ~55% in miRNA-transfected U251-MG cells (n=3). $^{*}$p<0.05 compared with control and negative control cells. (D) ASIC1 knockdown resulted in a decrease in U251-MG cell migration; either PcTX-1 or AIP failed to further decrease the migration ability in ASIC1 knockdown cells; however, PcTX-1 and AIP together led to a slight but significant decrease in migration in ASIC1 knockdown cells. NC represents the scramble miRNA transfected group; miR-ASIC1, miR-ASIC1 + PcTX-1, miR-ASIC1 + AIP, and miR-ASIC1 + PcTX-1 + AIP indicate specific miRNA against ASIC transfected group, specific miRNA against ASIC transfected group treated with PcTX-1, AIP, and AIP + PcTX-1, respectively. $^{*}$p<0.05 compared with negative control cells. $^{\#}$p<0.05 compared with miR-ASIC1 group (n=3 for each condition).

It has been reported that ASIC1a functions as a non-selective transient channel in rat C6 glioma cells; activation of ASIC1a induces a short depolarization or transient calcium influx in these cells, even if the acidic stimulus is persistent (22). Glioma cells appear to not exhibit the typical acid induced ASIC1 current (11,16), which may be due to the channel already being maximally activated by the native acidic condition (13). In this study, we recorded a constitutively activated, amiloride-sensitive current in U251-MG cells. This current is inhibited by treatment with PcTX-1, a specific ASIC1 blocker. Moreover, upon knockdown of ASIC1, the current was dramatically decreased. These data strongly suggest that this constitutively activated current recorded in U251-MG cells is carried by ASIC1.

CaMKII catalyzes the phosphorylation of ASIC1a at Ser478 and Ser479 residues, which activates the ASIC channels. This process may contribute to ischemia-induced cell death in rodent ischemic CNS neurons (17). GBM cells and ischemic CNS neurons share similar acidic and low oxygen microenvironments (23,24). Therefore, we hypothesized that CaMKII may regulate ASIC1 currents in GBM cells. We found that CaMKII interacted with ASIC1 and co-localized at the plasma membrane in U251-MG cells. Furthermore, we discovered that the ASIC1 currents were significantly decreased in U251-MG cells upon treatment with AIP, a specific CaMKII inhibitor. Moreover, AIP treatment did not further decrease ASIC1 currents in cells where ASIC1 was knocked down, suggesting that CaMKII specifically regulates ASIC1 currents. ASIC1 plays a role in GBM cell migration ability (11,20), cell cycle progression (20), and volume regulation (19). However, it is not known whether this CaMKII-mediated activation of ASIC1 affects the ability of GBM cells to migrate.

To this end, we tested whether inhibition of CaMKII and/or ASIC1 would lead to reduced GBM cell migration. We reasoned that if ASIC1 is the most relevant target for CaMKII, then downregulation of ASIC1 expression or pharmacological inhibition of its activity should result in significantly reduced migration of U251-MG. The migration assay results show that both reduced expression of ASIC1 or pharmacological inhibition of ASIC1 caused a significant reduction of cell migration. Furthermore, inhibition of CaMKII led to a greater reduction of cell migration, suggesting CaMKII-mediated ASIC1 contributes to a reduction of the cell's ability to migrate. Since it has been reported that the volume-gated chloride channel ClC-3 is involved in glioma invasion (25) and that phosphorylation of ClC-3 by CaMKII is important in glioma cell migration (26), the synergetic effect of PcTX-1 and AIP on cell migration might be attributed to inhibition of ClC-3. Although AIP treatment induced a greater reduction in cell migration, this treatment did not further reduce migration in cells where ASIC1 was knocked down.

Moreover, a combined PcTX-1 and AIP treatment exhibited an additive effect on the reduction of cell migration in cells where ASIC1 was knocked down. This additive effect might be a result of residual ASIC1s in these cells. Specifically, AIP treatment likely inhibits the activity of the residual ASIC1s and further reduces cell migration. Taken together, these data suggest that the migration of U251-MG cells is primarily regulated by CaMKII-mediated, constitutively activated ASIC1 channels. Since it has been suggested that ASIC1a is permeable to calcium (27), it is possible that these constitutively activated ASIC1 channels may also allow calcium to permeate and activate CaMKII in U251-MG cells, thereby regulating ASIC1 channels. Nevertheless, our data may provide potential therapeutic targets for preventing the invasiveness of gliomas.

## Acknowledgements

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